INVESTIGATION OF THE GENETICS OF RECOMBINATIONAL TELOMERE ELONGATION (RTE) AND ITS STEPS IN THE MILK YEAST *KLUYVEROMYCES LACTIS*

by

EVELINA YURIYEVNA BASENKO

(Under the Direction of Michael McEachern)

ABSTRACT

In the absence of telomerase certain cancers achieve immortalization through telomere elongation using Alternative Lengthening of Telomeres pathway, which is dependent on telomeric homologous recombination (HR). The process of recombinational telomere elongation (RTE) has been extensively studied in yeast. Upon deletion of telomerase, yeast cells lose most telomeric repeats and die, but rare survivors emerge with elongated telomeres. *Saccharomyces cerevisiae* can utilize either Type I or Type II RTE which rely on *RAD51*-dependent or *RAD50/RAD59/SGS1*-dependent recombination machinery, respectively. *Kluyveromyces lactis* telomerase deletion mutants generate Type II survivors only. Rare *K. lactis* telomerase deletion (*ter1*-Δ) survivors emerge using RTE that is thought to occur via a three-step mechanism called the roll- and-spread model. According to this model, a telomeric circle (t-circle) is formed and utilized as a template for DNA synthesis to produce a long telomere. The sequence from a lengthened telomere is then spread to other short telomeres through break-induced replication events. To study the genetics of RTE in *K. lactis*, mutants lacking telomerase and one or more recombination genes (*RAD50*, *RAD51*, *RAD59*, and *SGS1*) were created. Both, the *RAD51* and *RAD50/RAD59/SGS1* pathways act together in a single Type II survivor formation pathway in *K.*

lactis. *RAD51*, but not *RAD59*, was required for telomere copying events through recombination originating specifically in subtelomeric regions. *RAD59* is essential in the absence of *RAD51* as indicated by the absence of survivors in the *ter1*-Δ *rad51*-Δ *rad59*-Δ triple mutant. Furthermore, we have addressed the genetics of the steps postulated to be involved in the roll-and-spread model. Small t-circles were dependent on *RAD50*, while large t-circles were diminished in the absence of *RAD50* and *RAD52* indicating that an alternative pathway of t-circle formation may exist. Our results also demonstrate that RTE in phenotypically similar mutants, but carrying different telomere capping mutations, has different genetic requirements. We also found that RTE can occur in the presence of partially defective telomerase. As such, yeast cells can use recombination to either moderately elongate telomeres via the roll-and-spread mechanism or simply to maintain extremely short telomeres.

INDEX WORDS: telomere, recombination, RTE, senescence, survivors, t-circles, *Kluyveromyces lactis, RAD50, RAD51, RAD59, SGS1*.

INVESTIGATION OF THE GENETICS OF RECOMBINATIONAL TELOMERE ELONGATION (RTE) AND ITS STEPS IN THE MILK YEAST *KLUYVEROMYCES LACTIS*

by

EVELINA YURIYEVNA BASENKO

MS, Mississippi State University, 2003

BS, Kherson Agrarian State University, Ukraine, 2001

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2011

INVESTIGATION OF THE GENETICS OF RECOMBINATIONAL TELOMERE ELONGATION (RTE) AND ITS STEPS IN THE MILK YEAST *KLUYVEROMYCES LACTIS*

by

EVELINA YURIYEVNA BASENKO

Major Professor: Michael McEachern

Committee: Sidney Kushner
Marcus Fechheimer
Claiborne Glover
Ellen Neidle

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2011

ACKNOWLEDGEMENTS

Being in the Graduate School is not easy, but the knowledge, the experience, and the people you meet make it all worthwhile.

I would like to express gratitude to my advisor Dr. Michael McEachern for valuable discussions; for his patience, generosity, and kindness; and for his teachings and contributions to the development of my scientific career.

I am grateful to my committee members Dr. Sidney Kushner, Dr. Claiborne Glover, Dr. Ellen Neidle, and Dr. Marcus Fechheimer for insightful comments and constructive criticism. I am particularly thankful to my temporary advisor Dr. Kushner for his advice to not give up and always do my best. This advice has helped me to get through the first, the toughest, years of the Graduate School.

To past and present members of the lab and undergraduate students with whom I had a pleasure to work with, I want to say thank you for your help in research, for your delightful company, and your friendship.

On a personal note I would like to thank my wonderful family for their never diminishing support and love; for many laughs and unforgettable times. To all my friends- thank you for being my back up and travel companions through my life. To Jen and Paul- I will definitely miss sneaking up on you at your desks! Also, I want to particularly acknowledge my best friends Laura and Marly for making my bad days tolerable and the good times the best!

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

Introduction and Literature Review

Evolution of linear genomes poses species with two serious problems. First, chromosome ends must be differentiated from DNA breaks. Second, due to incomplete replication of DNA, chromosomal ends have to be replenished to counteract gradual DNA loss. In the majority of species with linear chromosomes, telomeres, from Greek "telos" for an end and "meros" for a part, protect chromosomal ends from being recognized as breaks and also buffer against DNA loss. Telomere maintenance is most frequently performed by telomerase, a specialized reverse transcriptase that synthesizes telomeres *de novo* using an RNA template (16). Telomere importance in genome protection was noted as early as the 1800s (69). However, it was not until the 1930s that the importance of telomeres in cell viability was truly appreciated with the discoveries made by Hermann Müller and Barbara McClintock that demonstrated that intact chromosomal ends (telomeres) prevented genomic instability (49).

Telomeres are typically composed of multiple repeats of 5-8 bp non-coding DNA sequences, although some exceptions exist such as yeast species that can have either degenerate or uniform, 16-31 bp repeats (188). Telomere length and integrity is tightly regulated by modulation of telomerase access to telomeres and by telomere associations with telomere-specific binding proteins (telomere capping) (53, 74). Telomere capping plays an essential function in telomere protection against inappropriate repair. Surprisingly, a number of proteins from repair pathways are normally found at telomeres ensuring their proper function.

If the proper functioning of the telomeric cap is altered, in addition to telomerase-dependent elongation, telomeres can also undergo repair through an error-prone non-homologous end joining (NHEJ) pathway or homologous recombination (HR), a process whereby a telomere copies a sequence from another telomeric source (115, 264). Yeast cells deficient in telomerase and certain human cancers can utilize HR to elongate telomeres (115, 175). This pathway is not understood well in human cancers, and yeasts are important model organisms for studying the genetics of this mechanism. This dissertation describes studies conducted in the milk yeast *Kluyveromyces lactis* to understand the mechanism of Recombinational Telomere Elongation (RTE) and its steps.

Structure of telomeres

The first telomeric sequence was obtained from ribosomal DNA isolated from the ciliate protozoan *Tetrahymena* (17). In the great majority of eukaryotes telomeres consist of multiple repeats of a short G-rich DNA sequence. Humans have a 6 bp (TTAGGG) telomeric repeat and so does the human parasite *Trypanosoma brucei* and some agriculturally important pathogens of *Aspergillus* spp (15, 119). However, some yeasts are exceptions to this rule. Baker's yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) has the variable telomeric repeat $TG_{2-3}(TG)_{1-6}$, while *K. lactis* has uniform 25 bp telomeric repeats (Table 1.1). The total length of telomeres varies greatly between various representatives of the eukaryotic kingdom, from a dozen bp in *Euplotes crassus* to several hundred bp in yeast or several thousand bp in humans.

Table 1.1. Telomere sequence and length vary between species.
Most eukaryotes with linear chromosomes have telomeric repeats capping chromosomal ends. The telomeric repeats consist of 6-8 bp repeats with some exceptions (24, 39, 188).

Species	Telomere length	Telomere sequence
Protozoa		
Tetrahymena thermophila	120-1000 bp	TTGGGG
Oxytricha	20-28 bp	GGGGTTTT
Trypanosoma brucei	3-10 kb	TTTAGGG
Yeast		
Saccharomyces cerevisiae	200-300 bp	$TG_{2-3}(TG)_{1-6}$
Kluyveromyces lactis	400-600 bp	GGTGTACGGATTTGATTAGGTATGT
Vertebrates		
Humans	5-15 kb	TTTAGGG
Mice	up to 150 kb	TTAGGG
Invertebrate		
Ants	9-13 kb	TTAGG
Honey bee	< 1kb	TTAGG
Plants		
Arabidopsis thaliana	2-5 kb	TTTAGGG
Tomato	20-50 kb	TT(T/A)AGGG

Telomeres consist of double-stranded DNA-protein structures terminating in a single-stranded 3' overhang on the G-rich strand. Both the double- and single- stranded telomeric DNA play important roles in telomere capping by providing binding substrates for telomere-specific binding proteins. Telomerase requires the 3' overhang for *de novo* telomere synthesis. The 3' overhang is protected against degradation by the binding of capping proteins and also by formation of a t-loop, a higher-order structure formed when a single-stranded telomeric end loops backwards and strand-invades upstream double-stranded telomeric sequences. Rap1 is capable of inducing DNA bending (172) as suggested by observations of looped telomeric DNA from 500 bp to 18 kb in length isolated from mouse and human cells (84). t-loops have been

identified in chickens, plants, and protozoans (30, 174, 184). The maintenance of the telomeric overhang is cell-cycle regulated and is achieved by careful orchestration of telomerase-dependent telomere elongation, Okazaki fragment synthesis, and resectioning of the C-rich telomeric strand.

While telomeres are present in the majority of eukaryotes, quite a few species have evolved non-canonical mechanisms of linear chromosomal end maintenance. For example, *Drosophila melanogaster* and the silk worm *Bombyx mori* maintain their chromosomal ends using gene conversion (DNA copying) and transposition of non-LTR retrotransposons (68, 155, 193, 218). The mosquito *Anopheles*, the vector of malaria, contains complex repeats at the chromosomal ends that are maintained by recombination. A marker placed within terminal chromosomal regions is found at different locations containing nucleotide polymorphisms (219). In addition, plants in the genera *Cestrum, Sessea*, *Vestia* of Solanaceae, and *Allium* (Asparagales) are completely devoid of a predominant telomeric motifs. Instead, a complex repeating pattern at the ends of their chromosomes is thought to be produced through recombination between chromosomes (242). Furthermore, mitochondrial genomes of yeast exhibit a striking variety in chromosomal end maintenance. In *Williopsis* and *Pichia* chromosomes terminate with covalently closed single-stranded hairpin loops, where nicking of the hairpin provides the 3'OH for DNA replication and, therefore, chromosome end elongation (185). The mitochondrial DNA of the human pathogen *Candida parapsilosis* exhibits telomeres with repeats of > 700 bp with sequence divergence between the left and right telomeres.

Telomerase-dependent mechanism of telomere elongation

Telomerase origins

An ancient origin of telomerase is supported by a discovery of a putative *TERT*, the catalytic protein subunit of telomerse, in the most primitive eukaryotic species, the parasite *Giardia* (147, 178). The telomerase RNA moiety, as a core enzymatic unit for DNA synthesis, suggests that telomerase could be a ribozyme remnant from the RNA-DNA transition where association of early ribozymes with protein particles improved ribozyme function and stability (14, 28, 190). Furthermore, the human enzyme can also become an RNA-dependent RNA polymerase, which is presumed to be the oldest form of polymerase enzymes (186). Comparison of reverse transcriptase protein coding sequences from *S. cerevisiae*, *Euplotes ardiculatus*, *S. pombe*, human, *Giardia lamblia*, *T. thermophila*, chicken, and *C. elegans* revealed universally conserved motifs (1, 2, A, B', C, D, and E). Mutations in any of these motifs lead to either telomere shortening or ablation of telomerase activity (89, 136, 179, 261).

Telomerase-dependent synthesis of telomeres

Telomerase-dependent telomere extensions are carried out in late S phase where telomerase is preferentially recruited to short telomeres (247). Normally the G-rich overhang length is about 10-15 nt in length in *S. cerevisiae* and over 200 nt in humans. The yeast (but not human) overhangs increase substantially in S phase of the cell cycle (12, 46, 146, 269). Telomerase alignment with and binding of the 3' overhang is the first step in the telomere synthesis reaction. This event is followed by elongation of the overhang by DNA synthesis from the RNA template, and eventually by translocation of telomerase to repeat the cycle again (Figure 1.1).

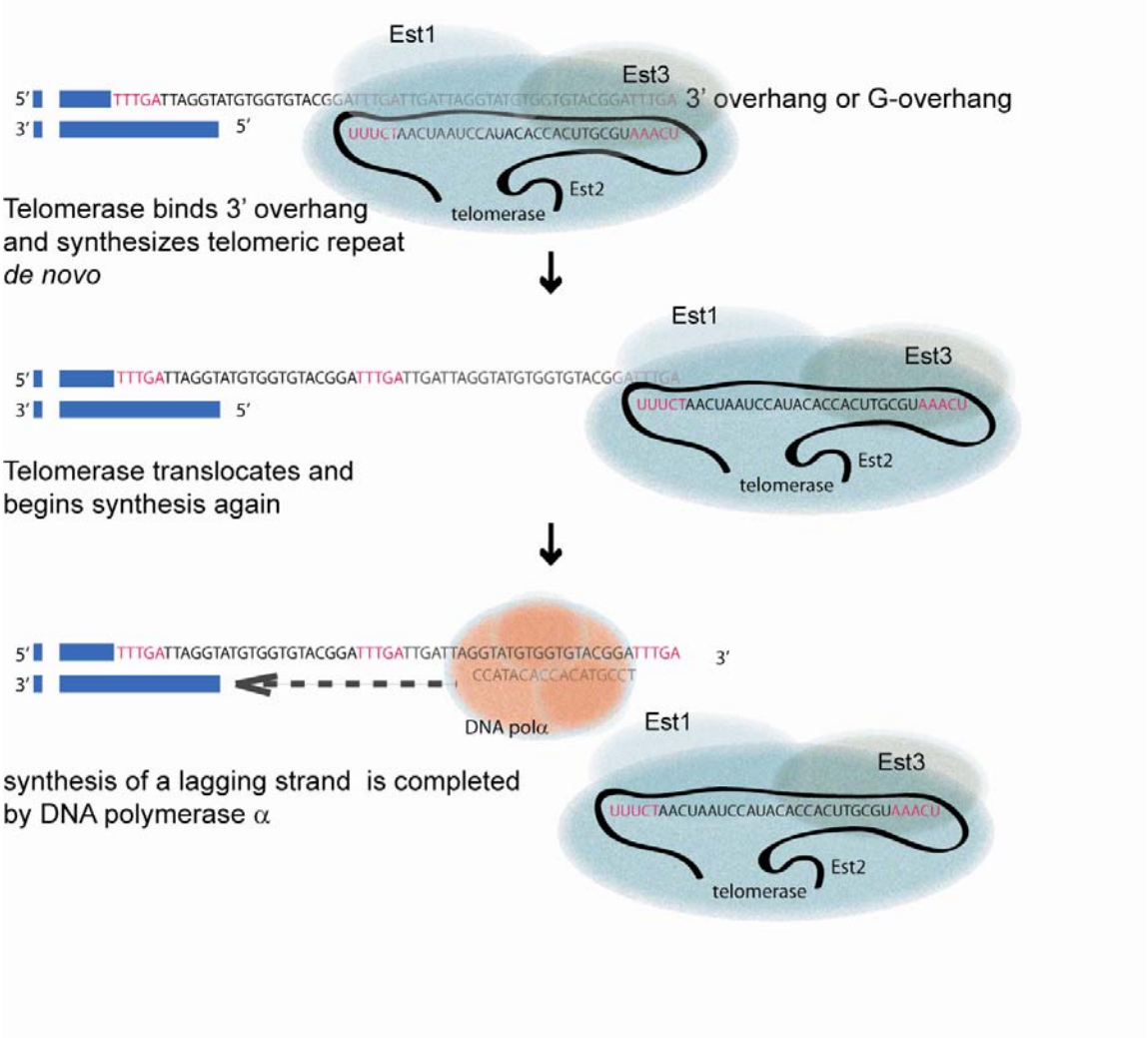

Figure 1.1. Telomere elongation by telomerase. Telomerase association with telomeres and successful rounds of synthesis and translocation lead to telomere lengthening. In yeast *S. cerevisiae* telomerase holoenzyme consists of Est1, Est2 (the catalytic unit), and Est3.

Telomerase holoenzyme in yeast is composed of RNA (the product of *TLC1* in *S. cerevisiae* and *TER1* in *K. lactis*) associated with three protein subunits (Est1, Est2, and Est3) (53). The catalytic subunit of telomerase (Est2) is found at telomeres throughout the cell cycle, but its peak accumulation occurs in late G1 and S phase (199, 232, 243) and is dependent on association of the RNA subunit with the end-binding Ku70/80 complex (11). Although the reverse transcriptase activity of telomerase is conferred by Est2, Est1 activates and recruits

telomerase to telomeres (126, 127, 135, 226). Est1 is a telomere overhang-specific binding protein which mediates interaction between the catalytic core of telomerase and telomere-binding protein and telomerase regulator Cdc13 (11, 63, 135, 137, 187, 198, 207, 243). Est2 is observed to associate with telomeres in G1 and this interaction is dependent on Ku protein (66). Est1 and Est3 are not required for catalysis *in vitro*, but deletion of any of the *EST* genes or *TLC1/TER1 in vivo* leads to telomere shortening and a growth decline also known as senescence (62, 127, 143). Est1, Est2, and Cdc13 exhibit maximal telomere saturation in S phase of the cell cycle (243).

Proper telomeric repeat synthesis requires telomerase alignment, and telomerase binding to telomeric repeats and is ensured by the template boundary elements as well as other secondary structures formed by the telomerase RNA moiety. Telomerase RNAs from ciliates, yeast, and vertebrates share four universal structural elements required for functional telomerase holoenzyme: the template, the 5' boundary element, the pseudoknot, and a long-range pairing element (255). The 5' boundary element prevents telomerase read-through past the RNA template for telomeric repeat. The *S. cerevisiae* conserved sequence motifs CS3 and CS4 of the telomerase pseudoknot are necessary for proper telomerase template usage and activity, while CS2 contains a binding site for Est1 (133). Mutations in each of these conserved sequences were shown to cause abnormalities in telomere synthesis or even to completely abolish telomerase activity.

In humans, the active telomerase holoenzyme consists of three major components: TERC (RNA component), TERT, and dyskerin (RNA-binding component). Immunoprecipitation assays identified the presence of two molecules of each component in the active telomerase complex. The proper assembly of this complex requires Cajal bodies which mediate pseudouridylation of

spliceosomal snRNAs. In addition, Cajal bodies are associated with other factors, including but not limited to Cajal body-associated protein TCAB1, pontin, and reptin. These proteins bind telomerase in a cell cycle-dependent manner and are involved in telomerase RNP assembly and trafficking of telomerase to telomeres (85, 156).

Evidence suggests that telomerase may exist as a dimeric complex in humans (262). It is unknown how and why telomerase forms dimers, but oligomerization may increase telomerase processivity through a template-switching mechanism where each telomerase unit alternates in *de novo* telomere synthesis. Alternatively, a dimeric telomerase could potentially synthesize telomeric DNA on both sister chromatids simultaneously (204).

Telomerase expression is tightly regulated. In *S. cerevisiae*, an estimated 29 telomerase RNA molecules per cell are responsible for maintenance of 32 telomeric ends. Yeast heterozygous for a telomerase deletion mutation exhibit a haplodeficiency phenotype characterized by establishment of stably short telomeres (171). These shorter telomeres are caused by changed telomere length maintenance equilibrium which may be due to the titration of telomerase subunits by mutant RNA templates. Upregulation of telomerase in cancer cells can be achieved by an increased copy number of *TERT/TERC* , but not by overexpression of the same genes. This suggests that strict regulation of telomerase expression may be an additional safeguard of genome integrity. This is further supported by observations that untimely expression of telomerase in mice leads to formation of tumors when exposed to carcinogens (273).

Telomerase is involved in processes other than telomere length homeostasis. TERT is involved in Wnt pathway signaling (194), which is important for stem cell self-renewal and cancer cell proliferation (9), as well as the prevention of neural and muscle apoptosis (224).

Telomerase also upregulates growth-promoting genes (228), downregulates apoptotic genes (55), coordinates chromatin structure, responds to DNA damage by association with telomeres in the absence of its catalytic activity (157, 222), and promotes proliferation of epidermal stem cells independent of its RNA moiety (67, 220).

Telomere binding proteins

Telomeres are a molecular catch 22. They protect a genome but at the same time they have to be protected from DNA degradation or inappropriate repair by either HR or non-homologous end joining (NHEJ) routes.

The essential Repressor Activator Protein 1 (Rap1) is a multifunctional protein found to bind yeast telomeres (Figure 1.2). Rap1 also participates in transcriptional regulation of many genes and is involved in mating type silencing (118). Additionally, Rap1 binds to double-stranded telomeric DNA and exerts both negative and positive regulation of telomere length (43, 114). Its association with telomeres is cell-cycle regulated with Rap1 maximal accumulation occurring in S/G2 phase. The degree of telomere saturation by Rap1 molecules has an inverse correlation with telomere length (76, 116, 120, 140, 151, 210, 218). Investigations into the structure of Rap1 indicated that it contains three domains: a Rap1 C-terminal (RCT) domain, a central MYB domain, and a non-essential N-terminal BRCT domain (26, 34, 246). The *S. cerevisiae* RCT domain enables Rap1 interaction with Rap1-associated factors (Rif1 and Rif2) as well as silent information regulator (Sir3 and Sir4) proteins. Together with Rif1 and Rif2, Rap1 negatively regulates telomere lengthening by a mechanism which is not well understood (129, 151).

Together with Sir3 and Sir4, Rap1 facilitates silencing near telomere regions shown to be important for adaptation and evolution of yeast species (37). The central domain consists of two linked homeodomains which each bind one half of a telomeric repeat each (65). Mutations within the binding site of these domains destabilize telomere length regulation to various degrees in *K. lactis* as well (86, 158). The Rap1 central domain together with Rif2 and Sir4 prevents telomere-telomere fusions (152, 192). These telomere fusions result from NHEJ repair, and deletions of NHEJ players ScKu70/80, Mre11 and Lig4 rescue certain rap1 mutants (192). Rap/Rif2-dependent inhibition of NHEJ is thought to be dependent on Rif2 competition with Tel1 for Xrs2 (92). Additionally, evidence suggests that Rap1 also blocks recruitment of a checkpoint kinase Mec1/Ddc2, MRX complex, and Rad52 to telomeres (111, 182).

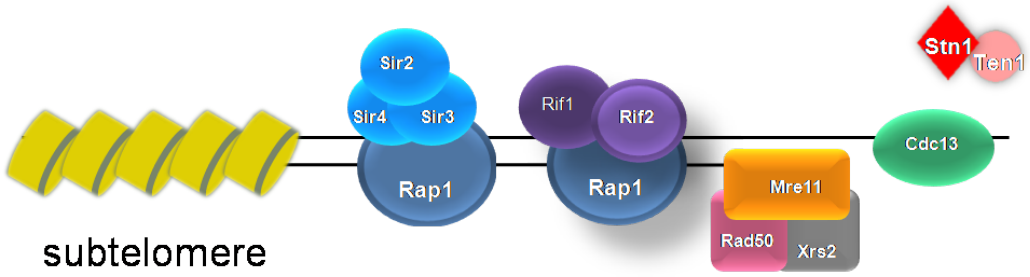

Figure 1.2. Telomere specific binding proteins in *S. cerevisiae.* Telomere protection against repair or degradation is conferred by telomere association with telomere specific double- and single-stranded DNA-binding proteins.

ScCdc13 is a G-overhang binding protein (99, 134, 167, 187). Just like Rap1, Cdc13 is both a positive and a negative regulator of telomere length (32, 71, 72, 87, 206). These two functions of Cdc13 have been genetically separated to different Cdc13 domains. The *S. cerevisiae* Cdc13 protein consists of 4 domains: an N-terminal domain; a recruitment domain (RD) interacting with Est1 (positive telomere length regulator); an OB-fold containing DNA

binding domain (DBD) and a C-terminal domain responsible for negative telomere length regulation (32, 97, 99, 198, 244). Positive and negative regulation of telomere length is ensured by competitive binding of Stn1 and Est1 (32). As a positive regulator of telomere length, Cdc13 recruits telomerase by interaction with Est1 upon its phosphorylation by Tel1/Mec1 or cyclin-dependent kinase Cdk1 (11, 63, 131, 198, 252). Phosphorylation of Cdc13 by Cdk1 also blocks recruitment of the negative telomere length regulators, Stn1 and Ten1 (79, 82, 131, 206, 243). Binding of Est1 by Cdc13 is abolished by the cdc13-2 missense mutation and leads to senescence (187). A deletion spanning several hundred terminal amino acids (*cdc13-5* mutation) leads to formation of long 3' overhangs as a result of compromised C-strand synthesis in response to telomerase-dependent telomere elongation (32).

Stn1 together with Cdc13 and Ten1 forms an essential capping complex of OB-fold-containing proteins known as the CST complex. Together they participate both in telomere capping and in telomere length regulation (80, 198). Stn1 was initially identified as a suppressor of cdc13 deficiency in yeast. *S. cerevisiae STN1* deletion mutants activate G2/M cell cycle arrest and become unviable (82). The N-terminus of Stn1 contains a Ten1 binding domain, while the C-terminus of Stn1 interacts with Pol12, a regulatory subunit of polymerase α-primase (196). As a capping protein, Stn1 also prevents recombination at telomeres. A mutation in the N-terminal domain of *K. lactis* Stn1 causes telomere instability and telomere hyperrecombination (106).

Components of the CST complex have been found in other species such as *Arabidopsis* suggesting that the role of OB-fold proteins may be evolutionarily conserved (230). A human Stn1 homolog, OBFC1, also binds single-stranded telomeric DNA and interacts with another telomere protein TPP1 (259). Ten1 is an essential protein identified as a dosage suppressor of the *stn1-13* mutation in *S. cerevisiae* causing change in telomere length (81). Overexpression of

Ten1 and the essential domain of Stn1 bypasses the requirement for Cdc13 in *S. cerevisiae* (206). At this time it is unknown whether *K. lactis* has a homolog of Ten1.

Nucleases and repair proteins are the least expected entities to be found at telomeres in their normal state; however, many repair proteins not only associate but are required for proper telomere length maintenance. Telomeres are bound by repair proteins from the both NHEJ and HR repair pathways. In *S. cerevisiae,* the Ku heterodimer is involved in regulation of the 3' overhang length as well as in the positive regulation of telomere length (21, 33, 106, 153). It interacts with telomerase RNA (54, 79, 232) acting as a positive enhancer of its interaction with telomeres. When Ku is deleted in *S. cerevisiae,* telomeres become vulnerable to shortening and exhibit elongation of their G-rich overhangs (22, 83, 203). Loss of Ku80 in *K. lactis* did not affect telomere length maintenance, but caused longer overhangs similarly to *S. cerevisiae* and more frequent subtelomeric recombination (27).

The yeast MRX complex consists of Mre11, Rad50, and Xrs2 (117) and is by far the most multifaceted protein complex associated with telomeres. It is implicated in DNA damage signaling, DNA replication, DNA repair, meiosis and mitosis, and telomere maintenance (121). Mre11 is a dimer acting as 3'-5' exo- and endonuclease on single- and double-stranded DNA substrates (197). Mre11 is a highly conserved protein and contains an N-terminal Mn^{2+}/Mg^{2+} phosphodiesterase domain and two DNA-binding domains located at the C-terminus (45, 267). This protein has been implicated in resection of 5' end of DSBs during repair although it appears to lack the actual 5'-3' exonucleolytic activity. It is possible that the polarity of Mre11 is altered by one of its binding partners. Mre11, however, is not the only nuclease acting at telomeric ends. Exo1 and Dna2 were shown to contribute to telomere resection after initial MRX action (64, 96). Mutants that bear a deletion of Mre11 or a double mutation of any of the MRX complex

members with ScTel1 exhibit very short telomeres (216). In addition, deletion of *mre11* causes compromised maintenance of telomeric overhang, but does not affect extension/resection of overhangs in S-phase-dependent telomere replication (122).

Rad50 belongs to the structural maintenance of chromosome (SMC) family of proteins. Rad50 is important for telomerase-dependent telomere elongation as indicated by telomere shortening in *S. cerevisiae rad50* deletion mutant (93, 266). Rad50's Walker A and Walker B motifs, present on polar sides of the protein molecule, associate into a structure with affinity for double-stranded DNA. Rad50 also has weak helicase activity and zinc-hook formation for interaction with another Rad50 molecule. The weak ATPase activity of Rad50 facilitates clustering of MRX at DSB and increases interactions between DNA molecules involved in DNA repair (95). It has been shown that ATP hydrolysis as a result of ATP association with the ATP-binding cassette (ABC)-type ATPase domain (94, 95) is not sufficient to induce helicase activity of this complex but may rather mediate affinity of MRX for dsDNA and therefore control the frequency and length of its association with DNA. While Mre11 is thought to mediate short-range DNA synapsis, Rad50 zinc-hook structures, joined by coiled-coils, can connect DNA ends ~1200 angstroms apart. These coiled coils allow enough flexibility to permit close positioning of DNA ends for repair (93). Rad50 is important for telomerase-dependent telomere elongation as indicated by shortened telomeres in an *S. cerevisiae rad50* deletion mutant (93, 266).

Xrs2 is poorly conserved among eukaryotes. The N-terminus of this protein has FHA and BRCT domains and a Mre11-interaction domain at the C-terminus. The FHA domain is necessary for phosphopeptide-specific protein-protein interactions in response to DNA damage signaling (45, 57). The FHA domain in Nbs1, the human homolog of Xrs2, interacts with the phosphorylated domain of the checkpoint mediator Mdc1 (homolog of ScRad9) and the nuclease

Ctp1 (homolog of ScSae2). Interaction between Nbs1 and repair/checkpoint proteins is required for proper repair responses at telomeres. Nbs1 is also responsible for transport of the MRN complex into the nucleus, and this function is dependent on the Mre11-binding domain of Nbs1 located at the C-terminus (52, 138, 265).The MRX complex was also found to bind G4-DNA and the non-Watson-Crick DNA pairing at the G-rich 3'-overhangs (73, 221). While mutations in MRX are tolerated by yeast, various hypomorphic mutations in higher vertebrates including humans lead to deleterious effects including predisposition to cancer and premature ageing (6).

Surprisingly, the signaling PI-3-kinase-related protein kinases, Tel1 and Mec1, are also found at telomeres but are recruited to telomeres in a mutually exclusive manner (245). The significance of this recruitment pattern is not fully understood, but it appears that ScTel1 and ScMec1 are involved in somewhat redundant pathways of telomere maintenance. Deletion of ScTel1 leads to stably short telomeres without loss of cell viability (145). The deletion of both ScTel1 and ScMec1 leads to telomere loss and growth senescence similar to that seen in telomerase deletion mutants (215). The Blackburn lab (31) found that a telomerase deletion *tel1* double mutant exhibit telomere-DSB fusions, suggesting that Tel1 functions not only in telomere elongation, but also participates in telomere protection against NHEJ-dependent fusions. ScTel1 recruitment to telomeres is thought to be promoted by the ScMRX (Mre11/Rad50/Xrs2) complex (176).

Another class of proteins found to associate with telomeres is RecQ helicases. They are conserved from bacteria to humans, and play an important role in genome integrity. Only one nucleolar DNA helicase, Sgs1, is present in *S. cerevisiae* and *K. lactis* (70). The RecQ helicase in yeast, ScSgs1 participates in genomic recombination (272), restart of stalled replication forks, suppression of recombination between homeologous substrates of aberrant BIR-intermediates in

mitosis and meiosis (3). Sgs1 helicase activity is dependent on ATP hydrolysis. In *S. cerevisiae*, Sgs1 interacts with the 3'-5' exonuclease Mre11 and recombination factor Rad51 (35). In the absence of telomerase, Sgs1 is recruited to telomeres during generation of survivors with long telomeric tracts (38, 109). RecQ helicase is structurally similar to BLM and WRN in humans (4).

Telomeres and human health

Senescence

In most human cells telomerase has a low or undetectable level of expression (13). Critical shortening of telomeres normally leads to a state of permanent cell cycle arrest also known as replicative senescence, it is induced by activation of p53 and Rb tumor suppression pathways (223). Cellular senescence is distinct from replicative senescence and is triggered by factors other than short telomeres such as activation of oncogenes or cell damage by cytotoxic drugs or genotoxic stress (88, 212). *In vitro*, senescent cells exhibit several hallmark phenotypes: they arrest in the G1 stage of a cell cycle, develop resistance to apoptosis (programmed cell death), attain flattened cell morphology, and at pH=6 stain positive for β-galactosidase activity. *In vivo*, senescence can be detected by positive β-galactosidase activity and by induction of telomere dysfunction-induced foci (TIFs) caused by DNA damage response proteins aggregating at critically shortened telomeres.

Short telomeres have a positive correlation with stress in women (59), and increased β-galactosidase activity and TIFs are linked to cell ageing. The extent to which senescence alone affects the onset of ageing is debatable. Studies of age-related pathologies did not find a high positive correlation between age-related pathologies and senescence. The issue is complicated by

the fact that cells from various organs undergo senescence in culture, but the actual rate of senescence in living organisms still remains unknown (166, 212).

Senescence has been suggested to be a powerful tumor suppression mechanism that is advantageous to an organism during its peak reproductive period (205), but senescent cells can also act as pro-tumorigenic factors (212). Persistent DNA damage signaling triggers secretion of chemokine and cytokine factors that generate reactive oxygen species and activate p53-dependent cell cycle arrest in the neighboring cells. This phenomenon is also known as Senescence-Associated Secretory Phenotype (SASP) (44). People with chronic inflammatory condition known as Barrett's esophagus are more prone to develop esophageal cancer possibly due to persistent cell exposure to inflammatory factors and oxidative stress (214). An interesting alternative hypothesis about the role of senescence in eukaryotic cells was proposed by Roger Reddel, who argues that a tumor suppressive quality of senescence is a by-product of an ancient antiviral mechanism (212). According to this hypothesis, senescence is advantageous as a response to abnormal activation of cell proliferation and β-interferon signaling as a result of viral infection. The SASP response in one cell will trigger senescence in the neighboring cells which are most likely to be infected by the same virus as well. This hypothesis is supported by the observation that viruses have developed a number of mechanisms designed to overcome senescence, apoptosis (programmed cell death), and immune responses of a host (212).

Disorders attributed to dysfunctions in telomere-binding proteins

Telomere dysfunction leads to a number of human diseases. Dysfunctions in telomerase lead to Zinsser-Cole-Engman syndrome or Dyskeratosis Congenita (DC or DKC), characterized by various somatic abnormalities including bone-marrow failure, abnormal skin pigmentation,

nail dystrophy, acquired aplastic anemia, pulmonary fibrosis, and liver disease, etc. (25). DC is a genetically inherited disorder that has several genetic subtypes: X-linked, autosomal dominant and autosomal recessive. The causes of autosomal recessive DC are unknown. X-linked DC stems from mutations in *DKC1* encoding a nucleolar protein Dyskerin, which is a part of telomerase catalytic holoenzyme (113). Haplodeficiency in telomerase RNA (TERC) in diploid cells causes the autosomal dominant form of DC. Because Dyskerin and TERC are crucial in telomerase function, all people with X-linked and autosomal dominant DC exhibit short telomeres, high rates of genomic instability, and predisposition to cancer. In addition, DC symptoms of various severities are also observed in people with homozygous mutations in NOP10 and NHP2, proteins necessary for proper telomerase function.

Mutations in telomere-binding proteins Mre11 and ATM lead to the onset of premature ageing syndrome known as Ataxia Telangiectasia (AT) (162). Patients with this disorder have short telomeres, high levels of genomic instability, increased occurrence of tumorigenesis, neurodegeneration, premature ageing, immunodeficiency, and high sensitivity to DNA damage (268). AT patients have dysfunction in ATM-dependent DNA damage signaling pathways leading to accumulation of unrepaired DNA lesions that trigger destabilization of the whole genome. A mutation in Rad50 is reported only in a single individual, and it causes symptoms similar to AT.

Human homologs of RecQ helicases, BLM and WRN, are important players in genome stability and cancer development at the same time. When mutated, they cause Bloom's syndrome (congenital telangiectatic erythema) and Werner's syndrome. These diseases are associated with higher rates of chromosomal aberrations, slow growth, and increased chance of cancer. In addition, people with Werner's syndrome exhibit signs of premature ageing (19).

An active mechanism of telomere maintenance is required for development of cancer

Replicative senescence can be bypassed if favorable mutations occur in cell cycle arrest pathways. Human cells that bypass senescence undergo significant genetic and epigenetic changes, critical telomere shortening, chromosomal damage, and genome rearrangements. Only a few cells, approximately 1 x 10^{-7}, escape mortality after bypass of senescence (211). All such survivors exhibit significant genomic alterations coupled with activation of a telomere maintenance pathway (40, 213). Activation of telomerase occurs in ~85% of cancers, including melanomas, lymphomas, neuroblastomas, leukemia, and various carcinomas. Detection of high levels of telomerase expression is normally linked to a poor prognosis (212). However, expression of telomerase per se does not induce malignant transformation (108), but rather is required to sustain cancer proliferation (102).

A subset of cancers, prevalently originating from mesenchyme and central nervous system tissues, utilizes an HR-dependent mechanism also called Alternative Lengthening of Telomeres (ALT). While ALT cancers comprise only about 15% of all known cancers, they are the most intriguing. For telomere elongation these cancers utilize some of the protein factors required for DNA repair in normal cells. ALT cancers exhibit telomeres that are more variable in length than those of telomerase-positive cancers or germ cells (2, 50, 90). These cancers also undergo frequent telomere sister chromatid exchange (T-SCE) (18). It is not clear how unequal T-SCE can contribute to telomere elongation. T-SCE may contribute to the telomere length heterogeneity visualized in ALT cells. ALT cells can elongate telomeres via intra- and inter-chromosome recombination (56, 173). The ALT-phenotype is also defined by the presence of extrachromosomal linear and circular telomeric DNA (t-circles) (260). An accurate determinant of the ALT phenotype is enrichment of cancerous cells for C-stand single-stranded t-circles

where thousands of C-strand t-circles can be contained in one ALT cell (90). T-circles can form upon aberrant resolution of t-loops leading to shortened telomeres. It has been demonstrated that t-loop stabilization mainly depends on the telomere-binding protein TRF2 and that NBS1- and XRCC3-dependent resolution of t-loops yields t-circles that can be double stranded, partially double-stranded, or single-stranded (42, 260).

Circular and linear DNA is sequestered in G2-dependent ALT-associated promyelocytic leukemia nuclear bodies (APBs) (213). PML bodies are regularly found in normal cells, and they were demonstrated to participate in DNA repair, apoptosis, proteosome degradation, tumor suppression, regulation of transcription, and response to viral infection. PML bodies in ALT cells, however, are distinct from those found in normal cells. In addition to circular and linear telomeric DNA, they also contain DNA repair proteins and telomere-specific binding proteins, TRF1, TRF2, MRN, ERCC1, XPF, 9-1-1 complex, BLM, WRN, BRCA1, RAD51, RAD52, and RPA (51, 91). Interestingly, NBS1 is required for APB formation as demonstrated by the observation that overexpression of the core PML protein with affinity to NBS1, Sp100, sequesters MRN complex and subsequently inhibits ALT (107).

Mechanisms of homologous recombination

There are various exogenous and endogenous agents that can cause DNA damage or even breakage. Certain wavelengths of radiation, such as gamma rays, X-rays, and UV-B and UV-C rays, are particularly harmful to DNA. Other sources of DNA damage include aflatoxins, various naturally and artificially produced chemical agents, erroneous DNA replication, oxygen reactive species produced by oxidative metabolism, or inflammation responses. These and other agents can cause deamination of nucleotides, crosslinks between nucleotides within the same DNA

strand or between different DNA strands, DNA nicks, and DSBs. DSBs are the most severe form of DNA damage and can be repaired by NHEJ or HR. NHEJ is the preferred pathway of DNA repair in humans and is thought to be a consequence of large genomes containing a high frequency of short microhomologies that can potentially initiate recombination between unrelated sequences. Consequently, in smaller genomes such as yeast, HR is more often the most prevalent pathway of DSB repair.

One of the first observations of homologous recombination at work was made in the early 1900s by Thomas Morgan who postulated that linked genes in *Drosophila* can undergo crossovers leading to changes in their inheritance pattern in the progeny (169). In meiosis the majority of DNA breaks are generated purposefully to create allelic crossovers for genetic variation within species. A model for the mitotic recombination was developed by Stern in order to explain a *Drosophila* mosaic eye phenotype, which is caused by recombination between linked recessive and dominant alleles (233). In addition to being involved in genetic variance in somatic cells and germ cells, recombination also is an essential player in genetic variability, development of immune responses, and cell viability.

Mitotic HR is distinct from the meiotic form because on most occasions it leads to non-crossover events (Figure 1.3). Evidence indicates that 5' end resectioning of DSBs may be a major trigger of HR, although there seems to be species-dependent regulation of NHEJ/HR rates. Delayed resectioning of DNA ends in human cells may allow plenty of time for NHEJ-dependent repair. However, in yeasts, which utilize mostly HR for DSB repair, only a moderate increase in the rate of NHEJ-dependent repair is observed in a background of mutations compromising HR (103, 256)

Mitotic recombination can be subdivided into synthesis-dependent strand annealing (SDSA), single-strand annealing (SSA), and break-induced replication (BIR) (117, 161). All types of recombination, whether it is meiotic or mitotic, share four major steps. These steps, however, have distinct genetic requirements and produce distinct DNA products. The first step is DSB sensing and repair response activation. The second step involves resectioning of 5' DNA to generate 3' overhangs sufficient for invasion of homologous sequence. The third step is the strand invasion of the donor sequence and new DNA synthesis. The final step involves resolution of repaired DNA containing recombinational structures through generation of crossover or non-crossover products.

Rad52 is the most important recombination factor for both meiotic and mitotic DSB repair in yeast. The Rad52 protein forms a peculiar multimeric protein ring with a large central channel (263). The full crystal structure of this protein is yet to be obtained, but studies of human *RAD52* demonstrate the existence of a deep groove running around the outside of the ring structure. The groove is made up of residues that are highly conserved across species, and it is this groove that is believed to bind ssDNA. The opening of the groove is too small to fit dsDNA, and therefore pairing of ssDNA with its complementary strand may cause Rad52 dissociation (195). Rad52 can recruit recombination factors or directly participate in ssDNA annealing in the Single Strand Annealing (SSA) pathway (for a of recombination proteins and their functions, see Table 1.2).

The Ku heterodimer and the MRX complex are the first entities to associate with a DSB sites (36). Ku is thought to form a ring structure around both broken ends (47). The multifunctional MRX may retain broken ends in close proximity aiding DNA repair. MRX initiates HR repair by recruitment of Tel1 (ATM) through the N-terminus of Xrs2 (Nbs1 in

vertebrates) (176, 177). Defects in components of the MRX complex lead to increased rates of spontaneous recombination thought to be due to defective DNA damage signaling and repair. Deletion mutants of MRX in *S. cerevisiae* exhibit retarded resection of HO-induced DSBs at the *MAT* locus, hypersensitivity to DNA-damaging agents, and defective meiosis, but do not obstruct mating type switching (105, 236). Nuclease-deficient mutants of Mre11 (*mre11-nd*) and *rad50S* are impaired in processing of hairpin structures and demonstrate sensitivity to IR and MMS similar to an *mre11* null allele. These results suggest that the integrity of the MRX is more important in processing of "bulky" DNA ends generated by IR or MMS than "clean" DSBs produced by endonuclease induction such as occur during mating type switching. Deletion of Mre11 does not dramatically affect resectioning of DSBs or DNA repair, suggesting the presence of other nucleases working cooperatively with the MRX complex.

Sae2 (ChIP in humans) is an additional nuclease participating in resectioning of DSBs (23, 100, 110). Sae2 exhibits several nucleolytic activities including endonucleolytic activity with preference for ss and ss/dsDNA transitions and ssDNA next to hairpin structures (128). Accumulation of unresolved recombination structures and large duplications between inverted repeats was observed in *sae2* mutants implicating its role in resolution of structures formed by resection (208). Such endonucleolytic processing of these hairpin structures is done by the MRX complex which interacts with Sae2 probably through associations with DNA at the DSB loci (112, 128). Sae2 recruitment to DSB releases Mre11 from DNA, while simultaneously facilitating recruitment of Rad52. In the absence of Sae2, Mre11 displacement from DNA is delayed, which indicates that the Mre11-Sae2 connection is an important factor at the crossroads between DSB signaling, resection, and initiation of recombination.

In addition to MRX and Sae2, Exo1, a 5'-3' exonuclease, also participates in DNA resection. Overexpression of Exo1 can overcome some repair deficiencies of *mrx* mutants in DNA resection (168). However, similarly to Sae2, deletion of Exo1 leads to a decrease but not negation of resection. Double deletion mutants of *exo1* with *sae2* exhibit increased sensitivity to DNA damaging agents (150). Therefore, Exo1 can replace the MRX complex to some extent, but an additional nuclease resects DNA ends in *exo1 mre11* and *exo1 sae2* double mutants (130, 150, 165, 168). This resection is carried out by the RecQ helicase Sgs1 acting together with ss-specific nuclease and helicase Dna2. The triple mutant *sae2 exo1 sgs1* is unable to resect DNA (165).

Interestingly, only the nuclease but not the helicase activity of Dna2 is required for DNA-end resection (274). Together, the MRX complex and Sae2 create short resectioning of the 5' DNA end while Exo1 and Sgs1/Dna2 are required for longer processive resectioning of the end. Inactivation of Sgs1 and Exo1 completely abrogates repair through the SSA mechanism (164). DNA resection, like DNA repair more generally, can only be effective in the restoration of the integrity of genetic information if initiated and regulated appropriately. Mitotic DSB repair occurs in the S and G2 phases of the cell cycle, and requires the cell cycle kinase Cdk1 (103). Checkpoint proteins Rad9 and Sae2 are targets of Cdk1. Inactivation of Rad9 increases DNA resection even in the absence of active CDKs, suggesting that Rad9 blocks inappropriate DNA-end resection (123). Sae2 is phosphorylated by Cdk1 on Ser267. Mutagenesis of this residue leads to decreased DNA resection, delayed HR, and an increase in NHEJ (98). When the same residue is modified to contain constitutively phosphorylated Ser, a *sae2* mutant resects in the absence of activated CDKs in the G1 phase.

Table 1.2. Recombinational proteins of *S. cerevisiae.*

Protein	mitotic HR	Function in HR	References
Rad52	All types of HR	Required for all HR; anneals ss DNA *in vitro*; interacts with RPA; recruits Rad51; promotes intra- and to some extent inter-molecular strand invasion; formation of D-loop in humans;	(7, 117, 229, 239)
Rad51	Gene conversion, spontaneous recombination, SDSA, BIR, RTE	Strand invasion in DNA repair Forms right-handed helical filament on ss- and ds-stranded DNA; Homology search;	(105, 209, 241, 254)
Rad54	SDSA, BIR, RTE	Interacts with Rad51; member of Swi2/Snf2 family; promotes chromatin remodeling; DNA unwinding, and strand annealing between donor and recipient DNA;	
Rad55/57	SDSA, BIR, RTE	Rad51 paralogs; mediate Rad51 binding and RPA displacement;	(117)
MRX complex	SDSA, BIR, RTE, NHEJ	DSB processing in DNA repair **Mre11, dimer:** 3'-5' exonuclease; exonuclease at circular and linear ssDNA; endonuclease at hairpin and 3' ssDNA at ss/ds DNA junction **Rad50, dimer:** SMC family, intra and inter DNA tethering **Xrs2:** Found only in eukaryotes, phosphorylated to activate checkpoint	(117, 235)
Srs2	SDSA	DNA helicase; reverses abnormal recombination intermediates by displacement of Rad51 *in vitro*;	(117, 217)
Rad59	SSA, BIR, RTE	Interacts with Rad52 and self; Recombination between direct repeast, especially with short homologies; spontaneous recombination between homeologous sequences; binds to ss and ds DNA; anneals ssDNA *in vitro*	(117, 231, 237)
Sgs1	DSBR, SDSA, RTE	Stalled replication fork restart, RTE	(4)

Processive DNA resection further creates RPA-coated ssDNA filament, and accumulation of ssDNA triggers Tel1 replacement by Mec1 (275) and initiation of cell cycle arrest (139). Upon Rad52 binding to ssDNA coated by single-strand binding protein (RPA), Rad52 facilitates recruitment of Rad51 and displacement of RPA (191). Rad51 is a universally conserved recombinase which forms right-handed nucleoprotein filaments on ssDNA (263). Rad51 has strong binding specificity to dsDNA and weakly binds ssDNA. The latter is greatly induced by Rad51 association with Rad52. It is the interaction with Rad52 that promotes strand invasion of a donor DNA molecule by a Rad51 filament (170, 201). Two Rad51 paralogs, Rad55 and Rad57, form a heterodimer which promotes Rad51-dependent reactions (240). Phosphorylation of Rad55 is required for efficient HR at stalled replication forks. While both Rad51 and Rad52 are required for spontaneous recombination, Rad55/57 are dismissible in interchromosomal DSB repair by gene conversion (202). However, Rad55 and Rad57 are more important in DNA repair at cold temperatures (240), and overexpression of Rad51 compensates for Rad55 and Rad57 deficiency (191). Rad51 filament invasion of a donor sequence is further promoted by Rad54, a protein from Swi2/Snf2 chromatin remodeling protein family. Homologous DNA pairing induces DNA-dependent ATPase activity of Rad54 and facilitates Rad51-mediated stand exchange *in vitro* (200).

Furthermore, Rad52, Rad59, and the MRX complex are important for the Rad51-independent mechanism of DNA repair. The Rad59 protein shares N-terminal domain sequence similarity with Rad52 (270). While Rad52 is required for all forms of recombination repair, Rad59 is dispensable for Rad51-dependent DNA repair but is required for certain types of HR including spontaneous recombination between repeated sequences via SSA, Rad51-independent BIR, and Type II RTE in yeast (189, 270). Rad59 binds Rad52 but not Rad51 *in vitro,* anneals

complementary ssDNA, and similarly to Rad52 has affinity to both ss- and dsDNA (48, 270). In addition, Rad59 acts in conjunction with the RSC (remodel structure of chromatin) complex to facilitate sister chromatid recombination (189).

Recombination has been studied in great detail in *S. cerevisiae*. During Synthesis-Dependent Strand Annealing repair (SDSA), a 3' DNA end of a DNA strand invades a donor DNA molecule (either a sister chromatid or less often, a homologous chromosome) (Figure 1.3). Strand invasion of a donor by a recipient molecule creates a D-loop. Rad52 and Rad51 epistasis group proteins facilitate the unwinding and invasion of the donor DNA helix, while DNA polymerase ε, DNA polymerase δ, and its processivity factor PCNA facilitate DNA synthesis. The invaded strand and its extended end are then unwound from the donor duplex where it can then anneal to the 3' tail of the other half of the DSB. Most SDSA events do not produce Holliday junctions (41). The reaction is finished by strand ligation and gap repair without generation of a crossover. In cells with a "clean" HO-induced DSB, SDSA was decreased 100-fold upon a *RAD52* deletion. Deletion of Rad51-group proteins decreased efficiency of repair to some extent, while *rad59* and *rad50* mutants remained SDSA proficient (148). SDSA can completely restore DNA integrity, or generate expansions or deletions in a recipient molecule due to shifted re-annealing after DNA synthesis and cleavage of non-homologous tails.

In an alternative model of DSB repair, DNA synthesis during repair can induce branch migration and capture of the other 3'end of a DSB in a DNA synthesis reaction. As a result, a structure known as a Holliday junction (HJ) is formed. Resolution of HJs in either the same or the opposite direction produces non-crossover or crossover DNA products. Crossover products predominantly occur in meiosis (180, 257). In prokaryotes HJs are resolved by the RuvABC resolvase complex that preferentially binds to and nicks four- and three-stranded DNA junctions

producing two independent double-stranded molecules. Homology searches did not reveal orthologs of resolvase RuvC in eukaryotes; however, several functionally similar factors were found in yeast. Cce1 and Ydc2 are *S. cerevisiae* and *S. pombe* mitochondrial HJ resolvases, respectively (132).

Another Rad52-dependent repair mechanism is the SSA pathway. It requires the strand annealing proteins Rad52 and Rad59, the DNA sequestering and DNA resectioning MRX complex (225), and the Rad1/Rad10 endonuclease complex (104). SSA utilizes direct repeats within the DNA molecule where DSB occurred (117). Repair through this pathway leads to loss of genetic information because single-stranded 3' ends that do not find homology upon strand annealing are removed by Rad1/10 endonuclease (1, 104).

Break-induced replication (BIR) is a Rad52-dependent pathway occurring through formation of a replication fork that generates a non-reciprocal translocation. BIR occurs occasionally during spontaneous mitotic recombination in yeast (60, 61), during re-start of broken or stalled replication forks, and during DSB repair when only one end is available (159). Some BIR events can extend for up to several thousand kb down the chromosome arm in *S. cerevisiae* (20, 60, 75). Mutants missing functional *RAD52* are essentially incapable of undergoing DNA repair via BIR. BIR can occur via several molecular mechanisms. A D-loop established at a point of strand invasion can prime DNA synthesis and then branch-migrate to gradually displace a newly formed DNA. Alternatively, a D-loop may initiate a uni-directional replication fork leading to semi-conservative DNA replication and resolution of a HJ. Finally, establishment of a uni-directional replication fork can also be accompanied by a displacement of both leading and lagging DNA strands (159).

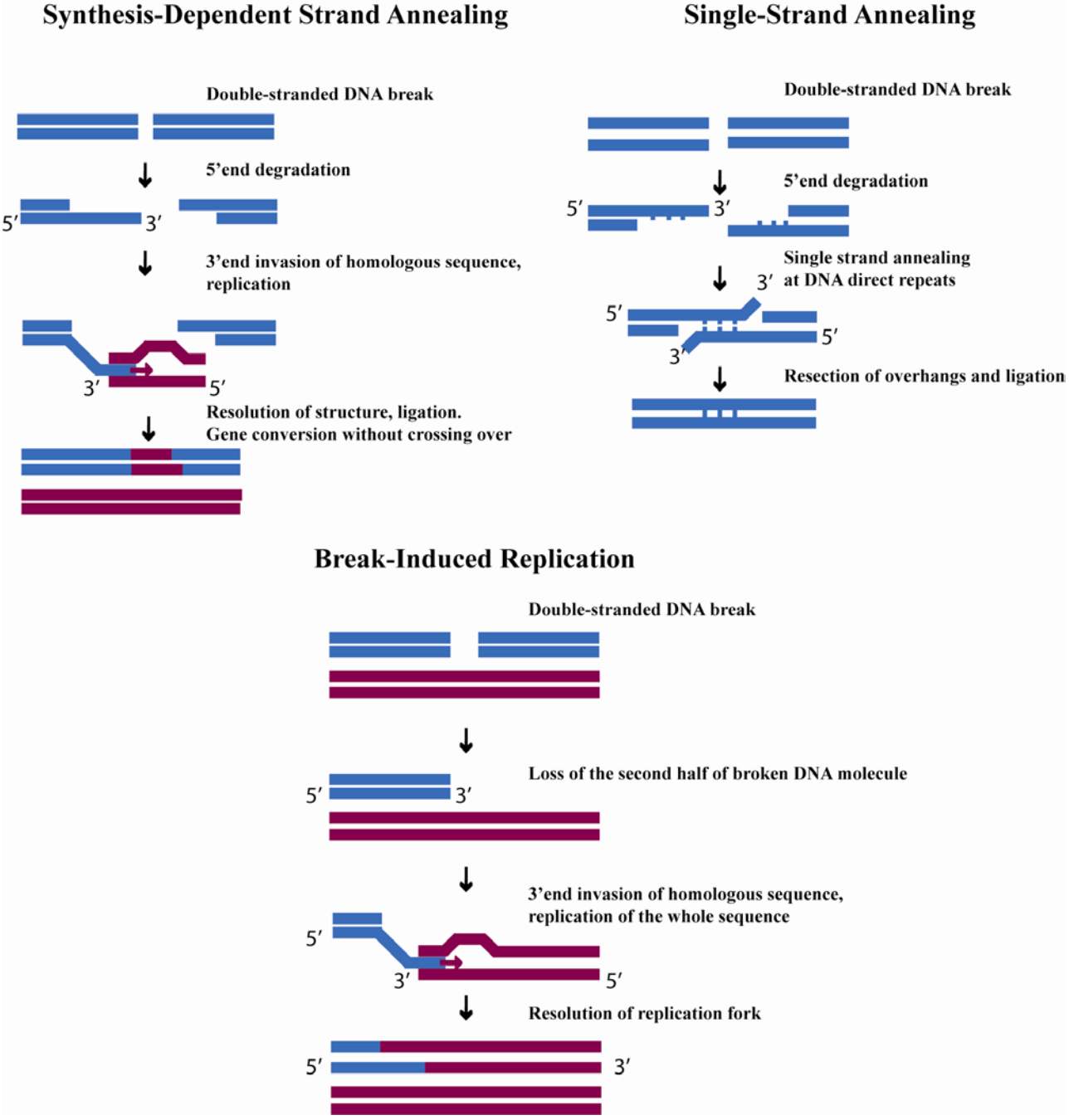

Figure 1.3. Pathways of DNA repair. The initial step of SDSA, SSA, and BIR pathways is resection of 5' DNA ends to create 3' single-stranded overhangs able to invade a donor sequence (SDSA and BIR) or to find exposed sequence from another DNA direct repeat near the same DNA molecule (SSA). Repair through SDSA leads to non-reciprocal gene conversion. SSA normally leads to loss of DNA between the two participating direct repeats due to resection and overhang cleavage. During BIR, the second part of a broken DNA molecule is lost and replication proceeds to the ends of the donor DNA molecule.

BIR-dependent repair of an HO-cleaved chromosome arm is diminished in yeast *rad51* mutants. However, some cells can initiate repair through *RAD51*-independent BIR, which involves *RAD50*, *RAD59*, MRX, and *TID1* (225). The extent of homologous sequence available for recombination seems to be a determining role in BIR choice. DNA ends with short homology (less than 70-100 bp) are repaired by *RAD51*-independent BIR, which can require a particular upstream BIR-promoting element (enhancer site) to promote formation of a processive replication fork capable of extending for more than several kilobases at the same time (149). *RAD51*-dependent BIR, on the other hand, requires more homology and is more efficient (159).

While most DSB repair events are initiated within several hours in yeast, BIR takes approximately three times longer. It is thought that in Rad51-dependent BIR strand invasion occurs via a mechanism similar to that occurring during repair of a DSB flanked by homologous sequences. BIR accounts for only a small percentage of repair events of DSBs. Experimental evidence suggests that DNA synthesis is delayed in BIR, but not in repair through gene conversion (149). It is possible that the default cellular function is to search for homology on both sides of a DSB to ensure chromosomal sequence preservation, and therefore one-sided DNA synthesis. BIR is somehow delayed to ensure proper reunion of broken ends. Alternatively, it is possible that BIR events are initiated and are carried out but only involve extensive DNA copying if the second broken end cannot be located. Consistent with this model, Smith et al. (227) demonstrated that a broken chromosome III undergoes limited DNA synthesis as indicated by pieces of sequence acquired from homologous and ectopic chromosome sites. Such limited DNA synthesis and dissociation is thought to allow SDSA to be completed if a second DNA end is found. However, in the case when a second broken end cannot be located and strand annealing

cannot be completed, kinetics of the reaction is shifted towards BIR, which is capable of replication of the whole chromosome arm.

In contrast to gene conversion, BIR requires factors for both leading and lagging strand synthesis (141). *POL1*, *POL2*, and *POL3* encode polymerases responsible for DNA synthesis in yeast, corresponding to Polα, Polε, and Polδ, respectively (5, 238). Polα associates with primase and initiates synthesis at leading and lagging stands. Rad51-dependent BIR also requires Polα-primase and Polδ. Polα-primase and Polδ are essential for the initial extension of the invading strand (144). Polε, and not Polδ, is required for BIR events involving copying a chromosomal arm (141). Recruitment of Polε might prevent template switching during extensive BIR; however the precise mechanism of this process is yet to be elucidated.

Both, Rad51-dependent and Rad51-independent BIR events have been shown to contribute to telomere repair in the absence of telomerase in the yeasts *S. cerevisiae* and *K. lactis*, and are further discussed below.

Recombinational telomere elongation (RTE) in *S. cerevisiae*

Deletion of telomerase components in *S. cerevisiae* causes senescence characterized by a decline in growth eventually leading to death for a majority of the cell population. The declining growth of telomerase-defective mutants is associated with changes in colony morphology, where round and smooth colonies are replaced by progressively rougher and smaller colonies with every streak. Rough colony morphology corresponds to an increased percentage of cells undergoing cell cycle arrest due to critical telomere shortening. While most yeast cells die within 50-100 cell divisions after deletion of telomerase, rare post-senescence survivors emerge with telomeres becoming elongated through RTE.

S. cerevisiae survivors can use two different RTE pathways referred to as Type I and Type II (142, 249) (Figure 1.4). Type I survivors show significant amplification of the subtelomeric Y'-elements interspersed between short stretches of telomeric repeats with shorter than wild-type terminal telomeric tracts. Type I RTE requires *RAD52* and *RAD51*-episatasis group genes also involved in mitotic DNA repair through SDSA and Rad51-dependent BIR (124, 249). Type II survivors, on the other hand, have little or no Y' amplification and exhibit long terminal telomeric tracts, up to 12 kb (158, 249). Type II survivors rely on the N-terminal domain of *RAD52, MRX,* and *RAD59* (genes also required for Rad51-independent BIR) as well as the helicase Sgs1 (4, 33).

The telomeres in *S. cerevisiae* senescing cells are short and telomeric repeats are variable in sequence (33, 253). Deletion of any of the Rad51-epistasis group factors is detrimental for Type I survivor formation and leads to Type II survivor formation only. It has been suggested that substantially shorter telomeric tracts may not completely restore telomere capping and therefore cause the characteristic slower growth of Type I survivors (10). Comparison of telomere movement across the bud neck in telomerase-deficient and wild type cells indicated that they have a similar movement pattern until telomeres become critically short. Type I survivors continue to move across the bud neck similarly to abnormal dicentric chromosomes during mitosis and as such may contain linked or even fused sister chromatids, while Type II survivors regain a wild type-like pattern of telomere movement (234, 250).

Deletion of any of the Rad51-epistasis group genes in the background of telomerase deletion increases the rate of survivor formation when compared to double mutants of a telomerase deletion with *rad50*, *mre11*, or *xrs2* (124). The observation that mutations in the MRX complex lead to delay in survivor formation may partially depend on delay in DNA

resection which relies partially on the MRX complex. When genes from both pathways are deleted simultaneously, no survivor formation is generally observed, indicating that the strand annealing abilities of Rad52 and Rad59 are not sufficient to produce significant telomere elongation (124). Deletion of *RAD52* abolishes survivor formation (154); however, LeBel *et al*. (2009) also demonstrated that rare survivors can form in the absence of *RAD52*, suggesting either a third survivor formation pathway or different epigenetic control of the Type I and Type II pathways (125).

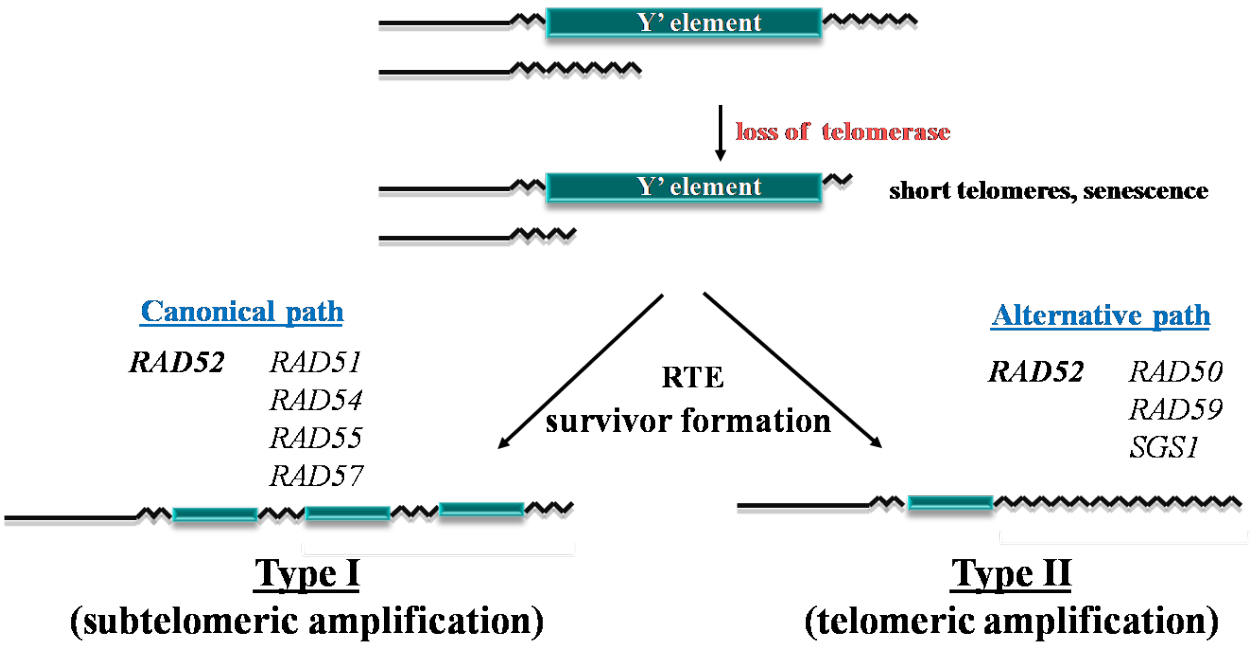

Figure 1.4. Model of Type I and Type II survivor formation in *S. cerevisiae*. *S. cerevisiae* has variable telomeric repeats with subtelomerically positioned Y' elements. Type I survivors rely on the *RAD51* epistasis gene group, also required for canonical DNA repair through SDSA. Type II survivors utilize *RAD52* as well as *RAD50*, *RAD59*, and *SGS1*.

It is not understood exactly how the Type I or Type II pathways are triggered. A single recombination event could potentially establish a pattern at an initially lengthened telomere, which is then spread to other telomeres or otherwise affects the choice of RTE path. Y' elements

encode a helicase which can facilitate recombination and therefore possibly the homogenization of terminal sequences (271). As such, Type I survivors are formed more frequently than Type II ones. In addition, when diploid strains of telomerase deletion mutants are created with Type I or Type II telomeres, sporulated cells tend to maintain inherited telomere structures (248).

Progressive telomere shortening triggers cell cycle arrest (258) by activation of Rad53 and phosphorylation of Rad9 or Mrc1 (58, 77, 101). However, signaling and activation of cell cycle arrest in senescent cells is not the same as the situation in which cells are experiencing chromosomal DSB (159). One of the contributing factors to that may be a difference in the number of DNA ends inducing DNA damage signaling (there are 32 telomeres in diploid *S. cerevisiae* and 12 in haploid *K. lactis*). Yeast telomeres become prone to recombination well before they lose all telomeric repeats (251). Most uncapped telomeres still contain the subtelomere and some telomeric repeats bound by telomere-specific binding proteins and, therefore, are potentially modifying DNA damage signals at telomeres. As such, short telomeres can suppress an activated G2/M cell cycle arrest or even suppress a damage response at a DSB located close to telomeres (78, 163).

Recombinational telomere elongation in *K. lactis*

RTE has also been extensively studied in *K. lactis*. *K. lactis* telomeres are composed of 15-25 repeats of a 25-bp uniform telomeric repeat and are missing Y' and X subtelomeric elements. Instead, 11 out of 12 *K. lactis* telomeres share subtelomeric homology, referred to as an R element, with differences due to polymorphisms (183). The uniform nature of the telomeric repeats in *K. lactis* is more reminiscent of human telomeres when compared to the variable repeats of *S. cerevisiae*. *K. lactis* telomerase deletion (*ter1*-Δ) survivors exhibit amplification of

telomeric repeats, consistent with their being Type II-like survivors (158) (Figure 1.5). However, the extent of telomere lengthening (usually 100s of bp) is typically considerably less than that seen in *S. cerevisiae* Type II survivors. *K. lactis* survivors are Rad52-dependent, consistent with being produced by HR.

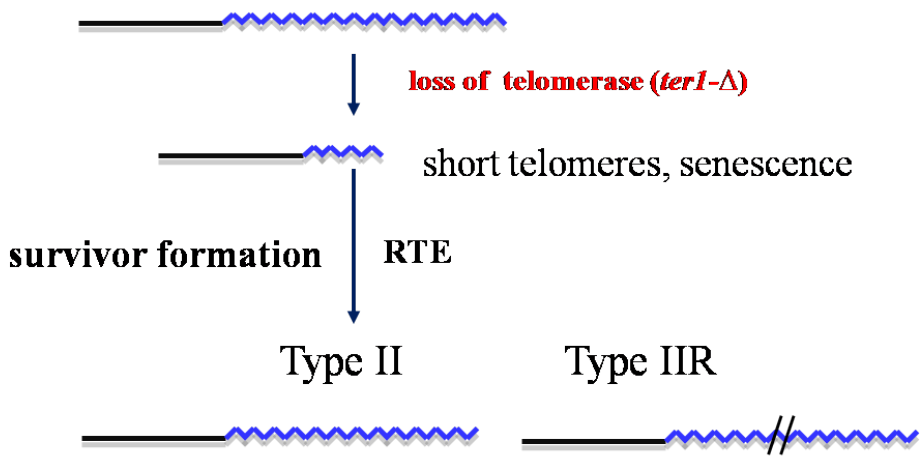

Figure 1. 5. *K. lactis* survivors lack Y' elements and produce Type II post-senescence survivors by telomeric amplification. Post-senescence survivors temporarily restore telomere capping and block recombination at telomeres until they become critically short again.

To investigate the mechanism of RTE in post-senescence survivors, Natarajan *et al*. (181) introduced a mutant telomerase into already senescing cells. The Bcl telomerase (*TER1-Bcl*) contains a silent mutation in the template that when reverse-transcribed into telomeric repeats creates a restriction site for *Bcl*I. Several rounds of serial passaging of these cells create chimeric telomeres containing two types of telomeric repeats: wild-type internal repeats flanked by outer Bcl repeats. Deletion of the *TER1-Bcl* telomerase leads to senescence and survivor formation. Digestion of genomic DNA from survivors with *Bcl*I (which cleaves Bcl telomeric repeats) showed that survivors that retained Bcl repeats apparently had repeating patterns of the two

repeat types within their telomeres (181). A survivor had the same pattern at all telomeres in a cell. Consequently, the roll-and-spread model was formulated to explain this result. According to this, a small t-circle can be utilized to produce an elongated telomere, the sequence of which is then copied by other short telomeres (Figure 1.6).

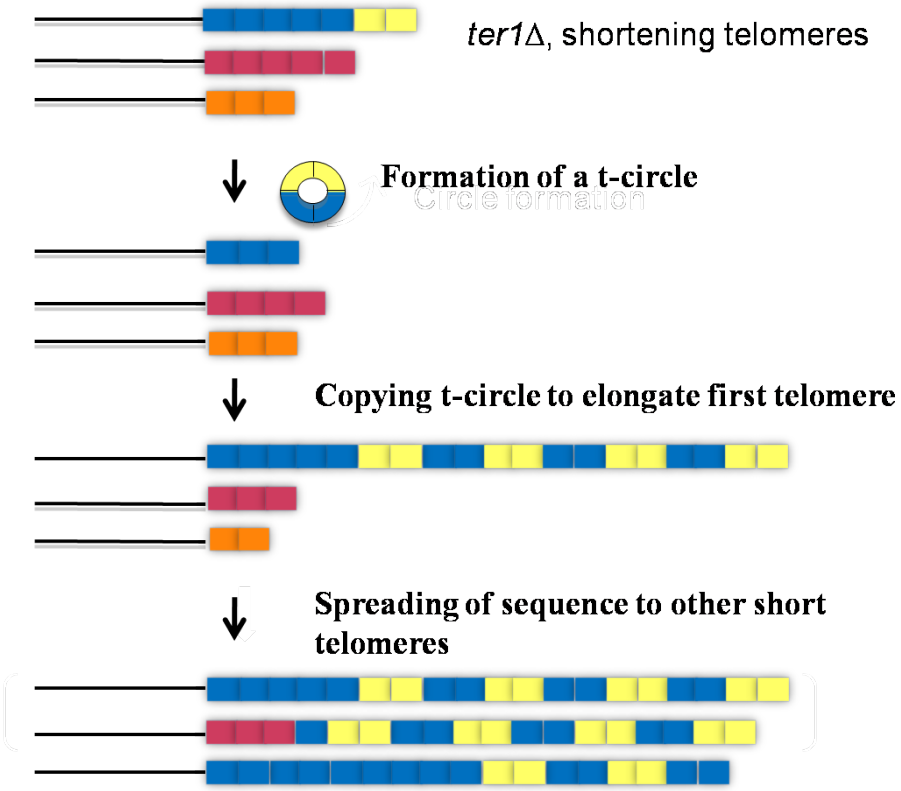

Figure 1.6. The roll-and-spread model of telomere elongation in *K. lactis*. Shortening telomeres may produce a t-circle which is then used as a template in rolling circle replication followed by spreading of copied sequence to all other telomeres.

Considerable evidence indicates that circles can be used as templates to produce long telomeres. In one study, a 1.6 kb t-circle was transformed into *ter1Δ* cells (181). This t-circle contained the *S. cerevisiae URA3* marker followed by *K. lactis* telomeric repeats. Yeast cells transformed with the 1.6 kb circle had elongated telomeres consisting of tandem repeats of the 1.6 kb circle

sequence (*URA3*-telomeric repeats). Although the telomere pattern in these cells was more reminiscent of Type I *S. cerevisiae* survivors, this experiment confirmed that double stranded t-circles can be successfully used in telomere elongation. Furthermore, t-circles were proposed to contribute to abrupt telomere elongation in *S. cerevisiae* through a *RAD50*-dependent mechanism (248). Natarajan *et al.* (181) co-transformed an artificially created 100 nt circle along with an ARS plasmid into *K. lactis* cells with short telomeres and after screening some hundreds of ARS plasmid transformants for incorporation of sequence from the small t-circle, demonstrated that cells could utilize the t-circle to achieve moderate telomere elongation reminiscent of "classical" *K. lactis* Type II survivors. t-circles have not been visualized in *ter1*-Δ cells, perhaps due to their transient and rare nature and limitations of current techniques.

Another key observation that supports the roll-and-spread model was provided by investigations of Topcu *et al*. (251). They utilized a *ter1Δ K. lactis* strain with one native telomere replaced by a *URA3*-marked telomere composed of Bcl telomeric repeats. When the Bcl telomere contained a wild-type number of repeats at the start of senescence, only ~10% of survivors exhibited spreading of Bcl repeats to all other telomeres while other survivors showed no obvious spreading or amplification of Bcl repeats. This frequency was consistent with the Bcl telomere being randomly selected among the 12 telomeres for being the source of amplified sequences. However, when the *URA3*-marked Bcl telomere was longer than other telomeres in the cell at the start of senescence, >90% of survivors had all of their telomeres copied from the *URA3*-marked Bcl telomere. Therefore, telomere sequence spreading is a conservative mechanism where once one telomere becomes long its sequence was readily copied to all other telomeres.

Short telomeres cause a high rate of subtelomeric gene conversion without telomere elongation in each of several *K. lactis* mutants containing short telomeres, whether or not those mutants undergo senescence (160). Direct evidence that t-circles are by-products of recombination comes from the *ter1-16T* mutant (86). *ter1-16T* harbors a mutation (C to T change at position 16 in the template region of *TER1*) that leads to the formation of mutant telomeric repeats with an altered Rap1 binding site. This mutant has a functional telomerase, but gradually produces highly lengthened telomeres, likely due to the disrupted Rap1 capping. Electron microscopy studies identified abundant small ds and ss t-circles of various sizes in this mutant. These t-circles are the byproduct of hyperrecombination and are abolished with the deletion of R*AD52* (86). The *ter1-16T* mutant has also been shown to produce telomeres with frequent t-loop structures (29, 86). The loops in these structures have a size distribution similar to that of the t-circles seen in the mutant. The t-loops are less common in a *rad52* double mutant or when telomeres are recapped by Rap1 overexpression, suggesting that they are recombination intermediates (29).

Another *K. lactis* mutant with abundant t-circles is *stn1-M1,* which carries a mutation in the N-terminal domain of *STN1* encoding one of the components of the CST complex. *stn1-M1* exhibits chronic rough colony morphology reminiscent of the one observed in senescing *ter1Δ* cells and also has ALT-like, very long and highly heterogeneous telomere lengths (106). Unlike *K. lactis ter1Δ* cells, where telomere elongation is rather moderate (total of 1-2 kb) (158), the *stn1-M1* mutation often produces telomeres greater than 12 kb (106). This type of RTE was termed Runaway Type II RTE (Type IIR RTE). In this mutant, much of the extrachromosomal DNA consists of t-circles of various sizes, some reaching up to 30kb in size (8). Inviability of

*stn1-M1 rad52-*Δ cells indicates that HR plays in an integral role in telomere elongation and maintenance in this mutant with a defective capping complex (106).

Focus of this Dissertation

The work presented here further investigates the genetics of RTE in *K. lactis*. More specifically, this study investigates the genetics of Type II survivor formation in a *K. lactis* telomerase deletion mutant as well as the genetics of the three major steps of RTE as postulated by the roll-and-spread model: 1) t-circle formation, 2) t-circle utilization, and 3) BIR-dependent spreading of sequence between telomeres.

References

1. **Aguilera, A.** 1995. Genetic evidence for different *RAD52*-dependent intrachromosomal recombination pathways in *Saccharomyces cerevisiae.* Curr. Genet. **27:**298-305.

2. **Allshire, R. C., M. Dempster, and N. D. Hastie.** 1989. Human telomeres contain at least three types of G–rich repeat distributed non-randomly. Nucleic Acids Res. **17:**4611-4627.

3. **Amin, A. D., A. B. H. Chaix, R. P. Mason, R. M. Badge, and R. H. Borts.** 2010. The roles of the *Saccharomyces cerevisiae* RecQ helicase *SGS1* in meiotic genome surveillance. PLoS ONE **5:**e15380.

4. **Ashton, T. M., and D. Hickson.** 2010. Yeast as a model system to study RecQ helicase function. DNA Repair **9:**303-314.

5. **Baker, T. A., and S. P. Bell.** 1998. Polymerases and the replisome: machines within machines. Cell **92:**295-305.

6. **Ball, L. G., and W. Xiao.** 2005. Molecular basis of ataxia telangiectasia and related diseases. Acta Pharmacol. Sin. **26:**897-907.

7. **Bärtsch, S., L. E. Kang, and L. S. Symington.** 2000. *RAD51* is required for the repair of plasmid double-stranded DNA gaps from either plasmid or chromosomal templates. Mol. Cell Biol. **20:**1194-1205.

8. **Basenko, E. Y., A. J. Cesare, S. Iyer, J. D. Griffith, and M. J. McEachern.** 2010. Telomeric circles are abundant in the *stn1-M1* mutant that maintains its telomeres through recombination. Nucleic Acids Res. **38:**182-189.

9. **Bayne, S., and J. Liu.** 2005. Hormones and growth factors regulate telomerase activity in ageing and cancer. Mol. Cell. Endocrinol. **240:**11-22.

10. **Bhattacharyya, M. K., and A. J. Lustig.** 2006. Telomere dynamics in genome stability. Trends Biochem. Sci. **31:**114-122.

11. **Bianchi, A., S. Negrini, and D. Shore.** 2004. Delivery of yeast telomerase to a DNA break depends on the recruitment functions of Cdc13 and Est1. Mol. Cell **16:**139-146.

12. **Bianchi, A., and D. Shore.** 2008. How telomerase reaches its end: mechanism of telomerase regulation by the telomeric complex. Mol. Cell **31:**153-165.

13. **Blackburn, E. H.** 2005. Telomerase and Cancer. Mol. Cancer Res. **3:**477-482.

14. **Blackburn, E. H.** 1990. Telomeres: structure and synthesis. J. Biol. Chem. **265:**5919-5921.

15. **Blackburn, E. H., and P. B. Challoner.** 1984. Identification of a telomeric DNA sequence in *Trypanosoma brucei.* Cell **36:**447-457.

16. **Blackburn, E. H., and K. Collins.** 2011. Telomerase: An RNP enzyme synthesizes DNA. Cold Spring Harb. Perspect. Biol. **3**.

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

18. **Blagoev, K. B., and E. H. Goodwin.** 2008. Telomere exchange and asymmetric segregation of chromosomes can account for the unlimited proliferative potential of ALT cell populations. DNA Repair **7:**199-204.

19. **Bohr, V. A.** 2008. Rising from the RecQ-age: the role of human RecQ helicases in genome maintenance. Trends Biochem. Sciences **33:**609-620.

20. **Bosco, G., and J. E. Haber.** 1998. Chromosome break-induced DNA replication leads to nonreciprocal translocations and telomere capture. Genetics **150:**1037-1047.

21. **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:**1819-1828.

22. **Boulton, S. J., and S. P. Jackson.** 1996. Identification of a *Saccharomyces cerevisiae* Ku80 homologue: roles double strand break rejoining and in telomeric maintenance. Nucleic Acids Res. **24:**4639-4648.

23. **Bressan, D. A., B. K. Baxter, and J. H. J. Petrini.** 1999. The Mre11-Rad50-Xrs2 protein complex facilitates homologous recombination-based double-strand break repair in *Saccharomyces cerevisiae.* Mol. Cell Biol. **19:**7681-7687.

24. **Broun, P., M. W. Ganal, and S. D. Tanksley.** 1992. Telomeric arrays display high levels of heritable polymorphism among closely related plant varieties. Proc. Nat. Acad. Sci. U.S.A. **89:**1354-1357.

25. **Calado, R. T., and N. S. Young.** 2009. Telomere Diseases. N. Engl. J. Med. **361:**2353-2365.

26. **Callebaut, I., and J. Mornon.** 1997. From BRCA1 to RAP1: a widespread BRCT module closely associated with DNA repair. FEBS Lett. **400:**25-30.

27. **Carter, S. D., S. Iyer, J. Xu, M. J. McEachern, and S. U. Astrom.** 2007. The role of nonhomologous end-joining components in telomere metabolism in *Kluyveromyces lactis*. Genetics **175:**1035-1045.

28. **Cech, T. R.** 1986. A model for the RNA-catalyzed replication of RNA. Proc. Natl. Acad. Sci. U.S.A. **83:**4360-4363.

29. **Cesare, A. J., C. Groff-Vindman, S. A. Compton, M. J. McEachern, and J. D. Griffith.** 2008. Telomere loops and homologous recombination-dependent telomeric circles in a *Kluyveromyces lactis* telomere mutant strain. Mol. Cell Biol. **28:**20-29.

30. **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:**271-279.

31. **Chan, S. W. L., and E. H. Blackburn.** 2003. Telomerase and ATM/Tel1p protect telomeres from nonhomologous end joining. Mol. Cell **11:**1379-1387.

32. **Chandra, A., T. R. Hughes, C. I. Nugent, and V. Lundblad.** 2001. Cdc13 both positively and negatively regulates telomere replication. Genes Dev. **15:**404-414.

33. **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:**1819-1827.

34. **Chen, Y. J., R. Rai, Z. Zhou, J. Kanoh, C. Ribeyre, Y. Yang, H. Y. Zheng, P. Damay, F. Wang, H. Tsujii, Y. Hiraoka, D. Shore, H. Hu, S. Chang, and M. Lei.** 2011. A conserved motif within RAP1 has diversified roles in telomere protection and regulation in different organisms. Nat. Struct. Mol. Biol. **18:**213-221.

35. **Chiolo, I., W. Carotenuto, G. Maffioletti, J. H. J. Petrini, M. Foiani, and G. Liberi.** 2005. Srs2 and Sgs1 DNA helicases associate with Mre11 in different subcomplexes following checkpoint activation and CDK1-mediated Srs2 phosphorylation. Mol. Cell Biol. **25:**5738-5751.

36. **Clerici, M., D. Mantiero, I. Guerini, G. Lucchini, and M. Longhese.** 2008. The Yku70-Yku80 complex contributes to regulate double-strand break processing and checkpoint activation during the cell cycle. EMBO Rep. **9:**810-818.

37. **Cockell, M., F. Palladino, T. Laroche, G. Kyrion, C. Liu, A. J. Lustig, and S. M. Gasser.** 1995. The carboxy termini of Sir4 and Rap1 affect Sir2 location: evidence for a multicomponent complex rquired for yeast telomeric silencing. J. Cell. Biol. **129:**909-912.

38. **Cohen, H., and D. A. Sinclair.** 2001. Recombination-mediated lengthening of terminal telomeric repeats requires the Sgs1 DNA helicase. Proc. Natl. Acad. Sci. U.S.A. **98:**3174-3179.

39. **Cohn, M., M. J. McEachern, and E. H. Blackburn.** 1998. Telomeric sequence diversity within the genus *Saccharomyces.* Curr. Genet. **33:**83-91.

40. **Colgin, L. M., and R. R. Reddel.** 1999. Telomere maintenance mechanisms and cellular immortalization. Curr. Opin. Genet. Dev. **9:**97-103.

41. **Collins, I., and C. S. Newlon.** 1994. Meiosis-specific formation of joint DNA molecules containing sequences from homologous chromosomes. Cell **76:**65-75.

42. **Compton, S. A., J. H. Choi, A. J. Cesare, S. Ozgür, and J. D. Griffith.** 2007. Xrcc3 and Nbs1 are required for the production of extrachromosomal telomeric circles in human alternative lengthening of telomere cells. Cancer Res. **67:**1513-1519.

43. **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:**739-750.

44. **Coppé, J., P. Desprez, A. Krtolica, and J. Campisi.** 2010. The senescence-associated secretory phenotype: The dark side of tumor suppression. Annu. Rev. Pathol. **5:**99-118.

45. **D'Amours, D., and S. P. Jackson.** 2002. The MRE11 complex: at the crossroads of DNA repair and checkpoint signalling. Nat. Rev. Mol. Cell Biol. **3:**317-327.

46. **Dai, X., C. Huang, A. Bhusari, S. Sampathi, K. Schubert, and W. Chai.** 2010. Molecular steps of G-overhang generation at human telomeres and its function in chromosome end protection. EMBO J. **29:**2788-2801.

47. **Daley, J. M., P. L. Palmbos, D. Wu, and T. E. Wilson.** 2005. Nonhomologous end joining in yeast. Annual Rev. Genetics **39:**431-451.

48. **Davis, A. P., and L. S. Symington.** 2001. The yeast recombinational repair protein Rad59 interacts with Rad52 and stimulates single-strand annealing. Genetics **159:**515–525. .

49. **de Lange, T., V. Lundblad, and E. H. Blackburn (ed.).** 2006. Telomeres, 2nd ed. Cold Spring Harbor Laboratory Press, New York.

50. **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:**518-527.

51. **Dellaire, G., and D. P. Bazett-Jones.** 2004. PML nuclear bodies: dynamic sensors of DNA damage and cellular stress. BioEssays **26:**963-977.

52. **Desai-Mehta, A., K. M. Cerosaletti, and P. Concannon.** 2001. Distinct functional domains of nibrin mediate Mre11 binding, focus formation, and nuclear localization. Mol. Cell Biol. **21:**2184-2191.

53. **DeZwaan, D. C., and B. C. Freeman.** 2010. Is there a telomore-bound 'EST' telomerase holoenzyme? Cell Cycle **9:**1913-1917.

54. **Driller, L., R. J. Wellinger, M. Larrivee, E. Kremmer, S. Jaklin, and H. M. Feldmann.** 2000. A short C-terminal domain of Yku70p is essential for telomere maintenance. J. Biol. Chem. **275:**24921-24927.

55. **Dudognon, C., F. Pendino, J. Hillion, A. Saumet, M. Lanotte, and E. Segal-Bendirdjian.** 2004. Death receptor signaling regulatory function for telomerase: hTERT abolishes TRAIL-induced apoptosis, independently of telomere maintenance. Oncogene **23:**7469-7474.

56. **Dunham, M. A., A. A. Neumann, C. L. Fasching, and R. R. Reddel.** 2000. Telomere maintenance by recombination in human cells. Nat. Genet. **26:**447-450.

57. **Durocher, D., I. A. Taylor, D. Sarbassova, L. F. Haire, S. L. Westcott, S. P. Jackson, S. J. Smerdon, and M. B. Yaffe.** 2000. The molecular basis of FHA domain: phosphopeptide binding specificity and implications for phospho-dependent signaling mechanisms. Mol. Cell **6:**1169-1182.

58. **Enomoto, S., L. Glowczewski, J. Lew-Smith, and J. G. Berman.** 2004. Telomere cap components influence the rate of senescence in telomerase-deficient yeast cells. Mol. Cell Biol. **24:**837-845.

59. **Epel, E. S., E. H. Blackburn, J. Lin, F. S. Dhabhar, N. E. Adler, J. D. Morrow, and R. M. Cawthon.** 2004. Accelerated telomere shortening in response to life stress. Proc. Natl. Acad. Sci. U.S.A **101:**17312-17615.

60. **Esposito, M. S.** 1978. Evidence that spontaneous mitotic recombination occurs at the two-strand stage. Proc. Natl. Acad. Sci. U.S.A **75:**4436–4440.

61. **Esposito, M. S., R. M. Ramirez, and C. V. Bruschi.** 1994 Nonrandomly-associated forward mutation and mitotic recombination yield yeast diploids homozygous for recessive mutations. Curr. Genet. **26:**302-307.

62. **Evans, S., K., and V. Lundblad.** 1999. Est1 and Cdc13 as comediators of telomerase access. Science **286:**117-120.

63. **Evans, S. K., and V. Lundblad.** 2002. The Est1 subunit of *Saccharomyces cerevisiae* telomerase makes multiple contributions to telomere length maintenance. Genetics **162:**1101-1115.

64. **Farah, J. A., G. A. Cromie, and G. R. Smith.** 2009. Ctp1 and Exonuclease 1, alternative nucleases regulated by the MRN complex, are required for efficient meiotic recombination. Proc. Nat. Acad. Sci. U.S.A. **106:**9356-9361.

65. **Feeser, E. A., and C. Wolberger.** 2008. Structural and functional studies of the Rap1 C-terminus reveal novel separation-of-function mutants. J. Mol. Biol. **380:**520-531.

66. **Fisher, T. S., A. K. P. Taggart, and V. A. Zakian.** 2004. Cell cycle-dependent regulation of yeast telomerase by Ku. Nat. Struct. Mol. Biol. **11:**1198-1205.

67. **Flores, I., M. L. Cayuela, and M. A. Blasco.** 2005. Effects of Telomerase and Telomere Length on Epidermal Stem Cell Behavior. Science **309:**1253-1256.

68. **Fujiwara, H., M. Osanai, T. Matsumoto, and K. K. Kojima.** 2005. Telomere-specific non-LTR retrotransposons and telomere maintenance in the silkworm, *Bombyx mori.* Chromosome Res. **13:**455-467.

69. **Gall, J. G.** 1996. Views of the cell: A pictoral history. The American Society for Microbiology, Bethesda, Maryland.

70. **Gangloff, S., J. P. McDonald, C. Bendixen, L. Arthur, and R. Rothstein.** 1994 The yeast type I topoisomerase Top3 interacts with Sgs1, a DNA helicase homolog: a potential eukaryotic reverse gyrase. Mol. Cell Biol. **14:**8391–8398.

71. **Gao, H., R. B. Cervantes, E. K. Mandell, J. H. Otero, and V. Lundblad.** 2007. RPA-like proteins mediate yeast telomere function. Nat. Struct. Mol. Biol. **14:**208-214.

72. **Gasparyan, H. J., L. Xu, R. C. Petreaca, A. E. Rex, V. Y. Small, N. S. Bhogal, J. A. Julius, T. H. Warsi, J. Bachant, O. M. Aparicio, and C. I. Nugent.** 2009. Yeast telomere capping protein Stn1 overrides DNA replication control through the S phase checkpoint. Proc. Natl. Acad. Sci. U.S.A. **106:**2206-2211.

73. **Ghosal, G., and K. Muniyappa.** 2005. *Saccharomyces cerevisiae* Mre11 is a high-affinity G4 DNA-binding protein and a G-rich DNA-specific endonuclease: implications for replication of telomeric DNA. Nucleic Acids Res. **33:**4692-4703.

74. **Giraud-Panis, M., M. T. Teixeira, V. Géli, and E. Gilson.** 2010. CST meets shelterin to keep telomeres in check. Mol. cell **39:**665-676.

75. **Golin, J. E., and M. S. Esposito.** 1984. Coincident gene conversion druing mitosis in *Saccharomyces.* Genetics **107:**355-365.

76. **Graham, I. R., R. A. Haw, K. G. Spink, K. A. Halden, and A. Chambers.** 1999. In vivo analysis of functional regions within yeast Rap1p. Mol. Cell Biol. **19:**7481-7490.

77. **Grandin, N., A. Bailly, and M. Charbonneau.** 2005. Activation of Mrc1, a mediator of the replication checkpoint, by telomere erosion. Biol. Cell **97:**799-814.

78. **Grandin, N., and M. Charbonneau.** 2008. Protection against chromosome degradation at the telomeres. Biochimie **90:**41-59.

79. **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:**8397-8408.

80. **Grandin, N., C. Damon, and M. Charbonneau.** 2001. Cdc13 prevents telomere uncapping and Rad50-dependent homologous recombination. EMBO J. **20:**6127-6139.

81. **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:**1173-1183.

82. **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:**512-527.

83. **Gravel, S., M. Larrivee, P. Labrecque, and R. J. Wellinger.** 1998. Yeast Ku as a regulator of chromosomal DNA end structure. Science **280:**741-744.

84. **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:**503-514.

85. **Grigoletto, A., P. Lestienne, and J. Rosenbaum.** 2011. The multifaceted proteins Reptin and Pontin as major players in cancer. Biochim. Biophys. Acta **1815:**147-157.

86. **Groff-Vindman, C., S. Natarajan, A. Cesare, J. D. Griffith, and M. J. McEachern.** 2005. Recombination at dysfunctional long telomeres forms tiny double- and single-stranded t-circles. Mol. Cell Biol. **25:**4406-4412.

87. **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.

88. **Harley, C. B., A. B. Futcher, and C. W. Greider.** 1990. Telomeres shorten during ageing of human fibroblasts. Nature **345:**458-460.

89. **Harrington, L. A., W. Zhou, T. McPhail, R. Oulton, D. S. Yeung, V. Mar, M. B. Bass, and M. O. Robinson.** 1997. Human telomerase contains evolutionarily conserved catalytic and structural subunits. Genes Dev. **11:**3109-3115.

90. **Henson, J. D., Y. Cao, L. I. Huschtscha, A. C. Chang, A. Y. M. Au, H. A. Pickett, and R. R. Reddel.** 2009. DNA C-circles are specific and quantifiable markers of alternative-lengthening-of-telomeres activity. Nat. Biotech. **27:**1181-1185.

91. **Henson, J. D., and R. R. Reddel.** 2010. Assaying and investigating Alternative Lengthening of Telomeres activity in human cells and cancers. FEBS Lett. **584:**3800-3811.

92. **Hirano, Y., K. Fukunaga, and K. Sugimoto.** 2009. Rif1 and Rif2 inhibit localization of Tel1 to DNA ends. Mol. Cell **33:**312-322.

93. **Hopfner, K., L. Craig, G. Moncalian, R. A. Zinkel, T. Usui, B. A. L. Owen, A. Karcher, B. Henderson, J. Bodmer, C. T. McMurray, J. P. Carney, J. H. J. Petrini, and J. A. Tainer.** 2002. The Rad50 zinc-hook is a structure joining Mre11 complexes in DNA recombination and repair. Nature **418:**562-566.

94. **Hopfner, K., A. Karcher, L. Craig, T. T. Woo, J. P. Carney, and J. A. Tainer.** 2001. Structural biochemistry and interaction architecture of the DNA double-strand break repair Mre11 nuclease and Rad50-ATPase. Cell **105:**473-485.

95. **Hopfner, K., A. Karcher, D. S. Shin, L. Craig, L. M. Arthur, J. P. Carney, and J. A. Tainer.** 2000. Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. Cell **101:**789-800.

96. **Hopkins, B. B., and T. T. Paull.** 2008. The *P. furiosus* Mre11/Rad50 complex promotes 5' strand resection at a DNA double-strand break. Cell **135:**250-260.

97. **Hsu, C.-L., Y.-S. Chen, S.-Y. Tsai, P.-J. Tu, M.-J. Wang, and J.-J. Lin.** 2004. Interaction of *Saccharomyces* Cdc13p with Pol1p, Imp4p, Sir4p and Zds2p is involved in telomere replication, telomere maintenance and cell growth control. Nucleic Acids Res. **32:**511-521.

98. **Huertas, P., F. Cortes-Ledesma, A. A. Sartori, A. Aguilera, and S. P. Jackson.** 2008. CDK targets Sae2 to control DNA-end resection and homologous recombination. Nature **455:**689-692.

99. **Hughes, T. R., R. G. Weilbaecher, M. Walterscheid, and V. Lundblad.** 2000. Identification of the single-strand telomeric DNA binding domain of the *Saccharomyces cerevisiae* Cdc13 protein. Proc. Natl. Acad. Sci. U.S.A. **97:**6457-6462.

100. **Iglesias, N., and J. Lingner.** 2009. Related mechanisms for end processing at telomeres and DNA double-strand breaks. Mol. Cell **35:**137-138.

101. **IJpma, A. S., and C. W. Greider.** 2003. Short telomeres induce a DNA damage response in *Saccharomyces cerevisiae*. Mol. Biol. Cell **14:**987-1001.

102. **Ikeda, S., T. Shibata, Y. Eishi, T. Takizawa, and M. Koike.** 2003. Correlation between the expression of telomerase reverse transcriptase and proliferative activity in breast cancer cells using an immunocytochemical restaining method. Pathol. Int. **53:**762-768.

103. **Ira, G., A. Pellicioli, A. Balijja, X. Wang, S. Fiorani, W. Carotenuto, G. Liberi, D. Bressan, L. Wan, N. M. Hollingsworth, J. E. Haber, and M. Foiani.** 2004. DNA end resection, homologous recombination and DNA damage checkpoint activation require *CDK1*. Nature **431:**1011-1017.

104. **Ivanov, E. L., N. Sugawara, J. Fishman-Lobell, and J. E. Haber.** 1996. Genetic requirements for the single-strand annealing pathway of double-strand break repair in *Saccharomyces cerevisiae.* Genetics **142:**693-704.

105. **Ivanov, E. L., N. Sugawara, C. I. White, F. Fabre, and J. E. Haber.** 1994. Mutations in *XRS2* and *RAD50* delay but do not prevent mating-type switching in *Saccharomyces cerevisiae.* Mol. Cell. Biol. **14:**3414-3425.

106. **Iyer, S., A. 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:**8064-8073.

107. **Jiang, W., Z. Zhong, J. D. Henson, A. A. Neumann, A. C. Chang, and R. R. Reddel.** 2005. Suppression of Alternative Lengthening of Telomeres by Sp100-mediated sequestration of the MRE11/RAD50/NBS1 complex. Mol. Cell Biol. **25:**2708-2721.

108. **Jiang, X., G. Jimenez, E. Chang, M. Frolkis, B. Kusler, M. Sage, M. Beeche, A. G. Bodnar, G. M. Wahl, T. D. Tlsty, and C. Chiu.** 1999. Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. Nat. Genet. **21:**111-114.

109. **Johnson, F. B., R. A. Marciniak, M. McVey, S. A. Stewart, W. C. Hahn, and L. Guarente.** 2001. The *Saccharomyces cerevisiae* WRN homolog Sgs1p participates in telomere maintenance in cells lacking telomerase. EMBO J. **20:**905-913.

110. **Keeney, S., and N. Kleckner.** 1995. Covalent protein-DNA complexes at the 5' strand termini of meiosis-specific double-strand breaks in yeast. Proc. Natl. Acad. Sci. U.S.A. **92:**11274-11278.

111. **Khadaroo, B., M. T. Teixeira, P. Luciano, N. Eckert-Boulet, S. M. Germann, M. N. Simon, I. Gallina, P. Abdallah, E. Gilson, V. Geli, and M. Lisby.** 2009. The DNA damage response at eroded telomeres and tethering to the nuclear pore complex. Nat. Cell Biol. **11:**980-987.

112. **Kim, H., S. Vijayakumar, M. Reger, J. C. Harrison, J. E. Haber, C. Weil, and J. H. J. Petrini.** 2008. Functional interactions between Sae2 and the Mre11 complex. Genetics **178:**711-723.

113. **Kirwan, M., and I. Dokal.** 2009. Dyskeratosis congenita, stem cells and telomeres. Biochim. Biophy. Acta **1792:**371-379.

114. **Klein, F., T. Laroche, M. E. Cardenas, J. F. Hofmann, D. Schweizer, and S. M. Gasser.** 1992. Localization of *RAP1* and topoisomerase II in nuclei and meiotic chromosomes of yeast. J. Cell Biol. **117:**935-948.

115. **Kraus, E., W. Leung, and J. E. Haber.** 2001. Break-induced replication: A review and an example in budding yeast. Proc. Natl. Acad. Sci. U.S.A. **98:**8255-8262.

116. **Krauskopf, A., and E. H. Blackburn.** 1996. Control of telomere growth by interactions of RAP1 with the most distal telomeric repeats. Nature **383:**354-357.

117. **Krogh, B. O., and L. S. Symington.** 2004. Recombination proteins in yeast. Annu. Rev. Genet. **38:**233-271.

118. **Kurtz, S., and D. Shore.** 1991. RAP1 protein activates and silences transcription of mating type gene in yeast. Genes Dev. **5:**616-628.

119. **Kusumoto, K. I., S. Suzuki, and Y. Kashiwagi.** 2003. Telomeric repeat sequence of *Aspergillus oryzae* consists of dodeca-nucleotides. Appl. Microbiol.Biotechnol. **61:**247-251.

120. **Kyrion, G., K. A. Boakye, and A. J. Lustig.** 1992. C-terminal truncation of RAP1 results in the deregulation of telomere size, stability, and function in *Saccharomyces cerevisiae*. Mol. Cell Biol. **12:**5159-5173.

121. **Lamarche, B. J., N. I. Orazio, and M. D. Weitzman.** 2010. The MRN complex in double-strand break repair and telomere maintenance. FEBS Lett. **584:**3682-3695.

122. **Larrivee, M., C. LeBel, and R. J. Wellinger.** 2004. The generation of proper constitutive G-tails on yeast telomeres is dependent on the MRX complex. Genes Dev. **18:**1391-1396.

123. **Lazzaro, F., V. Sapountzi, M. Granata, A. Pellicioli, M. Vaze, J. E. Haber, P. Plevani, D. Lydall, and M. Muzi-Falconi.** 2008. Histone methyltransferase Dot1 and Rad9 inhibit single-stranded DNA accumulation at DSBs and uncapped telomeres. EMBO J. **27:**1502-1512.

124. **Le, S., J. K. Moore, J. E. Haber, and C. W. Greider.** 1999. *RAD50* and *RAD51* define two pathways that collaborate to maintain telomeres in the absence of telomerase. Genetics **152:**143-152.

125. **LeBel, C., E. Rosonina, D. C. Sealey, F. Pryde, D. Lydall, L. Maringele, and L. A. Harrington.** 2009. Telomere maintenance and survival in *Saccharomyces cerevisiae* in the absence of telomerase and *RAD52*. Genetics **182:**671-684.

126. **Lee, J., E. K. Mandell, T. Rao, D. S. Wuttke, and V. Lundblad.** 2010. Investigating the role of the Est3 protein in yeast telomere replication. Nucleic Acids Res. **38:**2279-2290.

127. **Lendvay, T. S., D. K. Morris, J. Sah, B. Balasubramanian, and V. Lundblad.** 1996. Senescence mutants of *Saccharomyces cerevisiae* with a defect in telomere replication identify three additional EST genes. Genetics **144:**1399-1412.

128. **Lengsfeld, B. M., A. J. Rattray, V. Bhaskara, R. Ghirlando, and T. T. Paull.** 2007. Sae2 is an endonuclease that processes hairpin DNA cooperatively with the Mre11/Rad50/Xrs2 complex. Mol. Cell **28:**638-651.

129. **Levy, D. L., and E. H. Blackburn.** 2004. Counting of Rif1p and Rif2p on *Saccharomyces cerevisiae* telomeres regulates telomere length. Mol. Cell Biol. **24:**10857-10867.

130. **Lewis, L. K., G. Karthikeyan, J. W. Westmoreland, and M. A. Resnick.** 2002. Differential suppression of DNA repair seficiencies of yeast *rad50, mre11* and *xrs2* mutants by *EXO1* and *TLC1* (the RNA component of telomerase). Genetics **160:**49-62.

131. **Li, S., S. Makovets, T. Matsuguchi, J. D. Blethrow, K. M. Shokat, and E. H. Blackburn.** 2009. Cdk1-dependent phosphorylation of Cdc13 coordinates telomere elongation during cell-cycle progression. Cell **136:**50-61.

132. **Lilley, M. J., and M. F. White.** 2001. The junction-resolving enzymes. Nat. Rev. Mol. Cell Biol. **2:**433-443.

133. **Lin, J., H. Ly, A. Hussain, M. Abraham, S. Pearl, Y. Tzfati, T. G. Parslow, and E. H. Blackburn.** 2004. A universal telomerase RNA core structure includes structured motifs required for binding the telomerase reverse transcriptase protein. Proc. Natl. Acad. Sci. U.S.A. **101:**14713-14718.

134. **Lin, J. J., and V. A. Zakian.** 1996. The *Saccharomyces* CDC13 protein is a single-strand TG1-3 telomeric DNA-binding protein in vitro that affects telomere behavior *in vivo*. Proc. Natl. Acad. Sci. U.S.A. **93:**13760-13765.

135. **Lingner, J., T. R. Cech, T. R. Hughes, and V. Lundblad.** 1997. Three Ever Shorter Telomere (*EST*) genes are dispensable for in vitro yeast telomerase activity. Proc. Natl. Acad. Sci. U.S.A. **94:**11190-11195.

136. **Lingner, J., T. R. Hughes, A. Shevchenko, M. Mann, V. Lundblad, and T. R. Cech.** 1997. Reverse transcriptase motifs in the catalytic subunit of telomerase. Science **276:**561-567.

137. **Livengood, A. J., A. J. Zaug, and T. R. Cech.** 2002. Essential regions of *Saccharomyces cerevisiae* telomerase RNA: separate elements for Est1p and Est2p interaction. Mol. Cell Biol. **22:**2366-2374.

138. **Lloyd, J., J. R. Chapman, J. A. Clapperton, L. F. Haire, E. Hartsuiker, J. Li, A. M. Carr, S. P. Jackson, and S. J. Smerdon.** 2009. A supramodular FHA/BRCT-repeat architecture mediates Nbs1 adaptor function in response to DNA damage. Cell **139:**100-111.

139. **Longhese, M., D. Mantiero, and M. Clerici.** 2006. The cellular response to chromosome breakage. Mol. Microbiol. **60:**1099-1108.

140. **Longtine, M. S., N. M. Wilson, M. E. Petracek, and J. Berman.** 1989. A yeast telomere binding activity binds to two related telomere sequence motifs and is indistinguishable from RAP1. Curr. Genet. **16:**225-239.

141. **Lorente, B., C. E. Smith, and L. S. Symington.** 2008. Break-induced replication: What is it and what is it for? Cell Cycle **7:**859 - 864.

142. **Lundblad, V., and E. H. Blackburn.** 1993. An alternative pathway for yeast telomere maintenance rescues *est1*- senescence. Cell **73:**347-360.

143. **Lundblad, V., and J. W. Szostak.** 1989. A mutant with a defect in telomere elongation leads to senescence in yeast. Cell **57:**633-643.

144. **Lydeard, J. R., S. Jain, M. Yamaguchi, and J. E. Haber.** 2007. Break-induced replication and telomerase-independent telomere maintenance require Pol32. Nature **448:**820-823.

145. **Ma, Y., and C. W. Greider.** 2009. Kinase-independent functions of *TEL1* in telomere maintenance. Mol. Cell. Biol. **29:**5193-5202.

146. **Makarov, V. L., Y. Hirose, and J. P. Langmore.** 1997. Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. Cell **88:**657-666.

147. **Malik, H. S., W. D. Burke, and T. H. Eickbush.** 2000. Putative telomerase catalytic subunits from *Giardia lamblia* and *Caenorhabditis elegans*. Gene **251:**101-108.

148. **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:**7131-7136.

149. **Malkova, A., M. L. Naylor, M. Yamaguchi, G. Ira, and J. E. Haber.** 2005. *RAD51*-dependent break-induced replication differs in kinetics and checkpoint responses from *RAD51*-mediated gene conversion. Mol. Cell Biol. **25:**933-944.

150. **Mantiero, D., M. Clerici, G. Lucchini, and M. Longhese.** 2007. Dual role for *Saccharomyces cerevisiae* Tel1 in the checkpoint response to double-strand breaks. EMBO Rep. **8:**380-387.

151. **Marcand, S., E. Gilson, and D. Shore.** 1997. A protein-counting mechanism for telomere length regulation in yeast. Science **275:**986-990.

152. **Marcand, S., B. Pardo, A. Gratias, S. Cahun, and I. Callebaut.** 2008. Multiple pathways inhibit NHEJ at telomeres. Genes Dev. **22:**1153-1158.

153. **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 yku70 deletion mutants. Genes Dev. **16:**1919-1933.

154. **Maringele, L., and D. Lydall.** 2004. Telomerase- and recombination-independent immortalization of budding yeast. Genes Dev. **18:**2663-2675.

155. **Mason, J. M., and H. Biessmann.** 1995. The unusual telomeres of *Drosophila.* Trends in Genetics **11:**58-62.

156. **Mason, M., A. Schuller, and E. Skordalakes.** 2011. Telomerase structure function. Curr. Opin. Struct. Biol. **21:**92-100.

157. **Masutomi, K., R. Possemato, J. M. Y. Wong, J. L. Currier, Z. Tothova, J. B. Manola, S. Ganesan, P. M. Lansdorp, K. Collins, and W. C. Hahn.** 2005. The telomerase reverse transcriptase regulates chromatin state and DNA damage responses. Proc. Natl. Acad. Sci. U.S.A. **102:**8222-8227.

158. **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:**1822-1834.

159. **McEachern, M. J., and J. E. Haber.** 2006. Break-induced replication and recombinational telomere elongation in yeast. Annu. Rev. Biochem. **75:**111-135.

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

161. **McVey, M., and S. Lee.** 2008. MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. Trends in Genetics **24:**529-538.

162. **Metcalfe, J. A., J. Parkhill, L. Campbell, M. Stacey, P. Biggs, P. J. Byrd, A. Taylor, and M. R.** 1996. Accelerated telomere shortening in ataxia telangiectasia. Nat. Genet. **13:**350-353.

163. **Michelson, R. J., S. Rosenstein, and T. Weinert.** 2005. A telomeric repeat sequence adjacent to a DNA double-stranded break produces an anticheckpoint. Genes Dev. **19:**2546-2559.

164. **Mimitou, E., and L. Symington.** 2008. Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. Nature **455:**770-774.

165. **Mimitou, E. P., and L. S. Symington.** 2009. DNA end resection: many nucleases make light work. DNA Repair **8:**983-995.

166. **Mimura, T., and N. C. Joyce.** 2006. Replication competence and senescence in central and peripheral human corneal endothelium. Invest. Ophthalmol.Vis. Sci. **47:**1387-1396.

167. **Mitton-Fry, R. M., E. M. Anderson, D. L. Theobald, L. W. Glustrom, and D. S. Wuttke.** 2004. Structural basis for telomeric single-stranded DNA recognition by yeast Cdc13. J. Mol. Biol. **338:**241-255.

168. **Moreau, S., E. A. Morgan, and L. S. Symington.** 2001. Overlapping functions of the *Saccharomyces cerevisiae* Mre11, Exo1 and Rad27 nucleases in DNA metabolism. Genetics **159:**1423-1433.

169. **Morgan, T. H.** 1911. Random segregation versus coupling in mendelian inheritance. Science **34:**384.

170. **Mortensen, U. H., C. Bendixen, I. Sunjevaric, and R. Rothstein.** 1996. DNA strand annealing is promoted by the yeast Rad52 protein. Proc. Natl. Acad. Sci. U.S.A. **93:**10729-10734.

171. **Mozdy, A. D., and T. R. Cech.** 2006. Low abundance of telomerase in yeast: Implications for telomerase haploinsufficiency. RNA **12:**1721-1737.

172. **Müller, T., E. Gilson, R. Schmidt, R. Giraldo, J. Sogo, H. Gross, and S. M. Gasser.** 1994. Imaging the asymmetrical DNA bend induced by repressor activator protein 1 with scanning tunneling microscopy. J. Struct. Biol. **113:**1-12.

173. **Muntoni, A., A. A. Neumann, M. Hills, and R. R. Reddel.** 2009. Telomere elongation involves intra-molecular DNA replication in cells utilizing alternative lengthening of telomeres. Hum. Mol. Genet. **18:**1017-1027.

174. **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:**14436-14439.

175. **Nabetani, A., and F. Ishikawa.** 2011. Alternative lengthening of telomeres pathway: Recombination-mediated telomere maintenance mechanism in human cells. J. Biochem. **149:**5-14.

176. **Nakada, D., K. Matsumoto, and K. Sugimoto.** 2003. ATM-related Tel1 associates with double-strand breaks through an Xrs2-dependent mechanism. Genes Dev. **17:**1957–1962.

177. **Nakada, D., T. Shimomura, K. Matsumoto, and K. Sugimoto.** 2003. The ATM-related Tel1 protein of *Saccharomyces cerevisiae* controls a checkpoint response following phleomycin treatment. Nucleic Acids Res. **31:**1715-1724.

178. **Nakamura T. M., and C. T. R.** 1998. Reversing time: origin of telomerase. Cell **92:**587-590.

179. **Nakamura, T. M., G. B. Morin, K. B. Chapman, S. L. Weinrich, W. H. Andrews, J. Lingner, C. B. Harley, and T. R. Cech.** 1997. Telomerase catalytic subunit homologs from fission yeast and human. Science **277:**955-959.

180. **Nassif, N., J. Penney, S. Pal, W. R. Engels, and G. B. Gloor.** 1994. Efficient copying of nonhomologous sequences from ectopic sites via P-element-induced gap repair. Mol. Cell Biol. **14:**1613–1625.

181. **Natarajan, S., and M. J. McEachern.** 2002. Recombinational telomere elongation promoted by DNA circles. Mol. Cell Biol. **22:**4512-4521.

182. **Negrini, S., V. Ribaud, A. Bianchi, and D. Shore.** 2007. DNA breaks are masked by multiple Rap1 binding in yeast: implications for telomere capping and telomerase regulation. Genes Dev. **21:**292-302.

183. **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:**813-830.

184. **Nikitina, T., and C. L. Woodcock.** 2004. Closed chromatin loops at the ends of chromosomes. J. Cell Biol. **166:**161-165.

185. **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.

186. **Nosek, J., and L. Tomaska.** 2008. Origin and Evolution of Telomeres, vol. 1. Landes Bioscience, Austin, Texas.

187. **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:**249-252.

188. **Oeseburg, H., R. de Boer, W. van Gilst, and P. van der Harst.** 2010. Telomere biology in healthy aging and disease. Pflügers Arch. **459:**259-268.

189. **Oum, J., C. Seong, Y. Kwon, J. Ji, A. Sid, S. Ramakrishnan, G. Ira, A. Malkova, Sung, S. Lee, and E. Shim.** 2011. RSC facilitates Rad59-dependent homologous recombination between sister chromatids by promoting cohesin loading at DNA double-strand breaks. Mol. Cell Biol. **31:**3924-3937.

190. **Pace NR, M. T.** 1985. RNA catalysis and the origin of life. Orig. Life Evol. Biosph. **16:**97-116.

191. **Pâques, 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.

192. **Pardo, B., and S. Marcand.** 2005. Rap1 prevents telomere fusions by nonhomologous end joining. EMBO J. **24:**3117-3127.

193. **Pardue, M. L., S. Rashkova, C. E., D. P. G., G. J. A., and T. K. L.** 2005. Two retrotransposons maintain telomeres in *Drosophila*. Chromosome Res. **13**.

194. **Park, J.-I., A. S. Venteicher, J. Y. Hong, J. Choi, S. Jun, M. Shkreli, W. Chang, Z. Meng, P. Cheung, H. Ji, M. McLaughlin, T. D. Veenstra, R. Nusse, P. D. McCrea, and S. E. Artandi.** 2009. Telomerase modulates Wnt signalling by association with target gene chromatin. Nature **460:**66-72.

195. **Parsons, C. A., P. Baumann, E. Van Dyck, and S. C. West.** 2000. Precise binding of single-stranded DNA termini by human RAD52 protein. EMBO J. **19:**4175-4181.

196. **Paschini, M., E. K. Mandell, and V. Lundblad.** 2010. Structure prediction-driven genetics in *Saccharomyces cerevisiae* identifies an interface between the t-RPA Proteins Stn1 and Ten1. Genetics **185:**11-21.

197. **Paull, T. T., and M. Gellert.** 1999. Nbs1 potentiates ATP-driven DNA unwinding and endonuclease cleavage by the Mre11/Rad50 complex. Genes Dev. **13:**1276–1288.

198. **Pennock, E., K. Buckley, and V. Lundblad.** 2001. Cdc13 delivers separate complexes to the telomere for end protection and replication. Cell **104:**387-396.

199. **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:**64-67.

200. **Petukhova, G., S. Stratton, and P. Sung.** 1998. Catalysis of homologous DNA pairing by yeast Rad51 and Rad54 proteins. Nature **393:**91-94.

201. **Petukhova, G., S. A. Stratton, and P. Sung.** 1999. Single strand DNA binding and annealing activities in the yeast recombination factor Rad59. J. Biol. Chem. **274:**33839-33842.

202. **Pohl, T. J., and J. A. Nickoloff.** 2008. Rad51-independent interchromosomal double-strand break repair by gene conversion requires Rad52 but not Rad55, Rad57, or Dmc1. Mol. Cell Biol. **28:**897-906.

203. **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:**831-834.

204. **Prescott, J., and E. H. Blackburn.** 1997. Functionally interacting telomerase RNAs in the yeast telomerase complex. Genes Dev. **11:**2790-2800.

205. **Prieur, A., and D. S. Peeper.** 2008. Cellular senescence *in vivo*: a barrier to tumorigenesis. Curr. Opin. Cell Biol. **20:**150-155.

206. **Puglisi, A., A. Bianchi, L. Lemmens, P. Damay, and D. Shore.** 2008. Distinct roles for yeast Stn1 in telomere capping and telomerase inhibition. EMBO J. **27:**2328-2339.

207. **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:**1777-1788.

208. **Rattray, A. J., C. B. McGill, B. K. Shafer, and J. N. Strathern.** 2001. Fidelity of mitotic double-strand-break repair in *Saccharomyces cerevisiae*: A role for *SAE2/COM1*. Genetics **158:**109-122.

209. **Rattray, A. J., and L. S. Symington.** 1994. Use of a chromosomal inverted repeat to demonstrate that the Rad51 and Rad52 genes of *Saccharomyces cerevisiae* have different roles in mitotic recombination. Genetics **138:**587–595.

210. **Ray, A., and K. W. Runge.** 1998. The C terminus of the major yeast telomere binding protein Rap1p enhances telomere formation. Mol. Cell Biol. **18:**1284-1295.

211. **Reddel, R. R.** 2000. The role of senescence and immortalization in carcinogenesis. Carcinogenesis **21:**477-484.

212. **Reddel, R. R.** 2010. Senescence: an antiviral defense that is tumor suppressive? Carcinogenesis **31:**19-26.

213. **Reddel, R. R., and T. M. Bryan.** 2003. Alternative lengthening of telomeres: dangerous road less travelled. The Lancet **361:**1840-1841.

214. **Risques, R. A., T. L. Vaughan, X. Li, R. D. Odze, P. L. Blount, K. Ayub, J. L. Gallaher, B. Reid, and P. S. Rabinovitch.** 2007 Leukocyte telomere length predicts cancer risk in Barrett's esophagus. Cancer Epidemiol. Biomarkers Prev. **16:**2649-2255.

215. **Ritchie, K. B., J. C. Mallory, and T. D. Petes.** 1999. Interactions of *TLC1* (which encodes the RNA subunit of telomerase), *TEL1*, and *MEC1* in regulating telomere length in the yeast *Saccharomyces cerevisiae.* Mol. Cell Biol. **19:**6065-6075.

216. **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:**475-479.

217. **Rong, L., and H. L. Klein.** 1993. Purification and characterization of the *SRS2* DNA helicase of the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. **268:**1252-1259.

218. **Rong, Y. S.** 2008. Telomere capping in *Drosophila*: dealing with chromosome ends that most resemble DNA breaks. Chromosoma **117:**235-242.

219. **Roth, C. W., F. Kobeski, M. F. Walter, and H. Biessmann.** 1997. Chromosome end elongation by recombination in the mosquito *Anopheles gambiae.* Mol. Cell Biol. **17:**5176-5183.

220. **Sarin, K. Y., P. Cheung, D. Gilison, E. Lee, R. I. Tennen, E. Wang, M. K. Artandi, A. E. Oro, and S. E. Artandi.** 2005. Conditional telomerase induction causes proliferation of hair follicle stem cells. Nature **436:**1048-1052.

221. **Seb, D., and W. Gilbert.** 1992. Novel DNA superstructures formed by telomere-like oligomerns. Biochem. **31:**65-70.

222. **Sharma, G. G., A. Gupta, H. Wang, H. Scherthan, S. Dhar, V. Gandhi, G. Iliakis, J. W. Shay, C. S. H. Young, and T. j. K. Pandita.** 2003. hTERT associates with human telomeres and enhances genomic stability and DNA repair. Oncogene **22:**131-146.

223. **Shay, J. W., and W. E. Wright.** 2005. Senescence and immortalization: role of telomeres and telomerase. Carcinogenesis **26:**867-874.

224. **Sheila A. Stewart, William C. Hahn, Benjamin F. O'Connor, Elisa N. Banner, Ante S. Lundberg, Poonam Modha, Hana Mizuno, Mary W. Brooks, Mark Fleming, Drazen B. Zimonjic, Nicholas C. Popescu, and R. A. Weinberg.** 2002. Telomerase contributes to tumorigenesis by a telomere length-independent mechanism. Proc. Natl. Acad. Sci. U.S.A. **99:**12606-12611.

225. **Signon, L., A. Malkova, M. L. Naylor, H. Klein, and J. E. Haber.** 2001. Genetic requirements for *RAD51*- and *RAD54*-independent break-induced replication repair of a chromosomal double-strand break. Mol. Cell Biol. **21:**2048-2056.

226. **Singer, M. S., and D. E. Gottschling.** 1994. *TLC1:* template RNA component of *Saccharomyces cerevisiae* telomerase. Science **266:**404-409.

227. **Smith, C. D., D. L. Smith, J. L. DeRisi, and E. H. Blackburn.** 2003. Telomeric protein distributions and remodeling through the cell cycle in *Saccharomyces cerevisiae*. Mol. Biol. Cell **14:**556-570.

228. **Smith, L. L., H. A. Coller, and J. M. Roberts.** 2003. Telomerase modulates expression of growth-controlling genes and enhances cell proliferation. Nat. Cell Biol. **5:**474-479.

229. **Song, B., and P. Sung.** 2000. Functional interactions among yeast Rad51 recombinase, Rad52 mediator, and replication protein A in DNA strand exchange. J. Biol. Chem. **275:**15895-15904.

230. **Song, X., K. Leehy, R. T. Warrington, J. C. Lamb, Y. V. Surovtseva, and D. E. Shippen.** 2008. *STN1* protects chromosome ends in *Arabidopsis thaliana.* Proc. Nat. Acad. Sci. U.S.A. **105:**19815-19820.

231. **Spell, R. M., and S. Jinks-Robertson.** 2003. Role of mismatch repair in the fidelity of *RAD51*- and *RAD59*-dependent recombination in *Saccharomyces cerevisiae.* Genetics **165:**1733–1744.

232. **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:**2384-2395.

233. **Stern, C.** 1936. Somatic crossing over and segregation in *Drosophila melanogaster.* Genetics **21:**625-730.

234. **Straatman, K., and E. Louis.** 2007. Localization of telomeres and telomere-associated proteins in telomerase-negative *Saccharomyces cerevisiae.* Chromosome Res. **15:**1033-1050.

235. **Strunnikov, A. V., and R. Jessberger.** 1999. Structural maintenance of chromosomes (SMC) proteins. Eur. J. Biochem. **263:**6-13.

236. **Sugawara, N., and J. E. Haber.** 1992. Characterization of double-strand break-induced recombination: homology requirements and single-stranded DNA formation. Mol. Cell Biol. **12:**563-575.

237. **Sugawara, N., G. Ira, and J. E. Haber.** 2000. DNA length dependence of the single-strand annealing pathway and the role of S*accharomyces cerevisiae RAD59* in double-strand break repair. Mol. Cell Biol. **20:**5300–5309. .

238. **Sugino, A.** 1995. Yeast DNA polymerases and their role at the replication fork. Trends in Biochem. Sci. **20:**319-323.

239. **Sugiyama, T., and S. C. Kowalczykowski.** 2002. Rad52 protein associates with replication protein A (RPA)-single-stranded DNA to accelerate Rad51-mediated displacement of RPA and presynaptic complex formation. J. Biol. Chem. **277:**31663-31672.

240. **Sung, P.** 1997. Yeast Rad55 and Rad57 proteins form a heterodimer that functions with replication protein A to promote DNA strand exchange by Rad51 recombinase. Genes Dev. **11:**1111-1121.

241. **Sung, P., and D. L. Robberson.** 1995. DNA strand exchange mediated by a *RAD51*-ssDNA nucleoprotein filament with polarity opposite to that of RecA. Cell **82:**453-461.

242. **Sykorová, E., J. Fajkus, M. Mezníková, K. Y. Lim, K. Neplechová, F. R. Blattner, M. W. Chase, and A. R. Leitch.** 2006. Minisatellite telomeres occur in the family Alliaceae but are lost in *Allium.* Am. J. Bot. **93:**814-823.

243. **Taggart, A. K. P., S. Teng, and V. A. Zakian.** 2002. Est1p as a cell cycle-regulated activator of telomere-bound telomerase. Science **297:**1023-1026.

244. **Taggart, A. K. P., and V. A. Zakian.** 2003. Telomerase: what are the Est proteins doing? Curr. Opin. Cell Biol. **15:**275-280.

245. **Takata, H., Y. Kanoh, N. Gunge, K. Shirahige, and A. Matsuura.** 2004. Reciprocal association of the budding yeast ATM-related proteins Tel1 and Mec1 with telomeres in vivo. Mol. Cell **14:**515-522.

246. **Tan, M., C. Wei, and C. M. Price.** 2003. The telomeric protein Rap1 is conserved in vertebrates and is expressed from a bidirectional promoter positioned between the *Rap1* and *KARS* genes. Gene **323:**1-10.

247. **Teixeira, M. T., M. Arneric, P. Sperisen, and J. Lingner.** 2004. Telomere length homeostasis is achieved via a switch between telomerase- extendible and -nonextendible states. Cell **117:**323-335.

248. **Teng, S., J. Chang, B. McCowan, and V. A. Zakian.** 2000. Telomerase-independent lengthening of yeast telomeres occurs by an abrupt Rad50p-dependent, Rif-inhibited recombinational process. Mol. Cell **6:**947-952.

249. **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:**8083-8093.

250. **Thrower, D. A., J. Stemple, E. Yeh, and K. Bloom.** 2003. Nuclear oscillations and nuclear filament formation accompany single-strand annealing repair of a dicentric chromosome in *Saccharomyces cerevisiae.* J. Cell Sci. **116:**561-569.

251. **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:**3348-3353.

252. **Tseng, S. F., J. J. Lin, and S. C. Teng.** 2006. The telomerase-recruitment domain of the telomere binding protein Cdc13 is regulated by Mec1p/Tel1p-dependent phosphorylation. Nucleic Acids Res. **34:**6327-6336.

253. **Tsukamoto, M., K. Yamashita, T. Miyazaki, M. Shinohara, and A. Shinohara.** 2003. The N-Terminal DNA-binding domain of Rad52 promotes *RAD51*-independent recombination in *Saccharomyces cerevisiae.* Genetics **165:**1703-1715.

254. **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:**1328-1335.

255. **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:**1779-1788.

256. **Valencia, M., M. Bentele, M. r. B. Vaze, G. Herrmann, E. Kraus, S. Lee, P. Schar, and J. E. Haber.** 2001. NEJ1 controls non-homologous end joining in *Saccharomyces cerevisiae.* Nature **414:**666-669.

257. **Virgin, J. B., J. P. Bailey, F. Hasteh, J. Neville, A. Cole, and G. Tromp.** 2001. Crossing over is rarely associated with mitotic intragenic recombination in *Schizosaccharomyces pombe.* Genetics **157:**63-77.

258. **Viscardi, V., M. Clerici, H. Cartagena-Lirola, and M. Longhese.** 2005. Telomeres and DNA damage checkpoints. Biochimie **87:**613-624.

259. **Wan, M., J. Q. Qin, Z. Songyang, and D. Liu.** 2009. OB Fold-containing Protein 1 (OBFC1), a human homolog of yeast Stn1, associates with TPP1 and is implicated in telomere length regulation. J. Biol. Chem. **284:**26725-26731.

260. **Wang, R. C., A. Smogorzewska, and T. de Lange.** 2004. Homologous recombination generates T-loop-sized deletions at human telomeres. Cell **119:**355-368.

261. **Weinrich, S. L., R. Pruzan, L. Ma, M. Ouellette, V. M. Tesmer, S. E. Holt, A. G. Bodnar, S. Lichtsteiner, N. W. Kim, J. B. Trager, R. D. Taylor, R. Carlos, W. H. Andrews, W. E. Wright, J. W. Shay, C. B. Harley, and G. B. Morin.** 1997. Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. Nat. Genetics **17:**498-502.

262. **Wenz, C., B. Enenkel, M. Amacker, C. Kelleher, K. Damm, and J. Lingner.** 2001. Human telomerase contains two cooperating telomerase RNA molecules. EMBO J. **20:**3526-3534.

263. **West, S. C.** 2003. Molecular views of recombination proteins and their control. Nat. Rev. Mol. Cell Biol. **4:**435-445.

264. **Williams, B., and A. J. Lustig.** 2003. The paradoxical relationship between NHEJ and telomeric fusion. Mol. Cell **11:**1125-1126.

265. **Williams, R. S., G. E. Dodson, O. Limbo, Y. Yamada, J. S. Williams, G. Guenther, S. Classen, J. N. M. Glover, H. Iwasaki, P. Russell, and J. A. Tainer.** 2009. Nbs1 flexibly tethers Ctp1 and Mre11-Rad50 to coordinate DNA double-strand break processing and repair. Cell **139:**87-99.

266. **Williams, R. S., G. Moncalian, J. S. Williams, Y. Yamada, O. Limbo, D. S. Shin, L. M. Groocock, D. Cahill, C. Hitomi, G. Guenther, D. Moiani, J. P. Carney, P. Russell, and J. A. Tainer.** 2008. Mre11 dimers coordinate DNA end bridging and nuclease processing in double-strand-break repair. Cell **135:**97-109.

267. **Williams, R. S., J. S. Williams, and J. A. Tainer.** 2007. Mre11–Rad50–Nbs1 is a keystone complex connecting DNA repair machinery, double-strand break signaling, and the chromatin template. Biochem. Cell Biol. **85:**509-520.

268. **Wong, K. K., and R. A. DePinho.** 2003. Walking the telomere plank into cancer. J. Natl. Cancer Inst. **95:**1184-1186.

269. **Wright, W. E., V. M. Tesmer, K. E. Huffman, S. D. Levene, and J. W. Shay.** 1997. Normal human chromosomes have long G-rich telomeric overhangs at one end. Genes Dev. **11:**2801-2809.

270. **Wu, Y., J. S. Siino, T. Sugiyama, and S. C. Kowalczykowski.** 2006. The DNA binding preference of RAD52 and RAD59 proteins. J. Biol. Chem. **281:**40001-40009.

271. **Yamada, M., N. Hayatsu, A. Matsuura, and F. Ishikawa.** 1998. Y′-Help1, a DNA helicase encoded by the yeast subtelomeric Y′ element,iIs induced in survivors defective fortTelomerase. J. Biol. Chem. **273:**33360-33366.

272. **Yamagata, K., J. Kato, A. Shimamoto, M. Goto, Y. Furuichi, and H. Ikeda.** 1998. Bloom's and Werner's syndrome genes suppress hyperrecombination in yeast *sgs1* mutant: Implication for genomic instability in human diseases. Proc. Natl. Acad. Sci. U.S.A. **95:**8733–8738.

273. **Ying, C., T. M. Bryan, and R. R. Reddel.** 2008. Increased copy number of the TERT and TERC telomerase subunit genes in cancer cells. Cancer Science **99:**1092 - 1099.

274. **Zhu, Z., W. Chung, E. Shim, S. Lee, and G. Ira.** 2008. Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. Cell **134:**981-994.

275. **Zou, L., and S. J. Elledge.** 2003. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science **300:**1542-1548.

CHAPTER 2

Telomeric circles are abundant in the *stn1-M1* mutant that maintains its telomeres through recombination

[2] Basenko, E. Y., A. Cesare, S. Iyer, J. D. Griffith, and M. J. McEachern. 2010. *Nucleic Acids Research*. 31(8):182-189.

Reprinted here with permission of the publisher

Abstract

Some human cancers maintain their telomeres using the Alternative Lengthening of Telomeres (ALT) mechanism; a process thought to involve recombination. Different types of recombinational telomere elongation (RTE) pathways have been indentified in yeasts. In senescing yeast telomerase deletion (*ter1-Δ)* mutants with very short telomeres, it has been hypothesized that copying a tiny telomeric circle (t-circle) by a rolling circle mechanism is the key event in telomere elongation. In other cases more closely resembling ALT cells, such as the *stn1-M1* mutant of *Kluyveromyces lactis*, the telomeres appear to be continuously unstable and routinely reach very large sizes. By employing two-dimensional gel electrophoresis and electron microscopy, we show that *stn1-M1* cells contain abundant double stranded t-circles ranging from ~100-30,000 bp in size. We also observed small single stranded t-circles, specifically composed of the G-rich telomeric strand and tailed circles resembling rolling circle replication intermediates. The t-circles most likely arose from recombination events that also resulted in telomere truncations. The findings strengthen the possibility that t-circles contribute to telomere maintenance in *stn1-M1* and ALT cells.

Introduction

Telomeres protect chromosome ends from nucleolytic degradation, end-to-end fusion and other processes that could compromise genome integrity(35). They are composed of tandem short DNA repeats and the specialized proteins that bind them (32). Telomeres are normally maintained by the enzyme telomerase which compensates for gradual sequence loss due to incomplete DNA replication by adding telomeric repeats onto chromosome termini. In most human somatic cells, telomerase activity is very low (3, 19). This leads to gradual telomere shortening which in turn can trigger replicative senescence, a process where a cell with critically short telomeres permanently exits from the cycle of division (10, 31). In contrast, the great majority of cancers are able to maintain their telomere lengths indefinitely. In most cases this occurs because of an up-regulation of telomerase activity (18). However, some cancers maintain their telomere lengths through a telomerase-independent process termed Alternative Lengthening of Telomeres (ALT) (4). The telomeres in ALT cells are highly heterogeneous, often extremely long and appear to be maintained through homologous recombination (7).

Much of what is known about recombinational telomere elongation (RTE) comes from studies in yeast, particularly *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. Yeast mutants lacking telomerase undergo growth senescence and most cells eventually die (23, 25). The cells that survive senescence are found to have lengthened telomeres through a process dependent upon *RAD52* and other genes involved in homologous recombination (8, 22, 24). Recombination in and near telomeres is greatly increased when the telomeres become short (27, 34). Work in both *K. lactis* and *S. cerevisiae* has suggested that RTE lengthens telomeric repeat arrays (Type II RTE) through a "roll and spread" mechanism (21, 28, 29, 34). According to this model, a small duplex DNA circle consisting of telomeric repeats (t-circle) formed by recombination in

cells with critically short telomeres, is used as a template for extending at least one telomere, through a rolling circle copying event. Once one long telomere is formed, other telomeres become extended by copying its sequence. Appreciable evidence supports this model. We have shown that the sequence of a single long telomere is preferentially copied to all other telomeres during survivor formation (34). Cells transformed with t-circles routinely acquire telomeres extended by tandem copies of the sequence of the transformed circle (28, 29). Additionally, t-circles are abundant in at least some types of cells with dysfunctional telomeres including human ALT cells and a *K. lactis* mutant with altered telomeric repeats (5, 17, 36). For recent reviews of RTE and t-circles, see (26, 33).

It has recently been shown that certain *K. lactis* mutant cells display a form of RTE that is distinct from the RTE that occurs in *ter1*-Δ mutants. The *stn1-M1* mutant causes an amino acid substitution in Stn1, a protein that forms a complex with Cdc13 and Ten1 in *S. cerevisiae*. Stn1 was shown to bind G-rich telomeric substrates as well as to provide an essential protective function at the telomeres (11, 14). This mutation displays moderate growth defects and leads to the rapid formation of very long and heterogeneous telomeres. Unlike a *ter1*-Δ mutant that appears to have a telomere capping defect only when telomeres become very short, the *stn1-M1* mutant has a continuous capping defect that is independent of telomere length. Other examples of this type of RTE, now known as Type IIR (runaway) RTE, have been seen in *K. lactis* telomerase RNA mutants, which generate mutant telomeric repeats (34). The most notable of these appear to be due to alterations in the telomeric repeat that reduce the binding affinity of the double strand telomere binding protein Rap1 (1). Certain *S. cerevisiae cdc13* and *stn1* mutants provide similar examples of RTE independent of the length of the telomeres (12, 13, 30). The features of yeast Type IIR RTE are particularly similar to those observed in human ALT cells.

Learning more about how telomere maintenance occurs in *stn1-M1* cells is therefore of considerable interest. Whether t-circles contribute to either the formation or the maintenance of the long telomeres of Type IIR RTE or of ALT cells is currently unknown. In this study, we show that a broad range of sizes of t-circles are produced in the *stn1-M1* mutant.

Materials and methods

Yeast strains

The strain 7B520 (*ura3-1 his2-2 trp1*) was described previously (37). The *stn1-M1* and *stn1-M1 ter1*-Δ strains used here were also described previously (20). All strains were routinely grown at 30° C.

DNA isolation

Genomic DNA used to generate the telomere restriction fragments separated by one or two-dimensional gel electrophoresis was isolated from 96 (1.5 ml) YPD liquid overnight cultures. Low molecular weight extrachromosomal telomeric DNA for electron microscopic examination was treated with RNA at 37° C for an hour and then at isolated by running uncut genomic DNA on 0.8% agarose gels at 90 V for 60 min. DNA migrating between 500 bp and 3500 bp linear markers was excised from the gel and electro-eluted onto 12-14,000 MWCO Spectra/Por dialysis tubing (Spectrum Laboratories Incorporated, Ranch Dominguez, CA). Electro-eluted DNA was subsequently concentrated using microcon YM-10 spin columns as directed by manufacturer (Amicon Bioseperations, Raleigh, NC). High molecular weight DNA for electron microscopy examination was attained by spheroplasting and isolating nuclei as

described previously (6) with the following modification; the lytic enzyme used was 100 μg/ml Zymolyase 100T (Seikagaku).

Southern and in-gel hybridizations

For one dimensional gel electrophoresis, *Eco*RI (NEB Beverly, MA) digested or undigested genomic DNA was separated on a 0.8 % SeaKem LE agarose gel (Lonza, Rockland Inc., Rockland, ME). For two dimensional gel analysis of low molecular weight DNA, uncut, RNase-treated genomic DNA was separated in a 4% NuSIeve 3:1 agarose gel initially containing 0.6 μg/ml chloroquine (Lonza, Rockland, ME) as previously described (5). In the above experiments, the gels were blotted onto Hybond N+ membrane and probed with either Klac1-25 G-strand telomeric probe (5' ACGGATTTGATTAGGTATGTGGTGT-3') or the Klac-25-1 C-stranded telomeric probe (3'-ACACCACATACCTAATCAAATCCGT-5'). All hybridizations were carried out in the presence of 500 mM Na_2HPO_4 and 7% sodium dodecyl sulfate (SDS) and the washes were done in 100 mM Na_2HPO_4 and 2% SDS. For two-dimensional gel electrophoresis of high molecular weight DNA, *Eco*RI digested DNA was separated along with 50 ng of circularized *Hin*dIII λ fragments, as described previously (6). The gel was blotted, probed with ^{32}P-labeled *K. lactis* telomeric C-strand and then subsequently probed with ^{32}P-labeled *Hin*dIIIλ fragments.

Electron microscopy

Low molecular weight gel-extracted DNA from *stn1-M1* and *stn1-M1 ter1-*Δ cells was incubated with 20 μg/ml T4 gene 32 protein (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% glutaraldehyde 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 absorption onto negatively-charged carbon-coated grids in the presence of spermidine followed by dehydration through a series of gradated ethanol washes, air dying, and rotary shadowcasting with tungsten at $1x10^{-6}$ Torr (6, 15). For examination of high molecular weight telomeric DNA, isolated genomic DNA was digested with *Alu*I, *Hpa*II and *Nla*III (NEB Beverly MA), at enzyme concentration of 1 U/μg for 2 hours, and then supplemented with an equal amount of each enzyme for an additional 2 hours. The telomere restriction fragments were then separated by size exclusion chromatography and the eluted fractions monitored for DNA concentration and telomeric DNA abundance. Telomere enriched fractions were prepared for electron microscopy by surface spreading on a denatured protein film (6, 16). Samples were examined on an FEI Tecnai 12 instrument (Hillsboro, Oregon). 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).

Results

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

The *stn1-M1* mutant generates extremely long telomeres independently of telomerase using Type IIR RTE (Fig. 2.1A) (20). Some of the telomeric hybridization signal seen in uncut genomic DNA from the *stn1-M1* and *stn1-M1 ter1-Δ* cells (the latter deleted for the telomerase

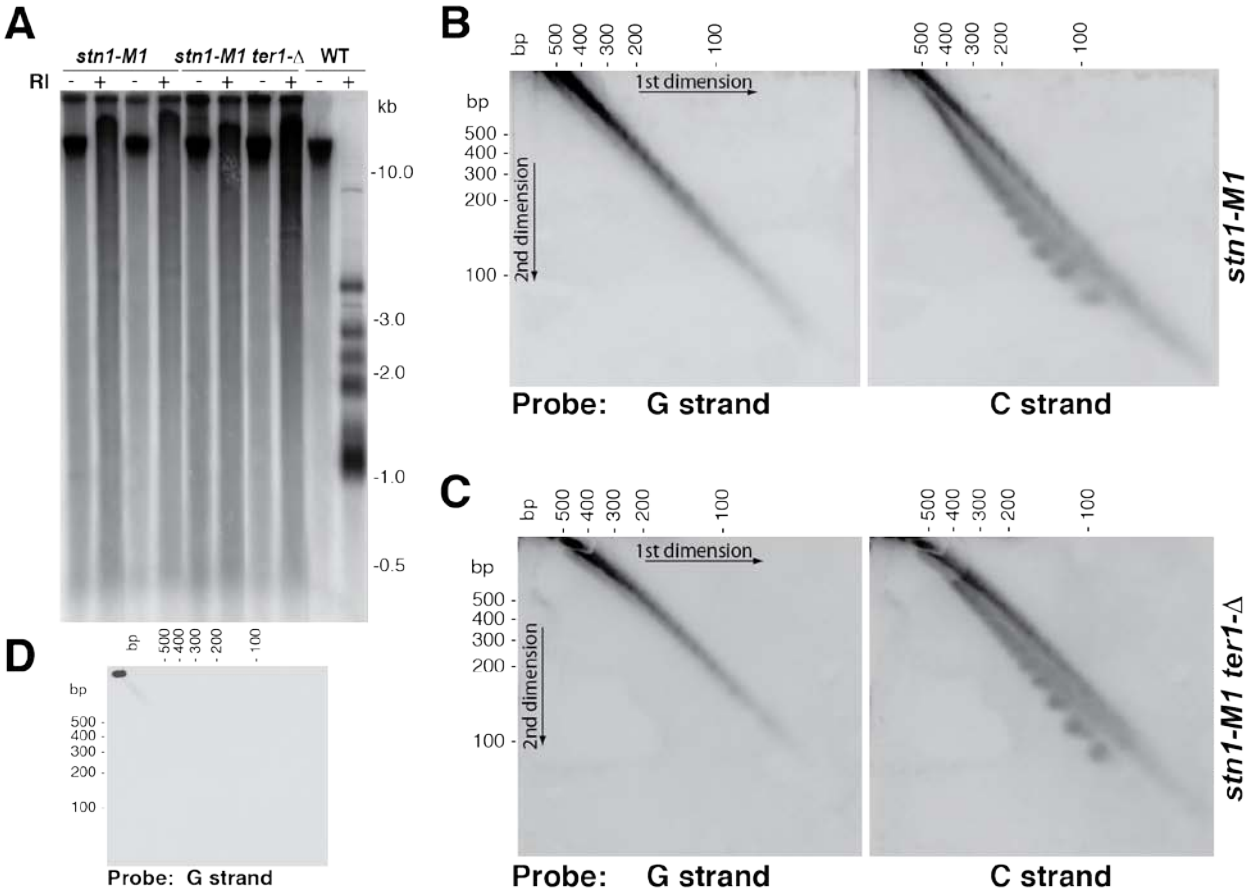

Figure 2. 1: Gel analysis of low molecular weight telomeric DNA from *stn1-M1* and *stn1-M1 ter1-Δ* cells. A) Southern blot of uncut and *Eco*RI digested genomic DNA from wild type (strain 7B520), *stn1-M1* and *stn1-M1 ter1-Δ* run on a one dimensional 0.8% agarose gel and hybridized to a telomeric probe. B-C) Southern blots of uncut genomic DNA from *stn1-M1* and *stn1-M1 ter1-Δ* run on 2D 4% agarose gels hybridized to either the C-strand or G-strand telomeric probes. D). Southern blot of uncut genomic DNA from a wild type control hybridized to G strand telomeric probe.

RNA gene) appeared throughout the length of the gel. This indicated that some of the smear of telomeric signal, particularly at low molecular weights, likely represented extrachromosomal telomeric sequences. Similar smears largely composed of double and single stranded t-circles that hybridized to telomere probes were seen in the long telomere mutant *ter1-16T* (17).

Therefore, we hypothesized that t-circles were being produced in *stn1-M1* cells. As a test of this, we electrophoresed uncut genomic DNA from *stn1-M1* and *stn1-M1 ter1-Δ* cells on 4% agarose two dimensional (2D) chloroquine gels to separate low molecular weight t-circles. Filters blotted from these gels were hybridized to oligonucleotide probes matching sequence from either the G-rich or the C-rich strands of *K. lactis* telomeric DNA (Fig. 2.1B-C). With a G-strand telomeric probe (right panels of Fig. 2.1B-C) we observed similar diagonals of closely spaced diffuse spots in both types of cells. With a C-strand telomeric probe (left panels of Fig. 2.1B-C), we observed the same diagonal plus an additional arc of spots. These results are very similar to the ladders formed from double stranded and single stranded t-circles that were observed previously in DNA from *ter1-16T* cells (17). The ladders of spots represented DNA species containing different integral numbers of the 25 bp *K. lactis* telomeric repeat. Our results suggested that *stn1-M1* and *stn1-M1 ter1-Δ* cells, like *ter1-16T*, produce very small double stranded t-circles as well as very small single stranded t-circles that were composed specifically of the G-rich strand of telomeric sequence. As expected, wild type cells produced no observable low molecular weight telomeric spots (Fig. 2.1D).

We next separated *Eco*RI-digested genomic DNA from *stn1-M1*, *stn1-M1 ter1-Δ* and a wild type control on low percentage agarose 2D gels (first dimension in 0.6% agarose gel and 1.1% agarose in second dimension) to test for the possible presence of high molecular weight t-circles. *Eco*RI does not cleave *K. lactis* telomeric DNA but does separate the rest of the genomic DNA into a variety of sizes that can be visualized on gels. *Hin*dIII fragments of phage λ DNA were ligated into circles and separated along with the *Eco*RI digested genomic DNA as a circular DNA control. The results of this experiment showed that a significant proportion of signal

produced from a telomeric probe in both *stn1-M1* and *stn1-M1 ter1-Δ* cells was present in an arc migrating with the double-stranded relaxed circle controls (2. 2 A-B). This was not observed with DNA from wild type cells (Fig. 2.2C). We conclude that *stn1-M1* and *stn1-M1 ter1-Δ* cells contain abundant high molecular weight t-circles.

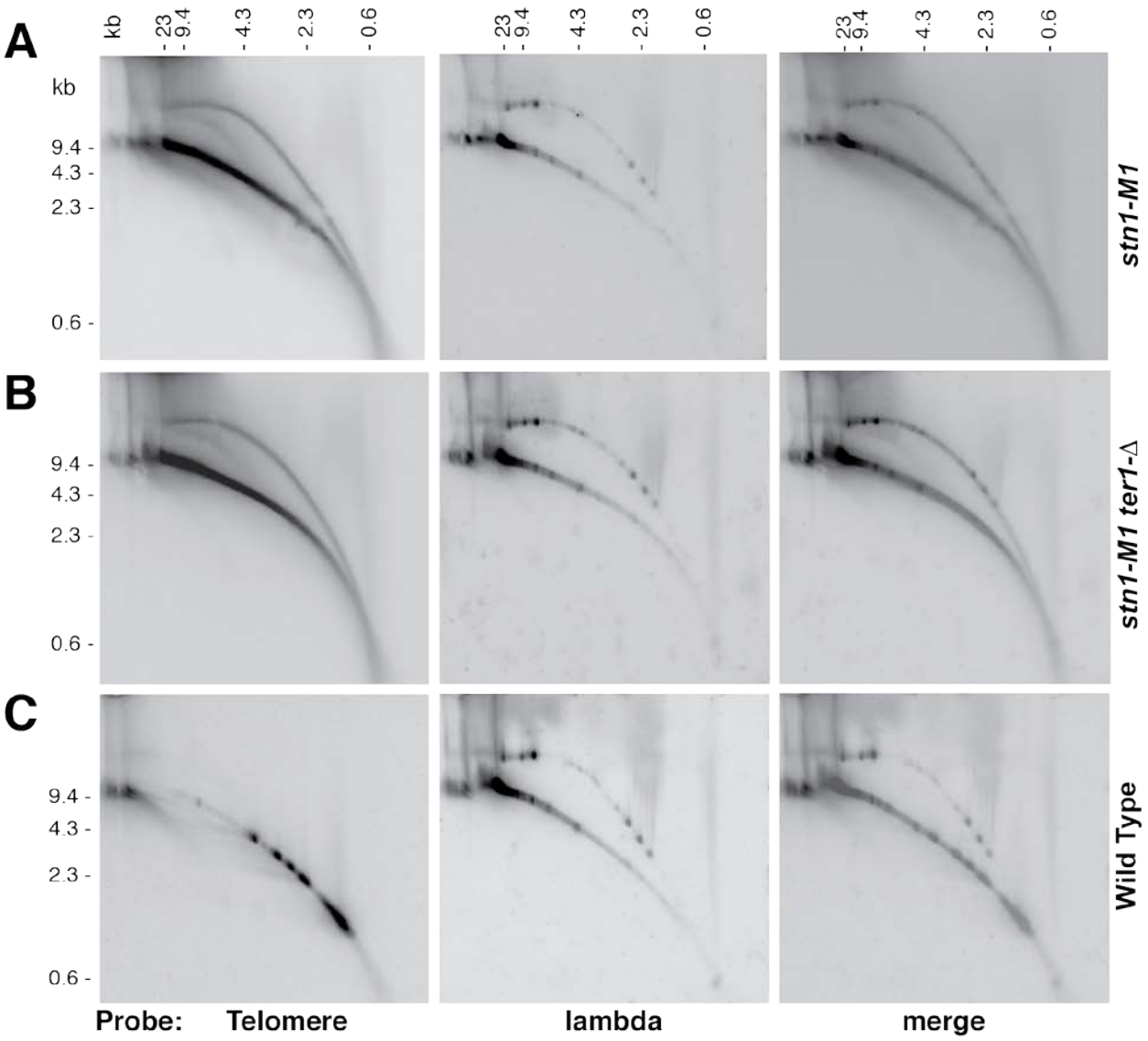

Figure 2. 2: Two -dimensional gel electrophoresis of high molecular weight telomeric DNA from *stn1-M1* and *stn1-M1 ter1-Δ* cells . *Eco*RI digested DNA (8 µg) from wild type (strain 7B520), *stn1-M1* and *stn1-M1 ter1*-Δ cells along with 50 ng of ligated phage λ *Hin*dIII fragments

were separated on low percentage 2D agarose gels (0.6% in the first dimension and 1.1% in the second dimension). Arrows represent arcs corresponding to linear and open circle DNA forms.

Visualization of t-circles by electron microscopy

To confirm the presence of small t-circles in *stn1-M1*, we examined extrachromosomal telomeric DNA from *stn1-M1* and *stn1-M1 ter1*-Δ by electron microscopy (EM). Undigested samples of *stn1-M1* and *stn1-M1 ter1*-Δ genomic DNA were separated on a 0.8% agarose gel and DNA running between 500 and 3500 bp (relative to linear DNA markers) was extracted. Once purified, this DNA was incubated with T4 gene 32 single strand DNA binding protein and visualized by EM (Figure 2.3A-F). In both the *stn1-M1* and *stn1-M1 ter1*-Δ samples we observed a high percentage of the DNA molecules to be circular. Scoring DNAs by EM, 19.5% ± 7.8% were circular in the *stn1-M1* sample and 30.0% ± 6.8% were circular in the *stn1-M1 ter1*-Δ sample. Surprisingly, less than 2% of the circles were single-stranded as judged by gp32 binding. Double-stranded circles lengths from the *stn1-M1* and *stn1-M1 ter1*-Δ samples were measured and the results are shown in Fig. 2.3G. Nearly all of the circles observed were less than 1 kb and most measured between 175-300 bp. This is very similar to the size distribution of t-circles observed by EM in DNA isolated from *ter1-16T* cells using the same protocol (17). The reason why single stranded circles were much less common in this experiment compared to the 4% agarose 2D gels is not clear.

In addition to fully circular DNA molecules, we also visualized a small number of double stranded DNA circles with tails of varying sizes, examples of which are shown in Fig. 2.4. These structures represented less than 3.5% of the total number of molecules visualized in the *stn1-M1* samples. In a few instances, these molecules were bound by T4 gene 32 protein at the base of the tail indicating the presence of a small region of single stranded DNA at the circle-tail

junction (Fig. 2.4C). These molecules conceivably represent intermediates in the formation or processing of t-circles, or circles undergoing rolling-circle replication.

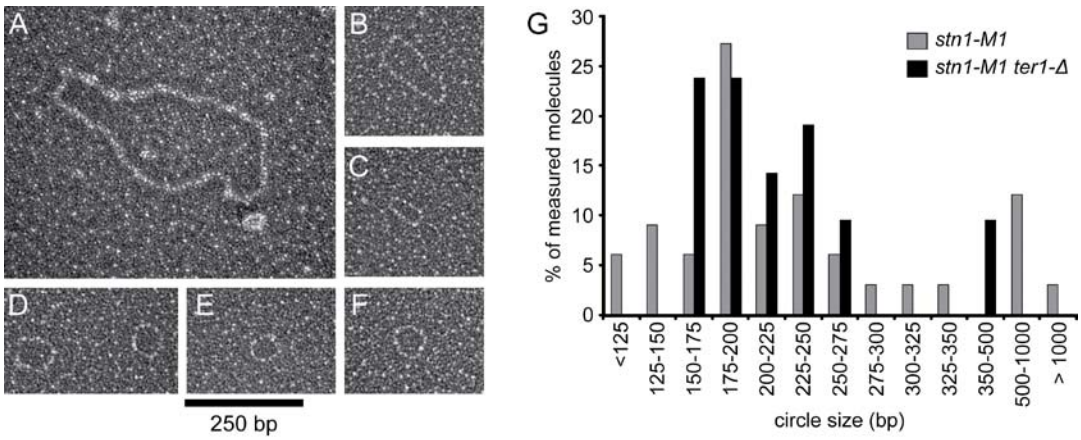

Figure 2. 3: Electron microscopy visualization of low molecular weight DNA circles from *stn1-M1* and *stn1-M1 ter1*-Δ cells. A-F) Electron micrographs of low molecular weight double strand t-circles. Circle sizes are 1320, 428, 191, 216 and 258, 187, and 201 bp respectively for A-F. Samples were mounted onto thin carbon foils, shadowcast with tungsten and are shown in negative contrast. Bar is equivalent to 250 bp. G) Size distribution of observed DNA circles from *stn1-M1* and *stn1-M1 ter1*-Δ. Measured double stranded circles observed in low molecular weight extrachromosomal DNA isolated from *stn1-M1* (n = 33) and *stn1-M1 ter1*-Δ (n = 21) cells.

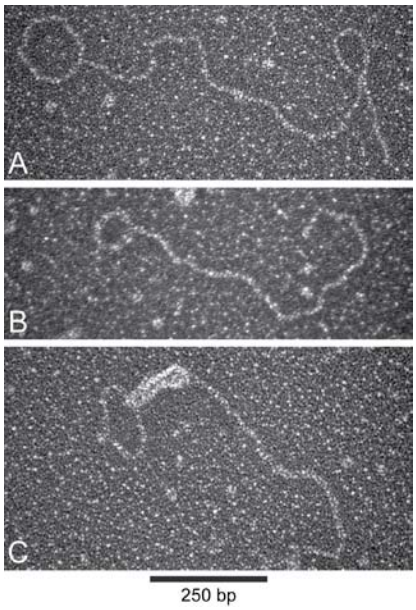

Figure 2. 4: Visualization of tailed circles from *stn1-M1* and *stn1-M1 ter1*-Δ mutant cells.

A-B) Electron micrographs of tailed circle DNA structures. Circular and tail portions of molecules shown in A-B are: 439 and 1361, and 252 and 918 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 in Figure 2.3. 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 796 bp. Bar is equivalent to 250 base pairs.

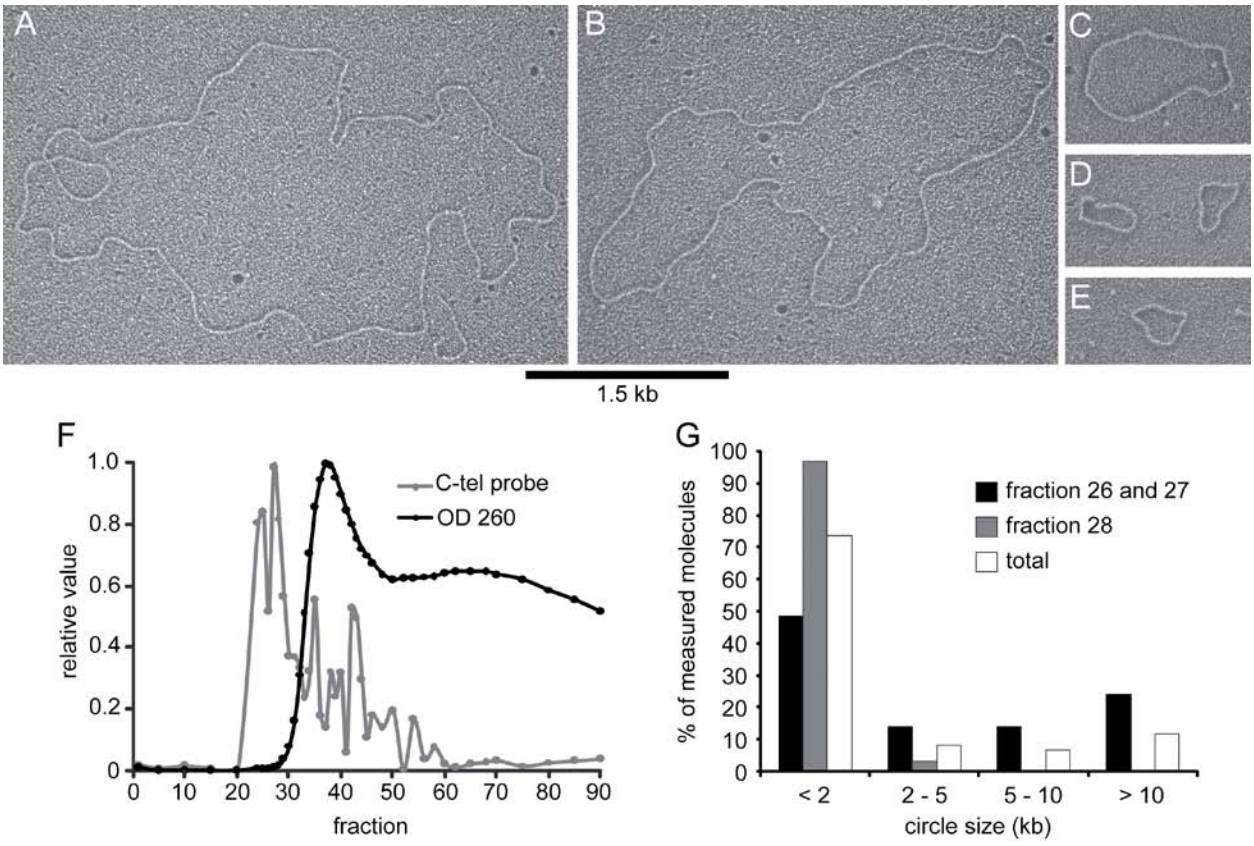

Figure 2. 5: Visualization of DNA circles from high molecular weight telomere-enriched DNA from *stn1-M1* A-E) Electron micrographs of DNA circles observed in the telomere enriched fractions from *stn1-M1*. Circle lengths for the molecules in A-E respectively are 15.7, 12.1, 3.1, 1.2 and 1.2, and 0.9 kb for fractions A-E respectively. Note that there is also a ~0.8 kb linear fragment in the lower right portion of panel A. Samples were prepared by coating the DNA in denatured cytochrome c and are shown in negative contrast. Bar is equivalent to 1.5 kb. F) Relative DNA abundance and telomeric DNA content of eluted fractions from gel chromatography separation of *Alu*I, *Hpa*II, and *Nla*III dirgest of *stn1-M1* genomic DNA. Molecules shown in A-E are from fractions 26-28. Size distribution of the measured circles from fractions 26-27 (n = 29) and 28 (n = 32).

To visualize the large t-circles by EM, high molecular weight telomere restriction fragments from *stn1-M1* cells were enriched by gel-exclusion chromatography, as described previously (6, 17). Briefly, crude nuclei from *stn1-M1* cells were isolated and total genomic DNA was digested with *Alu* I, *Hpa* II and *Nla* III restriction enzymes. This reduces the genomic DNA to very small sizes while leaving telomeric repeat tracts intact. The digested genomic DNA was then separated in a long gel-filtration chromatography column, and the eluted fractions were assayed for total and telomeric DNA content (Figure 2.5F). The fractions highly enriched for telomeric DNA were concentrated and examined by EM (15). In the telomere-enriched fractions, we observed a large number of t-circles ranging in size from 0.3 to 31 kb (Figure 2.5A-E). The majority of circles were small, with 73% of the circles from the telomeric enriched fractions measuring less than 3 kb in total length, and 11.5 % measuring over 10 kb (n=61). These results are similar to previous experiments in human ALT cells where the bulk of circular molecules were rather small compared to the average telomere size (5).

Discussion

The results presented here demonstrate that a broad size range of telomeric circles, from ~100 bp to >30 kb, are produced in the *K. lactis stn1-M1* mutant that maintains its telomeres using Type IIR RTE. Both small and large t-circles are formed independently of the presence of telomerase in the mutant cells. As telomeric DNA lacks the ability to initiate replication, the t-circles present in *stn1-M1* cells must be recent products of recombination rather than heritably replicating episomes. Because *stn1-M1 rad52* cells are inviable (20), it has not been possible to directly demonstrate that homologous recombination is required for formation of t-circles in these cells. However, t-circles in other systems, including *K. lactis ter1-16T* cells, have

previously been demonstrated to depend upon Rad52 or other homologous recombination genes (9, 17, 21, 36). The presence of the small t-circles from *stn1-M1* cells as a discrete series of spots on 2D gels, as seen previously with t-circles from the *ter1-16T* mutant (17), strongly favors the idea that these spots are composed of integral numbers of telomeric repeats, as expected for a recombination process dependent upon homology.

We conclude that the t-circle formation in *stn1-M1* cells is linked to the chronic telomere capping defects of this mutant. This defect results not only in highly elongated telomeres but also in abnormal cell and colony growth, large 3' telomeric overhangs and greatly increased rates of both subtelomeric recombination and telomeric truncation events (20). Unlike the Type II RTE of *ter1*-Δ mutants, where capping defects occur as a result of telomeres becoming too short, cells undergoing Type IIR RTE have capping defects believed to be independent of telomere length and are results of disruptions in the functioning of telomere proteins. These continuous capping defects presumably underlie the extreme telomere lengths and abundant products of telomeric recombination such as t-circles.

The abundance of t-circles in *stn1-M1* cells is compatible with them playing a role in the RTE that occurs in those cells. Although strong circumstantial evidence favors the hypothesis that small t-circles are often involved in the Type II RTE that occurs in yeast *ter1*-Δ mutants (28, 29), there is no evidence to date that t-circles are involved in the Type IIR RTE of *stn1-M1* cells or the apparently similar recombinational telomere maintenance of human ALT cells. The proposed role of t-circles in Type II RTE of *K. lactis ter1*-Δ mutants is in building the first relatively long telomere through a rolling circle copying event in a cell that contains only short telomeres. Once one long telomere is present, t-circles may no longer be necessary, as other telomeres may be lengthened by directly copying the sequence of the long telomere (34). We

hypothesize that the most significant possible role for t-circles in Type IIR RTE might be in the initial establishment of the long-telomere state (such as would occur in a newly germinated *stn1-M1* spore from a *STN1*/*stn1-M1* diploid). There, the formation of extremely long telomeres from the normal length telomeres initially present (~500 bp in *K. lactis*) could be accelerated by the rolling circle copying of a t-circle. However, even if not vital to the maintenance of Type IIR RTE, t-circles could play a significant role in telomere elongation due to their high abundance in the cell. It is believed that in *K. lactis ter1*-Δ mutants, the telomere lengthening from type II RTE (typically hundreds of bp) is thought to be limited by both the rarity of t-circles in those cells and the poorly processive copying of extremely small (~100 bp) t-circles. Many, if not most, of the larger t-circles in *stn1-M1* cells would likely be capable of producing much greater extensions. It is known, for example, that 1.6 kb *URA3*-telomere circles transformed into *K. lactis* cells routinely produce telomere extensions of >10,000 bp (28, 29).

The precise mechanism of t-circles formation in *stn1-M1* cells, or in other cells where they have been observed, is currently unknown. A previously proposed hypothesis is that a t-circle can be formed via a t-loop intermediate whereby the 3' end of the telomere strand invades a more internal region of the same telomere (17, 36). In favor of this possibility, t-loop structures have been observed in both human ALT cells and *K. lactis ter1-16T* cells (5, 6). Furthermore, the loop portions of these structures exhibit size distributions similar to those of t-circles observed in the same systems. T-circle formation from t-loops would also be expected to cause a deletion of the telomere involved in the process(2). Among the extrachromosomal structures observed in this study by EM were a small percentage of tailed circles. These likely represent either t-loops or rolling circle intermediates.

Small single stranded t-circles, specifically composed of the G-rich strand of telomeric sequence, were previously observed in *ter1-16T* (17) cells where they were approximately as abundant as double stranded circles, at least for circles <500 bps/nts. Both types of t-circles were absent in *ter1-16T rad52* cells, indicating they are generated by homologous recombination (17). It was suggested that the processing of a t-loop intermediate to form a t-circle often initially produced a transient t-circle that was partially double stranded and partially single stranded. Further processing then could produce more stable t-circles that were either fully double stranded or fully single stranded. The reason why the relative abundance of single stranded t-circles varied considerably between the EM and 2D gel analysis remains unclear. Variation in the amount lost during purification of yeast DNA is a possible contributing factor. The relatively small sizes of the single stranded t-circles likely renders them vulnerable to being lost during alcohol precipitations or dialysis. Additionally, we have observed considerable variation in both the total amount of telomeric signal and the amount of small extrachromosomal telomeric DNA from independent isogenic isolates of *stn1-M1* extracted by the same method (J. Xu, S. Iyer, E. Basenko and M. McEachern, unpublished data). It is quite possible that the proportion of single stranded t-circles may also be naturally variable.

The identification of t-circles in *stn1-M1* cells helps further underscore the similarities between the Type IIR RTE of yeast cells and the ALT phenomenon of some human cancers. Learning more about the mechanism of Type IIR RTE in yeast will certainly help to provide insights into how ALT occurs and what mutations underlie it.

Acknowledgements

This work was supported in part by grants from the National Institutes of Health to M.J.M (GM61645) and to J.D.G. (GM31819, ES13773) and a summer fellowship from the American Federation for Aging Research to S.I.

References

1. **Bechard, L. H., B. D. Butuner, G. J. Peterson, W. McRae, Z. Topcu, and M. J. McEachern.** 2009. Mutant telomeric repeats in yeast can disrupt the negative regulation of recombination-mediated telomere maintenance and create an alternative lengthening of telomeres-like phenotype. Mol. Cell. Biol. **29:**626-639.

2. **Bhattacharyya, M. K., and A. J. Lustig.** 2006. Telomere dynamics in genome stability. Trends in Biochem. Sci. **31:**114-122.

3. **Bodnar, A. G., M. Ouellette, M. Frolkis, S. E. Holt, C.-P. Chiu, G. B. Morin, C. B. Harley, J. W. Shay, S. Lichtsteiner, and W. E. Wright.** 1998. Extension of life-span by introduction of telomerase into normal human cells. Science **279:**349-352.

4. **Bryan, T. M., L. Marusic, S. Bacchetti, M. Namba, and R. R. Reddel.** 1997. The telomere lengthening mechanism in telomerase-negative immortal human cells does not involve the telomerase RNA subunit. Hum. Mol. Genet. **6:**921-926.

5. **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:**9948-9957.

6. **Cesare, A. J., C. Groff-Vindman, S. A. Compton, M. J. McEachern, and J. D. Griffith.** 2008. Telomere loops and homologous recombination-dependent telomeric circles in a *Kluyveromyces lactis* telomere mutant strain. Mol. Cell. Biol. **28:**20-29.

7. **Cesare, A. J., and R. R. Reddel.** Telomere uncapping and alternative lengthening of telomeres. Mech. Ageing Dev. **129:**99-108.

8. **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:**1819-1827.

9. **Compton, S. A., J.-H. Choi, A. J. Cesare, S. Ozgur, and J. D. Griffith.** 2007. Xrcc3 and Nbs1 are required for the production of extrachromosomal telomeric circles in human alternative lengthening of telomere cells. Cancer Res. **67:**1513-1519.

10. **Deng, Y., S. S. Chan, and S. Chang.** 2008. Telomere dysfunction and tumour suppression: the senescence connection. Nat. Rev. Cancer **8:**450-458.

11. **Gao, H., R. B. Cervantes, E. K. Mandell, J. H. Otero, and V. Lundblad.** 2007. RPA-like proteins mediate yeast telomere function. Nat. Struct. Mol. Biol. **14:**208-214.

12. **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:**3721-3734.

13. **Grandin, N., C. Damon, and M. Charbonneau.** 2001. Cdc13 prevents telomere uncapping and Rad50-dependent homologous recombination. the EMBO J. **20:**6127-6139.

14. **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:**512-527.

15. **Griffith, J. D., and G. Christiansen.** 1978. Electron microscope visualization of chromatin and other DNA-protein complexes. Ann. Rev. Biophys. Bioeng. **7:**19-35.

16. **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. **97:**503-514.

17. **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:**4406-4412.

18. **Harley, C. B., N. W. Kim, K. R. Prowse, S. L. Weinrich, K. S. Hirsch, M. D. West, S. Bacchetti, H. W. Hirte, C. M. Counter, C. W. Greider, and e. al.** 1994. Telomerase, cell immortality, and cancer. Cold Spring Harbor symposia on quantitative biology **59:**307-315.

19. **Hathcock, K. S., Y. J. Chiang, and R. J. Hodes.** 2005. In vivo regulation of telomerase activity and telomere length. Immun. Reviews **205:**104-113.

20. **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:**8064-8073.

21. **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 Δ-Mediated Telomere-Telomere Recombination in *Saccharomyces cerevisiae*. Eukaryot. Cell **4:**327-336.

22. **Lundblad, V., and E. H. Blackburn.** 1993. An alternative pathway for yeast telomere maintenance rescues est1- senescence. Cell **73:**347-360.

23. **Lundblad, V., and J. W. Szostak.** 1989. A mutant with a defect in telomere elongation leads to senescence in yeast. Cell **57:**633-643.

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

25. **McEachern, M. J., and E. H. Blackburn.** 1995. Runaway telomere elongation leads to senescence in yeast. Nature**:**403-409.

26. **McEachern, M. J., and J. E. Haber.** 2006. Break-induced replication and recombinational telomere elongation in yeast. Ann. Rev. of Biochem. **75:**111-135.

27. **McEachern, M. J., and S. Iyer.** 2001. Short Telomeres in Yeast Are Highly Recombinogenic. Mol. Cell **7:**695-704.

28. **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:**1115-1127.

29. **Natarajan, S., and M. J. McEachern.** 2002. Recombinational telomere elongation promoted by DNA circles. Mol. Cell. Biol. **22:**4512-4521.

30. **Petreaca, R. C., H.-C. Chiu, and C. I. Nugent.** 2007. The role of Stn1p in *Saccharomyces cerevisiae* telomere capping can be separated from its interaction with Cdc13p. Genetics **177:**1459-1474.

31. **Shawi, M., and C. Autexier.** Telomerase, senescence and ageing. Mech. Ageing Dev. **129:**3-10.

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

33. **Tomaska, L., J. Nozek, J. Kramara, and J. D. Griffith.** in press. Telomeric circles: Are they universal players in telomere maintenance? Nat. Struct. Biol.

34. **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. Nat. Acad. Sci. USA **102:**3348-3353.

35. **Verdun, R. E., and J. Karlseder.** 2007. Replication and protection of telomeres. Nature **447:**924-931.

36. **Wang, R. C., A. Smogorzewska, and T. de Lange.** 2004. Homologous Recombination Generates T-Loop-Sized Deletions at Human Telomeres. Cell **119:**355-368.

37. **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:**1111-1121.

CHAPTER 3

Recombination can either help maintain very short telomeres or generate longer telomeres in yeast cells with weak telomerase activity

[3] Basenko, E. Y., Z. Topcu, and M. J. McEachern. 2011. *Eukaryotic Cell*. 10(8): 1131-1142. Reprinted here with permission of the publisher

Abstract

Yeast mutants lacking telomerase are able to elongate their telomeres through processes involving homologous recombination. In this study, we investigated telomeric recombination in several mutants that normally maintain very short telomeres due to the presence of a partially functional telomerase. The abnormal colony morphology present in some mutants correlated with especially short average telomere length and with a requirement for *RAD52* for indefinite growth. Better growing derivatives of some of the mutants were occasionally observed and found to have substantially elongated telomeres. These telomeres were composed of alternating patterns of mutationally-tagged telomeric repeats and wild type repeats, an outcome consistent with amplification occurring via recombination rather than telomerase. Our results suggest that recombination at telomeres can produce two distinct outcomes in the mutants we studied. In occasional cells, recombination generates substantially longer telomeres, apparently through the roll-and-spread mechanism. However, in most cells, recombination appears limited to helping maintain very short telomeres. This latter outcome likely represents a simplified form of recombinational telomere maintenance that is independent of generating and copying telomeric circles.

Introduction

Telomeres are important guardians of chromosomal stability. In most human somatic cells, telomeres gradually become shorter due to incomplete replication of chromosomal ends and little or no telomerase activity (31). This can trigger replicative senescence or apoptosis that can serve to reduce the likelihood of cancer formation (26). However, abnormal cells that bypass cell cycle arrest can further develop into cancers and achieve cellular immortality through the activation of a telomere maintenance pathway (56). While telomere elongation in most cancers is dependent on the enzyme telomerase, a significant minority use a telomerase-independent pathway known as Alternative Lengthening of Telomeres (ALT) (10, 53). Considerable evidence suggests that ALT occurs through homologous recombination. ALT cells display high rates of telomeric recombination including sister chromatid exchange (1, 35) and the persistent presence of telomere-induced DNA damage foci (TIFs) (53). Most ALT cells have ALT-associated promyelocytic leukemia bodies (APBs) that contain telomere-specific binding and recombinational proteins and various forms of telomeric DNA (25, 68). Extrachromosomal telomeric DNA can occur in linear and circular forms (t-circles) which were suggested to contribute to telomere elongation in cancer cells (8, 24, 43). ALT tumors can sometimes test positive for both telomerase and ALT, but it is not definitively known if both pathways can coexist in individual cells (51, 62).

The budding yeasts *K. lactis* and *S. cerevisiae* have been used extensively as models for recombinational telomere elongation (RTE) (30, 36, 38, 39, 55). Deletion of telomerase in either species leads to a growth senescence characterized by a decline in growth rate and increasing irregularity in colony shape (27, 36, 37). This abnormal colony phenotype correlates with critically short telomeres becoming uncapped and triggering DNA damage responses including

cell cycle arrest. Many cells in late senescent cultures of mutants lacking telomerase are abnormally enlarged and have cell division defects (15, 27). The majority of senescing cells die within 50-100 cell divisions, but post-senescence survivors emerge with improved growth rates and telomeres that have been elongated by recombination (36, 37). In *S. cerevisiae*, telomerase deletion survivors are classified as Type I and Type II based on their telomere patterns and growth rate. Type I survivors exhibit amplification of subtelomeric Y'-elements with short telomeric tracts and have relatively slower growth and have short terminal telomeric tracts (11, 36, 57). Type II survivors, on the other hand, lack subtelomeric amplification, grow relatively rapidly and have long telomeric repeat tracts. Even though both pathways require *RAD52*, the requirements for other recombination proteins differ between the two pathways (32, 36, 39, 57). Mutations in proteins that act at telomeres and non-telomeric ends can influence the extent and speed of senescence as well as the structure of the telomeres produced by recombination (5, 18, 29, 48).

Post-senescence survivors of *K. lactis* telomerase deletion mutants (*ter1-Δ)* show moderate recombinational elongation of telomeric tracts and therefore are classified as Type II survivors. Considerable evidence, particularly from *K. lactis*, suggests that Type II RTE occurs through a roll-and-spread mechanism whereby a t-circle is used as a template in the rolling circle copying event to produce a substantially elongated telomere, which in turn becomes a template for break-induced replication (BIR) events that copy its sequence onto other short telomeres in the cell (34, 44, 45, 58). It was shown that post-senescence survivors generated in a *ter1-Δ* mutant with only terminal telomeric repeats being mutationally-tagged repeats often emerge with repeating patterns of the wild type and tagged repeats in their telomeres (45). This has been interpreted as stemming from a t-circle composed of both repeat types being a template in a rolling circle event

that generates the first long telomere. T-circles as small as 100 nt, when transformed into *K. lactis* cells, have been demonstrated to become incorporated at telomeres as tandem arrays (44, 45). Furthermore, single- and double stranded telomeric circles as small as ~100 nt/bp have been found in yeast mutants with dysfunctional telomeres (2, 9, 23, 34). Other evidence has verified a key prediction of the roll and spread model, namely that a single telomere can be the source of all elongated telomeric sequences in a post-senescence survivor (58).

Variations of type II RTE that occur in cells other than telomerase deletion mutants have also been documented in yeast. Mutations in the Cdc13/Stn1/Ten1 complex that binds and protects telomeric single strand overhangs, can lead to RTE that occurs without senescence and in the presence of telomerase (18-20, 28). The maintenance of telomeres in the *stn1-M1* mutant of *K. lactis* has been labeled as type IIR RTE because of the much longer 'runaway' telomeres that it produces relative to a telomerase deletion mutant (28). Other examples of Type IIR RTE were observed in telomerase mutants that had telomeres composed of certain mutant telomeric repeats, some with a disrupted ability to bind the double strand telomere binding protein Rap1 (3, 58). T-circles were shown to be present in cells undergoing Type IIR RTE (2, 3) but whether this type of RTE occurs through a roll-and-spread mechanism is currently unknown.

Here we show that certain strains of *K. lactis* with very short telomeres caused by compromised telomerase activity are capable of using recombination in the maintenance of their telomeres. In some cases, this recombination acts to simply help maintain the very short telomeres. However, in some cells of these mutants, recombination can generate substantially longer telomeres. Telomeric repeat tagging experiments suggest that this elongation occurs via a roll-and-spread mechanism.

Materials and Methods

Strains and growth conditions.

All yeast strains were streaked from a single colony every 3 days and grown at 30°C on solid rich, YPD, medium (yeast extract, peptone, dextrose). As a control strain we used wild type *K. lactis* strain 7B520 (*ura3-1*, *his2-2*, and *trp1*) (37, 67). All *ter1* mutants are derivatives of 7B520 and were constructed through the plasmid loop-in/loop-out procedure described previously (37, 38). The *ter1-28C(Taq), ter1-Dup21*-25, *ter1-24T(SnaB)*, *ter1-Bgl(fill)* were described previously (40) and are depicted in Figure 3.1. The base change at the -4 position in *ter1-Dup21*-25 and *ter1-ΔL-ApaL* causes slight telomere shortening by itself (63).

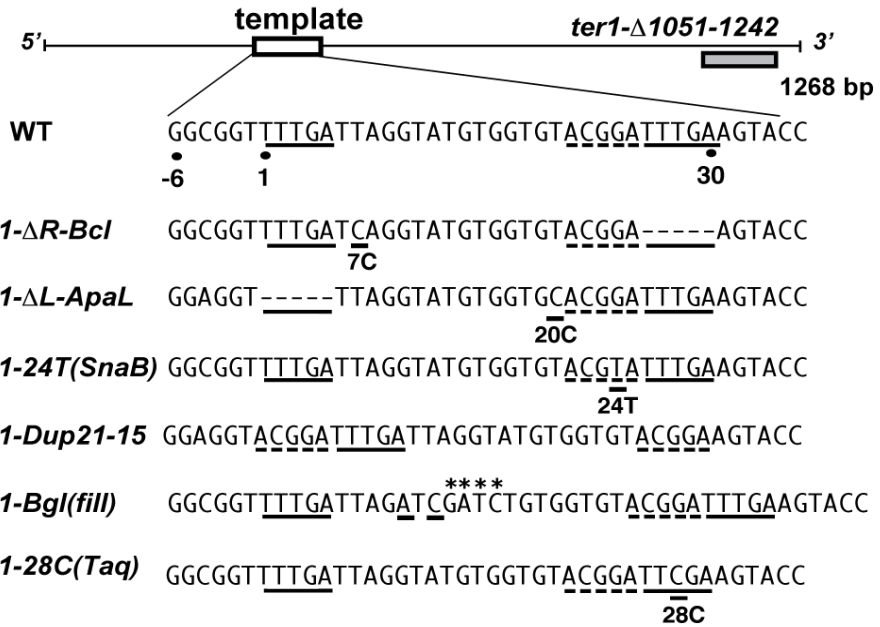

Figure 3. 1. *K. lactis* telomerase RNA and mutations used in this study. A map of the telomerase RNA gene (*TER1)* depicts the locations of the 30 bp template (white box) and the *ter1-Δ1051-1242* mutation (gray box). The sequence of telomeric DNA compliment to telomerase RNA is shown below for WT and *ter1* mutants. The first and last bases of the 30 bp template region are indicated by a black dot below the wild type (WT) sequence. The TTTGA terminal repeats of the template, indicated by solid line, are necessary for accurate telomerase alignment and binding during translocation events. Single nucleotide mutations are underlined by short black bars, deletions are defined by a hyphen, and insertions by asterisks. Phenotypically silent mutations, the *7C(Bcl) and 20C(ApaL),*create *Bcl*I and *Apa*LI restriction sites, respectively.

The *ter1-24T(SnaB)* strain carries a single base change creating *Sna*BI restriction site within telomeric the Rap1-binding site which also affects the translocation step of telomerase (65). The *ter1-Dup21-25* has a permutated template shifted 5 bp that is predicted to make wild-type telomeric repeats. The new 5 bp terminal repeats of this template are indicated by the dashed underlines. The equivalent single copy of this sequence is also indicated in the WT and other mutants. The *ter1-28C(Taq)* mutant contains a base change in the 5' terminal direct repeat of *TER1* template interfering with telomerase translocation and leading to the synthesis of 31 bp repeats (64).

The *K. lactis rad52-Δ* mutant was described previously (40). The *ter1 rad52-Δ* double mutants were generated by mating single mutant strains and sporulation of the resulting diploids. Matings were done on solid media containing malt extract. Diploids were grown on selective plates and their sporulation was performed on minimal sporulation media. Spores were dissected using a Zeiss Axiolab E microscope equipped with a micromanipulator (Carl Zeiss Inc, Thornwood, NY) and then grown on YPD solid media containing 1 M sorbitol. Confirmation of the presence of the *rad52-Δ* allele was done by Southern blotting, while *ter1* alleles were confirmed by visualization of rough colony morphology (which correlates with short telomeres) and/or Southern blotting.

Gel electrophoresis and Southern blotting.

Restriction digests of yeast genomic DNA were generally run on 0.8% agarose gels using 0.5X Tris Borate buffer. *Bsr*BI-digested DNA was resolved on 3.2% 3:1 NuSieve agarose gel (Cambrex Bio Science Rockland Inc., Rockland, ME) or on 6% polyacrylamide (Bio-Rad Laboratories, Hercules CA).

For Southern blotting, DNA from agarose gels was transferred onto Hybond N+ (Amersham Biosciences, Piscataway, N.J.) membranes using 0.4M NaOH. This was followed by UV-crosslinking membrane treatment in electronic ultraviolet crosslinker (Ultra Lum, Inc.

Paramount, CA). Telomeric signal was mostly visualized by hybridization to the Klac1-25 oligonucleotide (ACGGATTTGATTAGGTATGTGGTGT) labeled with γ-^{32}P ATP. To detect changes in *Bcl*I-marked telomeres a γ-^{32}P ATP-labeled Bcl oligo, Klac Bcl (GATCAGGTATGTGG) (61), was used, where the underlined nucleotide indicates the Bcl mutation. Hybridizations were carried out for 4-16 hours in 7% SDS, 0.5M EDTA, and 0.5M Na_2HPO_4 at 48°-55° C. Membranes were then washed three times for 5 min. each in 100 mM Na_2HPO_4 and 2% SDS at the same temperature. Southern blotting was visualized using a Storm Phosphoimager (Amersham Biosciences, Piscataway, N.J.).

Results

Homologous recombination contributes to the viability of some short telomere mutants.

Numerous mutations in the *K. lactis TER1* gene, including hypomorphic mutations, produced telomeres that stabilized at substantially shorter than wild type length (40, 60, 63). Some of these mutants exhibited colony morphologies ranging from slight to appreciable roughness reminiscent of those of senescing *ter1-Δ* cells but without the progressively worsening viability. This suggested that stable short telomeres can sometimes have telomere capping defects that are severe enough to cause growth deficiencies. Because highly shortened telomeres in yeast can frequently be repaired through homologous recombination, we sought to examine the role of recombination in the survival of three mutants with stable short telomeres. One of these, *ter1-Bgl(fill)*, produced a mild rough colony phenotype and two others, *ter1-ΔR-Bcl*, and *ter1-Δ1051-1242*, produced a pronounced rough colony phenotypes (Fig. 3.2A). The *ter1-Bgl(fill)* mutation contains insertion of GATC and two single base changes predicted to result in

the synthesis of 29 bp telomeric repeats (4 bp longer than wild type) having *Cla*I restriction sites (Fig. 3.1). The *ter1-ΔR-Bcl* mutant has two template alterations: a deletion of the 5 bp terminal repeat and a phenotypically silent point template mutation that creates a *Bcl*I restriction site. The terminal repeat deletion is predicted to render telomerase unable to carry out the translocation step of telomerase function and thus be unable to add more than a single repeat at a time onto a telomeric end. The *ter1-Δ1051-1242* strain has the silent *Bcl* template mutation as well as a large deletion near the 3' end of the *TER1,* which encompasses the region harboring phylogenetically conserved sequences CS5, CS6, and most of CS7 (52, 61). It has been previously shown that while CS5 and CS6 are partially dispensable for telomerase activity, CS7 is critical for Sm protein binding and therefore stability of telomerase enzymatic complex *in vivo* (54, 61). These three mutations were combined via mating and sporulation with a deletion of *RAD52*, a gene essential to most forms of homologous recombination in yeast. Three to nine independent single and double mutants of each strain were followed over several serial streaks on rich (YPD) medium. The control *TER1 rad52-Δ* mutants grew somewhat more slowly than *TER1 RAD52* strains but produced completely normal colony morphology that remained similar in size and appearance over each of the six serial streaks (data not shown). These results indicated that, as expected, the deletion of *RAD52* itself does not cause a rough colony phenotype or growth senescence. The control *ter1-Δ RAD52* strains senesced and produced better growing survivors by the 4^{th} or 5^{th} streaks as has been reported previously (37) (Fig. 3.2B). In contrast to *ter1-Δ RAD52*, all three *ter1-Δ rad52-Δ* clones produced smaller colonies and a more severe rough colony phenotype during senescence and became completely unviable by the 3^{rd} or 4^{th} streaks (Fig. 3.2B). This confirmed that in the absence of telomerase the elongation of short uncapped telomeres is dependent on *RAD52*. Deletion of *RAD52* in the *ter1* mutants with stably short

telomeres produced different responses. Each of eight of the *ter1-Bgl(fill) rad52-Δ* mutants exhibited almost no change in their very mild rough colony phenotype over the course of six serial re-streak times (data not shown). We conclude that recombination does not contribute significantly to the survival of *ter1-Bgl(fill)*.

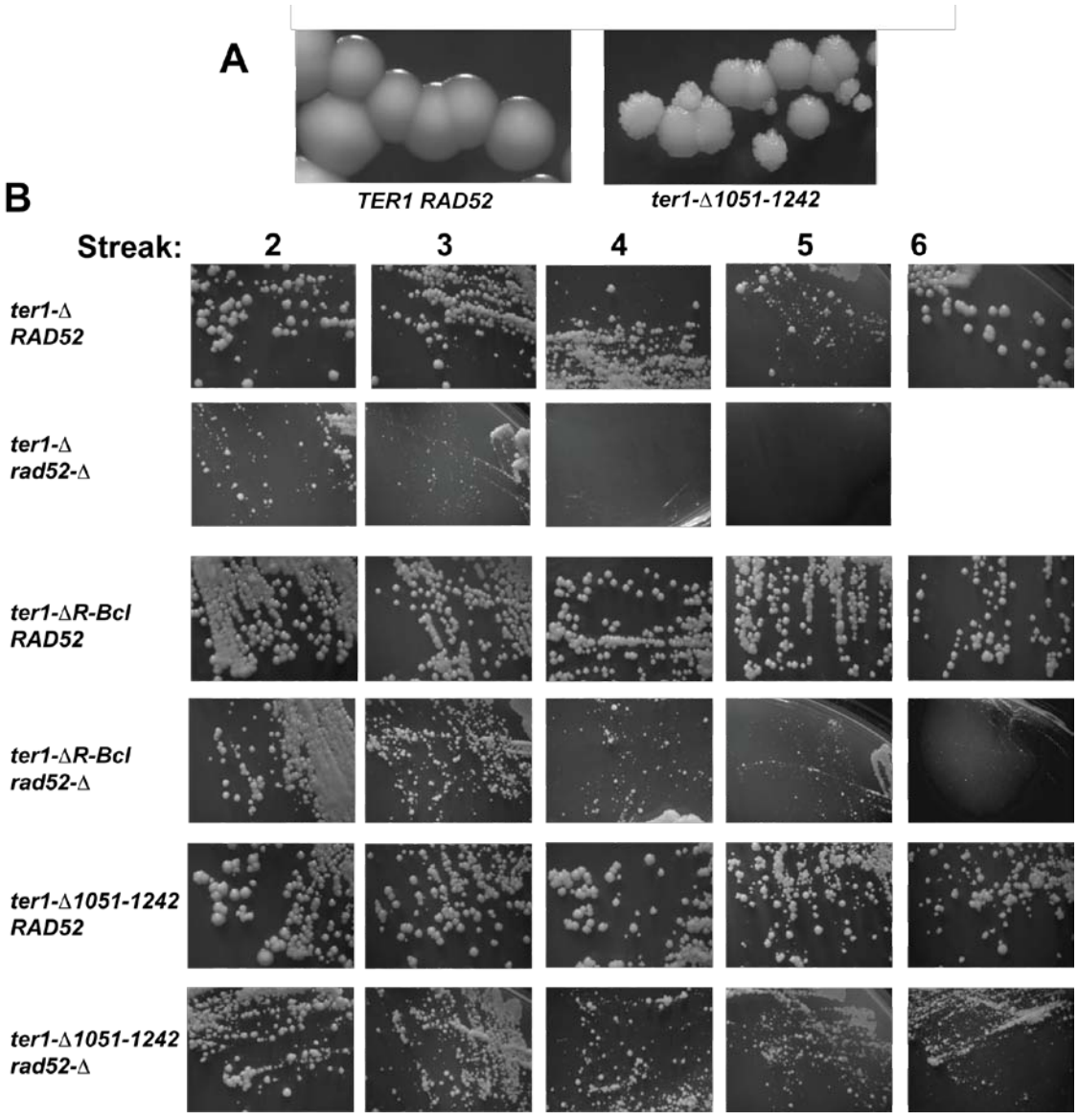

Figure 3. 2. Deletion of *RAD52* exacerbates growth defects in some *ter1* mutants with short telomeres. A) Colony growth of a wild type (WT) control strain and the *ter1-Δ1051-1242* mutant is shown as an example of normal and abnormal rough colony morphology, respectively.

B) Photographs of clonal passaging of *ter1-Δ*, *ter1-ΔR-Bcl***,** and *ter1-Δ1051-1242* single mutants and double mutants with *rad52-Δ* on rich YPD solid media.

In contrast, the growth defects of both the *ter1-ΔR-Bcl* and *ter1-Δ1051-1242* mutants were exacerbated by the deletion of *RAD52* (Fig. 3.2B)**.** Each of three *ter1-Δ1051-1242 rad52-Δ* mutant clones exhibited senescent-like colony phenotypes that steadily worsened over the streaks examined. Even more pronounced senescence was seen with the *ter1-ΔR-Bcl rad52-Δ* mutants, where all three clonal lineages ceased growing altogether by the 6^{th} streak (Fig. 3.2B). During these extended periods of senescence, neither mutant formed the better-growing survivors that occur through recombination in a telomerase deletion mutant ((27, 36, 37). These results suggest that homologous recombination is a vital contributor to telomere maintenance in *ter1-ΔR-Bcl and ter1-Δ1051-1242* mutants. The slower rate of growth decline in both of these mutants when compared to a *ter1-Δ rad52* mutant is presumably due to the presence of a partially active telomerase. The partial or complete growth dependence of some non-null telomerase mutants on *RAD52* strongly suggests that short telomeres in these mutants are frequently repaired and elongated by homologous recombination.

Particularly short telomeres are associated with the worst colony morphology defects in the non-null telomerase mutants.

We next examined the telomere lengths in a series of *ter1* mutants (described in Fig. 3.1) that produce stably short telomeres. Using a *Bsr*BI restriction digest that cleaves only 3 bp internal to the first telomeric repeat of 10 of 12 *K. lactis* telomeres we were able to resolve small telomere length differences among these mutants in two independent clones from each mutant (Fig. 3.3). Each of the seven *ter1* mutants examined had telomere lengths that averaged at least

300 bp shorter than the ~500 bp mean size of the wild type control strain. The telomeres in all the mutants examined were present primarily in a series of diffuse bands that were apparently multiples of the 25 bp *K. lactis* telomeric repeat. Such banding was reported recently and interpreted as indicating that *K. lactis* telomeres do not end at random positions but rather at preferred positions or regions on one or both strands within the 25 bp telomeric repeat (65).

Our results also demonstrated that mutants with the roughest colony morphology, *ter1-ΔL-ApaL* and *ter1-ΔR-Bcl*, also had exceptionally short telomeres, with most of their telomeric signal being at the ~100 bp position. The *ter1-ΔL-ApaL* mutation, like the *ter1-ΔR-Bcl* mutation, deletes one of the 5 bp terminal repeats of the template (Fig. 3.1) and probably blocks the ability of telomerase from adding more than a single repeat. This may also account for the lack of telomeres of greater than five repeats in length in these two mutants. Notably, ~100 bp of telomeric sequence (3-4 telomeric repeats) was previously found to be the minimum telomere length needed to block telomeric recombination in *K. lactis* (58).

The other short telomere mutants examined, *ter1-Δ1051-1242*, *ter1-Bgl(fill)*, *ter1-Dup21-25*, *ter1-24T(SnaB)* and *ter1-28C(Taq)*, had mean telomere lengths between 100-200 bp (Fig. 3.3). Among these, only *ter1-Δ1051-1242* produced colonies with more than slight roughness. Although the telomeres of this mutant were very short (with a mean size of ~125 bp), they appeared to be slightly longer than those of one mutant, *ter1-28C(Taq)*, that produced near-normal colonies. This may indicate that factors other than telomere length contribute to the tendency of mutant telomeres to promote the formation of abnormal colonies. Alternatively, we cannot dismiss the possibility that telomeres of the *ter1-Δ1051-1242* cells that we examined may have been partially elongated by recombination and therefore are not representative of their typical size. Overall, however, our results suggest that particularly short telomeres in *ter1*

mutants, particularly in the absence of appreciable length heterogeneity, are associated with both the rough colony phenotype and a stronger involvement of recombination in telomere repair and cell survival.

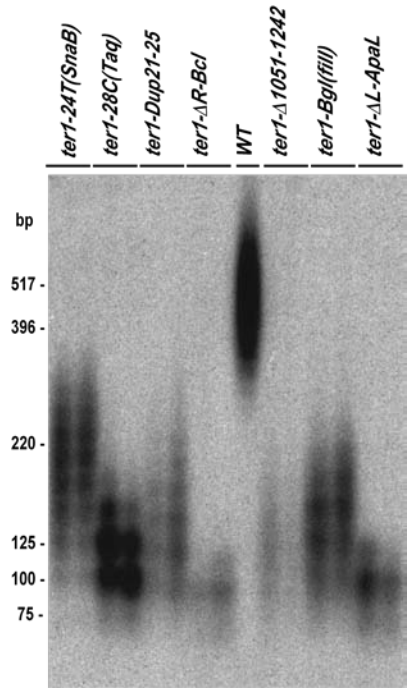

Figure 3. 3. Poorest colony morphology correlates with the shortest telomere length. Shown is a Southern blot of genomic DNA, hybridized to a telomeric probe, from the parental wild type (WT) strain and two independent clones of each mutant (indicated by black bars). DNA was digested with *Bsr*BI and resolved on a 3.2% agarose gel.

RTE can occasionally produce much longer telomeres in the *ter1-ΔR-Bcl, ter1-Δ1051-1242* and *ter1-Bgl(fill) mutants.*

During the course of passaging of short telomere *ter1* mutants on solid YPD medium by serial restreaking, it was noticed that some mutants that normally produced abnormally rough colonies, including the *ter1-ΔR-Bcl, ter1-Δ1051-1242* and *ter1-Bgl(fill),* occasionally produced colonies of normal smooth morphology. This was reminiscent of the formation of post-

senescence survivors in *ter1-Δ* cells that utilize RTE for telomere elongation (45). With the *ter1-Bgl(fill)* mutant, which normally displayed a slight to moderate rough colony phenotype, three instances of smooth colonies were observed during the course of passaging of four independent clones over a period of over 20 serial streaks. Two instances of smooth colony formation occurred 11 streaks apart in one *ter1-Bgl(fill)* isolate while the other occurred in a second, independent, *ter1-Bgl(fill)* isolate. We examined these three independent clones with smooth colonies for telomere length at the point of their isolation and at one or more later points during serial re-streaking on solid YPD medium. The results of this analysis are shown in Fig. 3.4A. Whereas the typical *ter1-Bgl(fill)* isolate designated as "control" exhibited the expected short telomeres, each of the three smooth-colony derivatives of *ter1-Bgl(fill)* were found to have greatly elongated telomeres. The long telomeres did not represent a new set point in telomere length regulation (as can occur with some mutations affecting telomere function) (63). This was evident from the fact that telomere length in these clones greatly decreased over the course of subsequent streaks. Over the course of 16 streaks, clone 1 was observed to return to near the typical telomere length of *ter1-Bgl(fill)* mutant, while clones 2 and 3 showed large reductions in average telomere size after four or five additional streaks. Concomitant with this telomere shortening, the colony phenotypes of the clones also gradually returned to the slightly rough phenotype which is a characteristic of typical of *ter1-Bgl(fill)* (data not shown).

If the lengthening of telomeric sequences from the smooth-colony clones occurred entirely from telomerase-mediated DNA synthesis, digestion with *Cla*I (which cleaves the mutant repeats synthesized by *ter1-Bgl(fill)* telomerase) should completely remove all of the additional sequence that had been added onto telomeric ends. As shown in the *Cla*I digested samples in Fig. 3.4A, this is not the case. Instead, there are several small bands at the bottom of

the gel that hybridize intensely to a wild type telomeric probe. These bands, shown better resolved on a 6% polyacrylamide gel (shown as the insert to the figure), are apparently

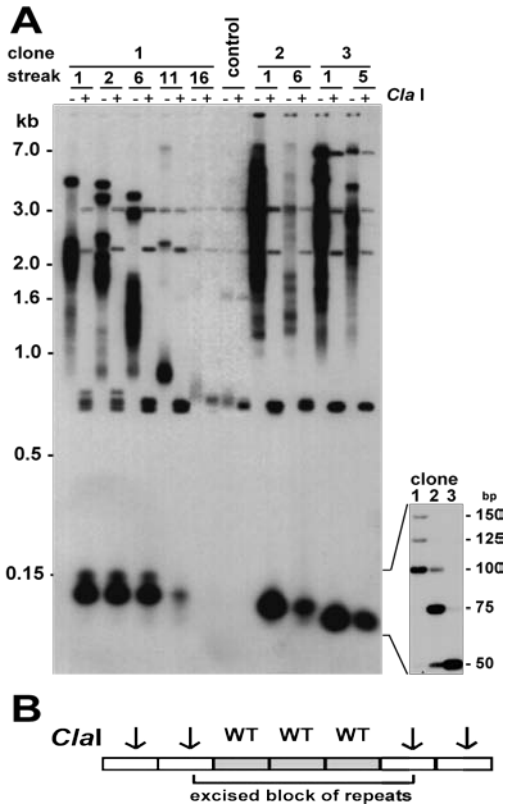

Figure 3. 4. Smooth colony derivatives of *ter1-Bgl(fill)* have telomeres lengthened by recombination. A) Genomic DNA was isolated from three independent clones of *ter1-Bgl(fill)* with smooth colony morphology and a control *ter1-Bgl(fill)* mutant with the typical slightly rough colony morphology. DNA was digested with *Eco*RI and also additionally with *Cla*I (indicated by +) prior to Southern blotting and hybridization to a telomeric probe. An inset at the bottom right depicts further resolution of bands released by *Eco*RI+*Cla*I digest in the three clones using a 6% polyacrylamide gel. B) Diagram showing a segment of telomeric DNA composed of wild type and mutant repeats (grey and white boxes, respectively). Cleavage of Bgl(fill) mutant repeats by *Cla*I (indicated by arrows) excises fragments composed of wild type repeats with terminal halves of mutant repeats as well as 29 bp mutant repeat fragments. The latter on not retained on the hybridization membrane.

composed of small blocks of wild type repeats that have become amplified in the smooth-colony clones and were not evident in the *ter1-Bgl(fill)* control. We estimate that the predominant small

*Cla*I fragments produced in the smooth-colony *ter1-Bgl(fill)* clones to be 54 bp, 79 bp and 104 bp in clones 1-3, respectively. These would be sizes expected if one, two, or three consecutive 25 bp wild type repeats are excised by cleavage within flanking 29 bp repeats (containing *Cla*I sites) derived from the *ter1-Bgl(fill)* telomerase (Fig. 3.4B). Our results indicated that much of the telomere elongation in the *ter1-Bgl(fill)* mutant is due to amplification of wild type telomeric repeats, rather than addition of *Cla*I repeats by telomerase. This amplification of small blocks of wild type repeats is highly reminiscent of that produced during post-senescence survivor formation in *ter1-Δ* cells engineered to initially have telomeres composed of basal wild type repeats and terminal Bcl repeats (45). In that case, repeating patterns were proposed to be the result of amplification of telomeric sequence derived from copying a t-circle composed of both wild type and mutationally-tagged repeats. We conclude that although *ter1-Bgl(fill)* cells do not require recombination to maintain telomeres, they are capable of occasionally undergoing episodes of RTE that can lead to most or all telomeres in the cell becoming grossly elongated.

The partial or complete dependence of *ter1-ΔR-Bcl* and *ter1-Δ1051-1242* on *RAD52* for long term growth strongly suggested that short telomeres in these mutants are commonly elongated by recombination. As described above, isolates of both of these mutants normally exhibit a steady phenotype of rough colonies when serially passaged on solid YPD medium. However, both mutants also produced occasional large smooth colonies and colony sectors in streaks. These were candidates for being clones that had lengthened their telomeres via recombination. The presence of the phenotypically silent *Bcl* base change in the *TER1* template of both mutants allowed for testing for the amplification of wild type repeats as was done in *ter1-Bgl(fill)* mutant.

The telomeres of several independent clones of *ter1-ΔR-Bcl* exhibiting improved colony morphology were investigated using *Eco*RI and *Eco*RI+*Bcl*I digests (Fig. 3.5). As anticipated, each of the eight clones displayed longer telomeres than were present in typical isolates of rough-colony *ter1-ΔR-Bcl* clones. In two out of five mutants (mutants 3 and 5), *Bcl*I digestion

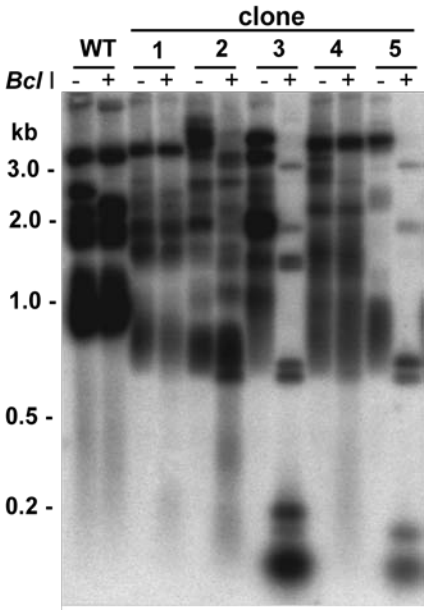

Figure 3. 5. Smooth colony derivatives of the *ter1-ΔR-Bcl* mutant exhibit lengthened telomeres, some with amplification of small blocks of wild type repeats. Shown is a Southern blot of genomic DNA, hybridized to a telomeric probe, from five independent *ter1-ΔR-Bcl* clones, initially identified as having smoother than normal colonies. DNA was digested with *Eco*RI and then additionally with *Bcl*I (indicated by +).

cleaved all telomeric fragments and released intensely hybridizing blocks of wild type telomeric repeats. As with *ter1-Bgl(fill)*, this result strongly suggests that RTE involving a t-circle containing both wild type and mutant *Bcl* repeats had occurred. Interestingly, the remaining three better-growing clones contained elongated telomeres that were largely or entirely resistant to *Bcl*I cleavage. This indicated that telomeric elongation had occurred through the addition of only wild type repeats, a result consistent with RTE- rather than telomerase-mediated elongation. In a few cases (clones 1, 2, and 4), a faint smear of telomeric signal was visible at or below ~500

bp in size. These are likely to be a result from one or a few telomeres retaining a Bcl mutant repeat at a near-basal position of telomeres otherwise composed of wild type repeats. The general lack of Bcl repeats in the smooth-colony subclones of *ter1-ΔR-Bcl* probably indicate that, prior to RTE, this mutant frequently had many of its telomeres lacking repeats with the Bcl base change. Overall, our results strongly support the belief that *ter1-ΔR-Bcl* can frequently generate long telomeres through Type II RTE.

Next, we passaged four *ter1-ΔR-Bcl* smooth-colony isolates for seven serial streaks and examined telomere length and colony growth at each streak (Fig. 3.6). The *Eco*RI pattern of telomeres from these mutants indicated that the lengthened telomeres exhibited a steady decline in length over the course of passaging. The decline continued until telomeres reached the very short sizes that characterized the short telomeres of typical *ter1-ΔR-Bcl* cells. This result is again fully expected for clones that had undergone Type II RTE. In at least two instances, some secondary telomere elongation events were observed (Fig. 3. 6, clones 5 and 6; indicated by arrows). These are likely to represent subsets of cells that had undergone RTE in the cell populations being examined. In both instances, the lengthened telomeres were no longer present in cells of later streaks. This is likely due to the fact that passaging involved streaking to single cells and a colony with cells containing elongated telomeres at streak 6 was presumably not used for streak 7. The gradual shortening of telomeres in the *ter1-ΔR-Bcl* clones was accompanied by a gradual decline in the ability of these clones to grow as indicated by the growth graphs shown above Southern the blots in Fig. 3.6. Growth was scored using an arbitrary scale developed previously to score senescence in *ter1-Δ* mutants (37). As shown in Fig. 3.6, clones started with growth scores of 3-4 (slightly rough to normal smooth) but gradually declined until, in three of four cases, they reached scores of ~1.5 (small and very rough colonies) that characterize growth

of typical *ter1-ΔR-Bcl* cells. We interpret this as indicating that telomere length is the critical determinant for colony growth ability in the *ter1-ΔR-Bcl* mutant.

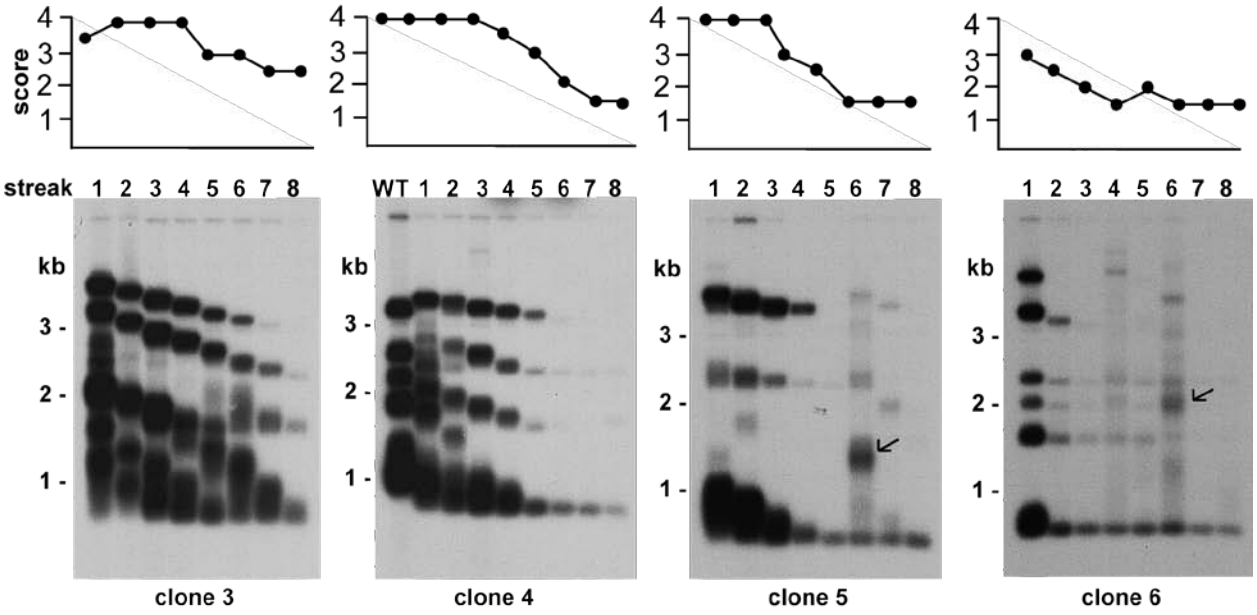

Figure 3. 6. Smooth colony derivatives of *ter1-ΔR-Bcl* undergo gradual telomere shortening and return to a rough colony phenotype with continued passaging. Four clones of *ter1-ΔR-Bcl* with elongated telomeres were serially passaged over eight streaks. DNA was cut with *Eco*RI prior to Southern blotting and hybridization to a telomeric probe. Graphs above Southern blotting depict colony morphology. Score of 1 represents the poorest growth with rough colonies, while a score of 4 defines a wild type-like colony phenotype with completely smooth colonies (37). Arrows represent instances where sizable increases in the length of one or more telomere has occurred in a cell population but apparently not in the cell from that streak that became the source of cells for the next streak. The wild type parental strain is shown in the panel with clone 4.

We next analyzed the *ter1-Δ1051-1242* mutant. Fig. 3.7A-B shows the typical short telomere phenotype of this mutant. Two independent clones are shown at each of two consecutive streaks after growth on solid medium. Telomeric fragments from both clones showed hybridization to a Bcl repeat-specific probe that was stronger at the later streak than it was for the earlier streak (Fig. 3.7B). This signal, but not signal to a wild type telomeric probe

(Fig. 3.7A), is removed upon digestion with *Bcl*I. This indicates that, as expected, the mutant telomerase is functional and has added Bcl telomeric repeats onto telomeric termini.

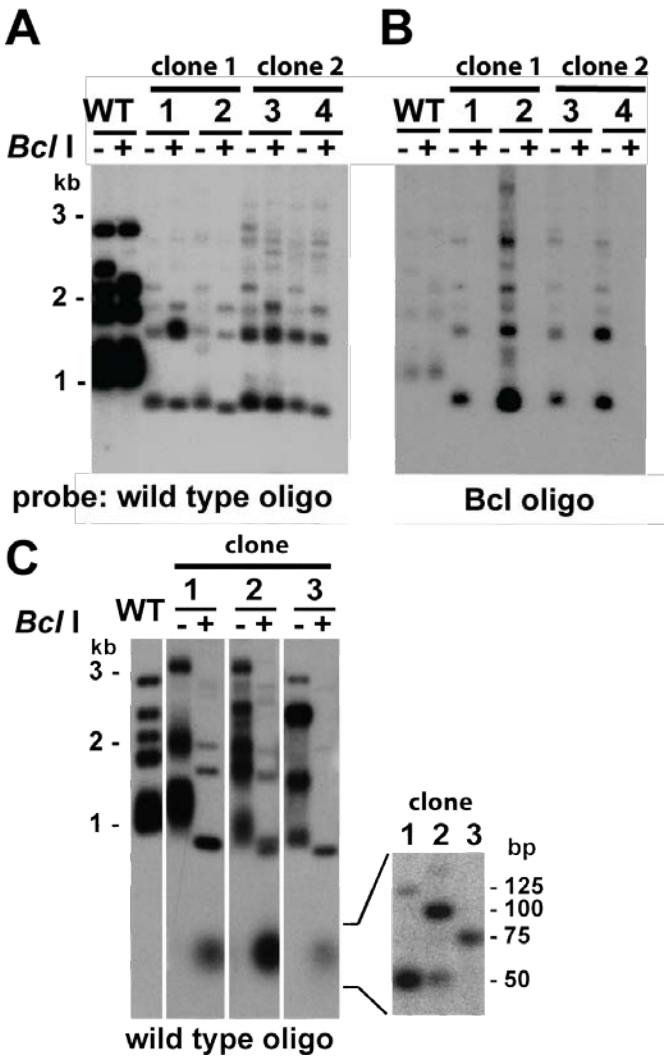

Figure 3. 7. Smooth colony derivatives of *ter1-Δ1051-1242* have telomeres lengthened by recombination. Southern blowing A) Shown are Southern blots of two clones of *ter1-Δ1051-1242* with typical rough colonies. Genomic DNA was digested with *Eco*RI and then also additionally with *Bcl*I (indicated by +) prior to hybridization with a telomeric oligo nucleotide that hybridizes to wild type (an mutant) telomeric repeats. B) The filter shown in A) was re-hybridized with Klac Bcl probe specific to Bcl telomeric repeats. C) Three better growing clones with smooth colony morphology were examined using the same digests and probe as in 7A. An inset at the bottom of the gel depicts further resolution of the bands released by *Eco*RI+*Bcl*I digest in three clones using 3.2% agarose gel.

To address the question whether RTE also plays a role in establishment of better growing colonies in the *ter1-Δ1051-1242* mutant we examined the telomeres of several independent clones with improved colony morphology by Southern blotting, three of which are shown in Fig. 3.7C. *Eco*RI digests revealed that, in each clone, many telomeres were substantially elongated relative to those of the normal rough-colony form of the same mutant (compare Fig. 3.7C to 3.7A). This is consistent with the improvement in colony phenotype being caused by the generation of longer telomeres. *Eco*RI + *Bcl*I digestions of these same DNAs showed that in each case, all telomeric fragments in the cell were cut much shorter with *Bcl*I. This cleavage also released prominent small fragments that ran near the bottom of the gel. Running these small DNA fragments on a high percentage agarose gel (inset to the right of Figure 3.7C) showed that they primarily consisted of a fragment of a single size within a given clone; 50 bp in clone 1, 100 bp in clone 2 and 75 bp in clone3. In subsequent passaging of the clones, telomeres were observed to begin to shorten (data not shown). We conclude that the elongated telomeres of the *ter1-Δ1051-1242* clones with improved colony morphology amplified both Bcl and wild type repeats in a manner consistent with Type II RTE.

Assessment of telomere lengths in mutants undergoing RTE in liquid culture.

To further assess the similarities between the RTE of *ter1-Δ* cells and that of mutants examined in this study, we passaged the mutants by serial dilutions in liquid medium. Unlike our passaging on plates, which goes through a bottleneck of a single cell at each streak, culturing in liquid is likely to produce a more heterogeneous populations of cells that will often have mixtures of independent cell lineages in various states of losing and rebuilding their telomeres. Two independent clones each of *ter1-Δ*, *ter1-ΔR-Bcl* and *ter1-Bgl(fill)* were inoculated into

liquid YPD medium and passaged serially by 1:1000 dilutions daily for 20 days with DNA being extracted at each passage. A single *TER1* wild type clone was used as a control. Mutant clones chosen for the initial inoculation had been grown for multiple streaks on plates prior to the start of the experiment. This allowed them to be non-identical in terms of their starting telomere length depending on whether they had already undergone RTE. As shown in Fig. 3.8, a wild type control showed little or no differences in telomere length or heterogeneity during the 20 liquid passages. The two *ter1-Δ* mutants examined started with telomeres of somewhat different size ranges. Clone 1 had telomeres at passage 1 ranging from very short to wild type length, while clone 2 started with the major group of telomeres that were all much shorter than those of the wild type. As is typical with *K. lactis* mutants with short telomeres that have been passaged for some time both *ter1-Δ* clones had undergone subtelomeric gene conversions that homogenized subtelomeric polymorphisms resulting in a display of fewer than the 5-6 different sized telomeric fragments of the wild type (40). The notable result with the prolonged passaging is that by dilution 20 the two clones have very similar overall telomere profiles, with the major telomere cluster primarily containing telomeres shorter than wild type length but with a tail of signal extending up to 1.5-2 kb in length. This can be interpreted as meaning that the telomeric repeat arrays in post-senescent *ter1-Δ* cells can be extended at least occasionally to lengths at least triple the ~500 bp size of wild type telomeres by RTE. These results are completely consistent with the properties of individual *ter1-Δ* mutants that have been clonally passaged on solid medium (37).

The two clones of *ter1-ΔR-Bcl* examined each started with very different telomere patterns (Fig. 3.8). Clone 1 had only very short telomeres and a greatly reduced pattern at passage 1 while clone 2 had a complex pattern that included many telomeres that were as long,

or much longer than those of wild type cells, presumably as a result of RTE. However, by the 20th passage, the telomere fragment profiles of the two clones were very similar to one another as well as similar to profiles of the telomeres of *ter1-Δ* clones at passage 20. The most noticeable difference was the presence of some longer telomeric fragments present in *ter1-ΔR-Bcl* clone 2. These, however, likely stem from subtelomeric sequence differences rather than from telomere length differences. These results provide further evidence that *ter1-ΔR-Bcl* cells can undergo a Type II RTE that closely resembles that which occurs in *ter1-Δ* cells.

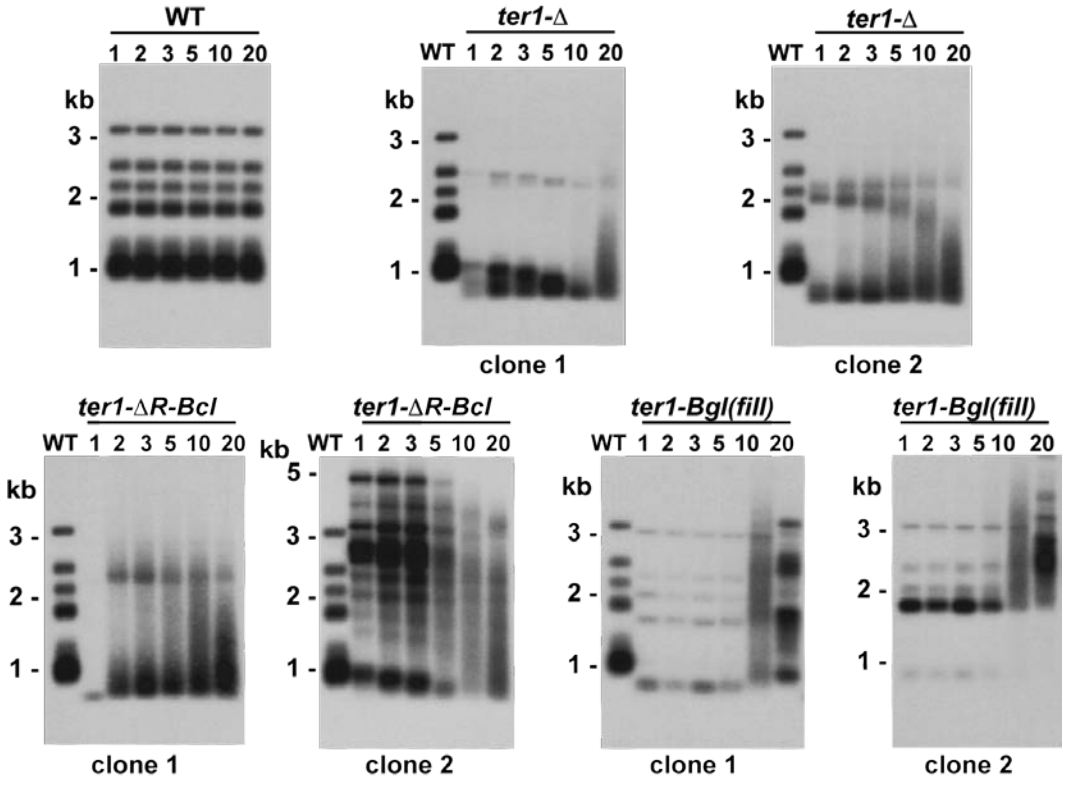

Figure 3. 8. Mutant cells undergoing RTE passaged in liquid medium. A wild type (WT) control and two clones of *ter1-Δ*, *ter1-ΔR-Bcl* and *ter1-Bgl(fill)* were passaged non-clonally for 20 serial dilutions in liquid YPD . Shown are Southern blotting of genomic DNA isolated from these strains at different passages (indicated by number above lanes), digested with *Eco*RI and subsequently hybridized to a telomeric probe.

The two clones of *ter1-Bgl(fill)* that were passaged in liquid both started with the very short telomeres that typify the normal state of that mutant (Fig. 3.8). Clone 2, however, was

observed to have a rather different profile of telomeric *Eco*RI fragments at passage 1. While *ter1-Bgl(fill)* clone1 and all the other mutant clones shown in Fig. 3.8 had a major group of telomeric fragments at a size of ~1 kb, the *ter1-Bgl(fill)* clone 2 had its most intense band at a size between 1.5-2 kb. This difference arose from abundant subtelomeric gene conversions occurring in clone 2 prior to the start of the experiment. The telomere fragment profiles in both *ter1-Bgl(fill)* clones were observed to be stable through 5 passages but showed substantial telomere elongations at passage 10 that persisted until passage 20. The telomere length patterns at passage 20 of *ter1-Bgl(fill)* were thus not as uniform as those exhibited by *ter1-Δ* or *ter1-ΔR-Bcl* cells. This failure of *ter1-Bgl(fill)* to reach a stable distribution of telomere length in serial liquid cultures probably stems largely from the lower rate of RTE in this mutant coupled with a presumably insufficiently long experimental growth course. Nonetheless, we conclude that passaging by dilutions in liquid culture provides a promising way to evaluate telomere length profiles in *K. lactis* mutants that undergo RTE.

Discussion

Our work here studied telomere maintenance in *K. lactis ter1* mutants with abnormally short telomeres. In all cases, these mutants retained at least some telomerase activity as indicated by the fact that none undergo the same growth senescence that is characteristic of a *ter1-Δ* mutant. However, in spite of this, telomeric recombination was found to often be extensive. In certain mutants, for example, long term propagation was severely impaired in the absence of the Rad52 protein, presumably because the crippled telomerase was not able to maintain telomeres without aid from recombination. We observed that in at least three mutants, *ter1-ΔR-Bcl*, *ter1-Δ1051-1242* and *ter1-Bgl(fill)*, clonal isolates could be readily found that

containing telomeres that had been elongated by recombination. This could be deduced from the fact that telomere lengthening was accompanied, in part, by amplification of wild type repeats in cells where telomerase could make only mutationally-tagged repeats.

Considerable evidence, particularly from work done with *K. lactis*, now supports the belief that the RTE in yeast cells lacking telomerase can occur through a roll-and-spread mechanism (39, 45). According to this hypothesis, shortened telomeres become prone to greatly increased levels of homologous recombination, some events of which can occasionally produce a small telomeric circle (t-circle). Rolling circle replication using a t-circle as a template, and perhaps initiated at an invading 3' telomeric end, is then thought to create a substantially lengthened telomere in a single step. Once cells contain one long telomere, other telomeres can then become elongated by copying its sequence, thereby spreading the sequence present in the original t-circle to most or all telomeres in the cell.

A suite of shared characteristics argue that the elongated telomeres observed in mutants studied here are produced in a manner that is mechanistically very similar to that observed in *K. lactis* cells that lack telomerase. In both situations, long telomere formation occurs in cells that have very short telomeres as well as abnormal cell and colony morphologies that are characteristic of cells with unrepaired DNA damage. In both situations, the telomere elongations are closely associated with an improved ability to grow. Additionally, the lengthened telomeres arising in *ter1-Δ* and the mutants studied here share a number of characteristics. The extent of lengthening observed is similar; typically limited to hundreds of base pairs. Further, in both situations, RTE is found to lengthen most or all telomeres in the cell. This is consistent with it being a concerted process, where once one telomere is elongated, all other short telomeres are much more likely to be lengthened as well. Previous work has shown that the sequence of a

single long telomere present in senescing *ter1-Δ* cells is copied to all other telomeres at >90% frequency (58). The elongated telomeres are not stable, but are instead subject to gradual shortening that can eventually reduce them back to very short sizes. Finally, the amplification of interspersed blocks of wild type repeats, which often are of a single predominant size within the telomeres in a given clone, was remarkably similar between the *ter1* mutants studied here and *ter1*-Δ cells. Such blocks are consistent with the first telomere lengthening event involving copying a small t-circle composed of both wild type and mutationally-tagged repeats.

The three examples of *ter1-Bgl(fill)* clones that had acquired elongated telomeres through RTE are exceptional because of the especially large size increases (one to several kb) in their telomeres. This is superficially similar to the Type IIR 'runaway' RTE that has been found to occur in the *stn1-M1* mutant and certain other *K. lactis* mutants with abnormal telomeric repeats (3, 28, 58). Significantly, the base changes within the Bgl(fill) telomeric repeat map to a short region that, when mutated, can lead to extreme telomere elongation either from telomerase-mediated or recombinationally-mediated elongation (58, 63). This makes it possible and perhaps likely, that the Bgl(fill) repeat is deficient at blocking telomeric sequences from initiating recombination. However, because the long telomeres in the *ter1-Bgl(fill)* clones are subject to gradual shortening, we conclude that they are not the product of full blown Type IIR RTE. It should be kept in mind though that the telomeres in the long telomere *ter1-Bgl(fill)* clones are composed of mixtures of both wild type and mutant telomeric repeats. Thus, we can not rule out the possibility that telomeres composed entirely of Bgl(fill) repeats would be capable of undergoing Type IIR RTE. One possible explanation for the long telomeres in the three *ter1-Bgl(fill)* clones is that capping defects in the Bgl(fill) repeats can occasionally lead to telomeric recombination occurring at slightly greater lengths and producing slightly larger t-circles than

would otherwise be the case. Large (1.5 kb) t-circles lead to much greater telomere elongation than do small (100 nt) ones (44). It is conceivable that slight differences in the sizes of the very small t-circles thought to trigger Type II RTE can cause large differences in how processively those molecules can be copied by a DNA polymerase. Such a phenomenon potentially could explain why Type II RTE in *S. cerevisiae* telomerase deletion mutants produces substantially longer telomeres than that seen in equivalent *K. lactis* mutants (11, 36, 57). *S. cerevisiae* telomeric repeats are highly heterogeneous in sequence and might be expected to lead to shortening telomeres that became prone to recombination at heterogeneous sizes. This in turn could lead to the production of t-circles of a larger size distribution than occurs in senescing *K. lactis ter1-Δ* cells.

One conclusion of our work is that the ability of *K. lactis* cells to undergo Type II RTE is linked to the presence of exceptionally short (<~100 bp) telomeres. This is consistent with the past observation that *K. lactis* telomeres up to ~100 bp in length were capable of recombining with other telomeres during *ter1-Δ* post-senescence survivor formation (58). One interpretation of this is that ~100 bp represents the minimum telomere length that prevents a telomere from initiating recombination. However, it is clear from past work that many *ter1* mutants that have not been observed to undergo RTE, such as *ter1-28C(Taq)* and *ter1-Dup21-25*, nonetheless experience highly elevated rates of subtelomeric BIR events as a consequence of telomere dysfunction (40, 46). This leads to the question of how telomere uncapping can lead to widespread BIR events at telomeres without RTE being observed. One possibility is that t-circle formation, presumably the rate limiting event of RTE, is always far rarer than other recombinational outcomes triggered by telomere uncapping such as BIR events. In this view, BIR events represent a standard, programmed response of the cell to short telomeres entering a

recombinational repair pathway while RTE from t-circle formation and copying may instead be a rare and stochastic outcome to such repair. Alternatively, or in addition, the uncapping events that trigger RTE could be qualitatively different in some manner from those occurring in cells that undergo BIR but not RTE. Conceivably, an altered cellular state associated with severe DNA damage, such as a checkpoint-activated or a damage-adapted state (13), might lead to alterations in how DNA damage, including uncapped telomeres, are repaired (66). Consistent with this possibility, evidence from *S. cerevisiae* indicates that efficient Type II RTE in that organism is dependent upon checkpoint sensor proteins (17, 59). It has also been demonstrated that DNA ends with partial telomere function do not elicit repair responses that are identical to non-telomeric ends. For example, senescing telomerase deletion mutants produce a different gene expression profile than other DNA damage responses (41, 47). Also, checkpoint signaling at DSBs near telomeric repeats is reduced relative to ends without nearby telomeric repeats (41, 47).

Another important inference from our work concerns the nature of the telomeric recombination that occurs in those cells of the *ter1-Δ1051-1242* and *ter1-ΔR-Bcl* mutants that are not lengthening telomeres through recombination but rather just maintaining short telomeres. Both of these mutants require *RAD52* for long term viability yet can be passaged on plates apparently indefinitely without either net telomere lengthening or net telomere shortening occurring in the bulk of cells. It therefore seems highly probable that most homologous recombination events occurring at telomeres in these mutants involves very modest lengthening events that act to maintain telomeres at short sizes. These HR processes could, in most cells, exclusively involve events where the shortest telomeres strand invade and copy sequence from other telomeres only slightly longer than themselves rather than include copying events

templated from t-circles. These may be mechanistically similar to the BIR events that can replace subtelomeric sequence described above. The purely telomere-copying mode of recombination that is independent of t-circles is likely to represent the simplest form of recombinational telomere maintenance. In *K. lactis ter1-Δ* mutants, such pure telomere copying is either unable to maintain telomeres by itself or occurs in such poorly-growing cells that it is difficult to observe once better growing post-senescence survivors with substantially elongated telomeres emerge through the roll-and-spread process with its added use of copying t-circles. Some previous evidence is consistent with RTE being able to occur in the absence of telomeric circles. Senescing *K. lactis* telomerase deletion cells with a single long telomere will specifically copy sequence from that telomere to all other telomeres in the cell (58). Additionally, human ALT cells with knocked down expression of Nbs1 or XRCC3, proteins that appear essential to t-circle formation, do not block continued proliferation of these cells (14).

Our identification of recombinational telomere maintenance that does not involve net elongation of very short telomeres has some important implications. First, it expands the range of phenomena that constitute RTE. Although it would best be described as a form of Type II RTE, the maintenance of very short telomeric tracts is reminiscent of Type I RTE in *S. cerevisiae*. However, it is not Type I RTE as there is no evidence for amplification of subtelomeric sequences. It could be noted that this also implies that the presence of only short telomeric tracts in *S. cerevisiae* cells using recombination to maintain telomeres is not sufficient to conclude that Type I RTE is occurring. Secondly, we would predict from our results that the genetic requirements for RTE occurring without net telomere lengthening, lacking the need for formation and use of a t-circle, might be different from standard Type II RTE. Interestingly, the maintenance of short telomeres by recombination in telomerase-negative mouse cells has

recently been reported (42). This implies that RTE in mammalian cells does not necessarily generate the long heterogenous telomeres characteristic of most ALT cancers.

A variety of other examples of telomeric recombination occurring in the presence of telomerase or compromised telomere capping have been documented in recent years. Among the more dramatic of these are yeast mutants that produce Type IIR RTE as a result of defects affecting the function of telomere capping proteins (21, 28, 49). Similarly, the ALT pathway present in some human cancers, which closely resembles Type IIR RTE in yeast, is not typically repressed if telomerase is exogenously expressed (7, 16, 22, 50). Additionaly, recombination can also play a role at telomeres in at least some cells with no known telomere dysfunction. In the yeast *Candida albicans*, telomere lengths become lengthened in a *rad52-Δ* background suggesting a role for recombination in normal telomere length regulation (12). In *K. lactis*, wild type telomeres can readily become greatly elongated when transformed with t-circles (45). Recombination was also shown cause dramatic shortening of abnormally long telomeres in otherwise wild type *S. cerevisiae* and *K. lactis* (4, 6, 33). Decreased turnover of internal repeats in a *K. lactis rad52-*Δ mutant argues that similar truncations occur even at normal length telomeres (4).

As purturbed telomere capping leads to alteration in normal telomere functioning, further investigation of circumstances at which both, telomerase and RTE contribute to telomere maintenance will allow better understanding of the mechanisms underlying carcinogenesis in humans and potentially help to further understand the peculiarities of telomere elongation in ALT cancers.

Acknowledgments

This work was supported a grant to M.J.M. from the National Institutes of Health (RO1GM061645). The authors would also like to thank Dr. Elizabeth Blackburn, in whose lab the initial stages of this work were done.

References

1. **Bailey, S. M., M. A. Brenneman, and E. H. Goodwin.** 2004. Frequent recombination in telomeric DNA may extend the proliferative life of telomerase-negative cells. Nucl. Acids Res. **32:**3743-3751.

2. **Basenko, E. Y., A. J. Cesare, S. Iyer, J. D. Griffith, and M. J. McEachern.** 2010. Telomeric circles are abundant in the *stn1-M1* mutant that maintains its telomeres through recombination. Nucl. Acids Res. **38:**182-189.

3. **Bechard, L. H., B. D. Butuner, G. J. Peterson, W. McRae, Z. Topcu, and M. J. McEachern.** 2009. Mutant telomeric repeats in yeast can disrupt the negative regulation of recombination-mediated telomere maintenance and create an Alternative Lengthening of Telomeres-like phenotype. Mol. Cell. Biol. **29:**626-639.

4. **Bechard, L. H., N. Jamieson, and M. J. McEachern.** 2010. Recombination can cause telomere elongations as well as truncations deep within telomeres in the yeast *Kluyveromyces lactis.* Eukaryot. Cell **10:**226-236.

5. **Bertuch, A. A., and V. Lundblad.** 2004. Exo1 contributes to telomere maintenance in both telomerase-proficient and telomerase-deficient *Saccharomyces cerevisiae.* Genetics **166:**1651-1659.

6. **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:**6559-6573.

7. **Cerone, M. A., J. A. Londono-Vallejo, and S. Bacchetti.** 2001. Telomere maintenance by telomerase and by recombination can coexist in human cells. Hum. Mol. Genet. **10:**1945-1952.

8. **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:**9948-9957.

9. **Cesare, A. J., C. Groff-Vindman, S. A. Compton, M. J. McEachern, and J. D. Griffith.** 2008. Telomere loops and homologous recombination-dependent telomeric circles in a *Kluyveromyces lactis* telomere mutant strain. Mol. Cell. Biol. **28:**20-29.

10. **Cesare, A. J., and R. R. Reddel.** 2010. Alternative lengthening of telomeres: models, mechanisms and implications. Nat. Rev. Genet. **11:**319-330.

11. **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:**1819-1827.

12. **Ciudad, T., E. Andaluz, O. Steinberg-Neifach, N. F. Lue, R. A. Calderone, and G. Larriba.** 2004. Homologous recombination in *Candida albicans*: role of CaRad52p in DNA repair, integration of linear DNA fragments and telomere length. Mol. Microbiol. **53:**1177-1194.

13. **Clémenson, C., and M. Marsolier-Kergoat.** 2009. DNA damage checkpoint inactivation: adaptation and recovery. DNA Repair **8:**1101-1109.

14. **Compton, S. A., J. Choi, A. J. Cesare, S. Ozgur, and J. D. Griffith.** 2007. Xrcc3 and Nbs1 are required for the production of extrachromosomal telomeric circles in human alternative lengthening of telomere cells. Cancer Res. **67:**1513-1519.

15. **Enomoto, S., L. Glowczewski, J. Lew-Smith, and J. G. Berman.** 2004. Telomere cap components influence the rate of senescence in telomerase-deficient yeast cells. Mol. Cell. Biol. **24:**837-845.

16. **Ford, L. P., Y. Zou, K. Pongracz, S. M. Gryaznov, J. W. Shay, and W. E. Wright.** 2001. Telomerase can inhibit the recombination-based pathway of telomere maintenance in human cells. J. Biol. Chem. **276:**32198-32203.

17. **Grandin, N., and M. Charbonneau.** 2007. Mrc1, a non-essential DNA replication protein, is required for telomere end protection following loss of capping by Cdc13, Yku or telomerase. Mol. Genet. Genomics **277:**685-699.

18. **Grandin, N., and M. Charbonneau.** 2003. The Rad51 pathway of telomerase-independent maintenance of telomeres can amplify $TG_{1\text{-}3}$ sequences in yku and cdc13 mutants of *Saccharomyces cerevisiae.* Mol. Cell. Biol. **23:**3721-3734.

19. **Grandin, N., C. Damon, and M. Charbonneau.** 2001. Cdc13 prevents telomere uncapping and Rad50-dependent homologous recombiantion. EMBO J. **20:**6127-6139.

20. **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:**1173-1183.

21. **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:**512-527.

22. **Grobelny, J. V., M. Kulp-McEliece, and D. Broccoli.** 2001. Effects of reconstitution of telomerase activity on telomere maintenance by the alternative lengthening of telomeres (ALT) pathway. Hum. Mol. Genet. **10:**1953-1961.

23. **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:**4406-4412.

24. **Henson, J. D., Y. Cao, L. I. Huschtscha, A. C. Chang, A. Y. M. Au, H. A. Pickett, and R. R. Reddel.** 2009. DNA C-circles are specific and quantifiable markers of alternative-lengthening-of-telomeres activity. Nat. Biotech. **27:**1181-1185.

25. **Henson, J. D., and R. R. Reddel.** 2011. Assaying and investigating Alternative Lengthening of Telomeres activity in human cells and cancers. FEBS Letters **584:**3800-3811.

26. **Hornsby, P.** 2010. Senescence and life span. Pflügers Arch. Europ. J. Physiol. **459:**291-299.

27. **IJpma, A. S., and C. W. Greider.** 2003. Short telomeres tnduce a DNA damage response in *Saccharomyces cerevisiae*. Mol. Biol. Cell **14:**987-1001.

28. **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:**8064-8073.

29. **Joseph, I. S., A. Kumari, M. K. Bhattacharyya, H. Gao, B. Li, and A. J. Lustig.** 2010. An mre11 mutation that promotes telomere recombination and an efficient bypass of senescence. Genetics **185:**761-770.

30. **Kass-Eisler, A., and C. W. Greider.** 2000. Recombination in telomere-length maintenance. Trends Biochem. Sci. **25:**200-204.

31. **Lansdorp, P. M.** 2009. Telomeres and disease. EMBO J. **28:**2532-2540.

32. **Le, S., J. K. Moore, and J. E. Haber.** 1999. *RAD50* and *RAD51* define two pathways that collaborate to maintain telomeres in the absence of telomerase. Genetics**:**143-152.

33. **Li, B., and A. J. Lustig.** 1996. A novel mechanism for telomere size control in *Saccharomyces cerevisiae.* Genes Dev. **10:**1310-1326.

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 δ-mediated telomere-telomere recombination in *Saccharomyces cerevisiae***.** Eukaryot. Cell **4:**327-336.

35. **Londono-Vallejo, J., H. Der-Sarkissian, L. Cazes, S. Bacchetti, and R. R. Reddel.** 2004. Alternative lengthening of telomeres is characterized by high rates of telomeric exchange. Cancer Res. **64:**2324-2327.

36. **Lundblad, V., and E. H. Blackburn.** 1993. An alternative pathway for yeast telomere maintenance rescues *est1*- senescence. Cell **73:**347-360.

37. **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:**1822-1834.

38. **McEachern, M. J., and E. H. Blackburn.** 1995. Runaway telomere elongation caused by telomerase RNA gene mutations. Nature **376:**403-409.

39. **McEachern, M. J., and J. E. Haber.** 2006. Break-induced replication and recombinational telomere elongation in yeast. Ann. Rev. Biochem. **75:**111-135.

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

41. **Michelson, R. J., S. Rosenstein, and T. Weinert.** 2005. A telomeric repeat sequence adjacent to a DNA double-stranded break produces an anticheckpoint. Genes Dev. **19:**2546-2559.

42. **Morrish, T. A., and C. W. Greider.** 2009. Short telomeres initiate telomere recombination in primary and tumor cells. PLoS Genet. **5:**e1000357.

43. **Nabetani, A., and F. Ishikawa.** 2009. Unusual telomeric DNAs in human telomerase-negative immortalized cells. Mol. Cell. Biol. **29:**703-713.

44. **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:**1115-1127.

45. **Natarajan, S., and M. J. McEachern.** 2002. Recombinational telomere elongation promoted by DNA circles. Mol. Cell. Biol. **22:**4512-4521.

46. **Natarajan, S., K. Nickles, and M. J. McEachern.** 2006. Screening for telomeric recombination in wild-type *Kluyveromyces lactis.* FEMS Yeast Res. **6:**442-448.

47. **Nautiyal, S., J. L. DeRisi, and E. H. Blackburn.** 2002. The genome-wide expression response to telomerase deletion in *Saccharomyces cerevisiae.* Proc. Nat. Acad. Sci. U S A **99:**9316-9321.

48. **Nugent, C. I., G. Bosco, L. O. Ross, S. K. Evans, A. P. Salinger, J. K. Moore, J. E. Haber, and V. Lundblad.** 1998. Telomere maintenance is dependent on activities required for end repair of double-strand breaks. Curr. Biol. **8:**657-60.

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:**249-252.

50. **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:**3862-3875.

51. **Reddel, R. R.** 2010. Senescence:an antiviral defence that is tumor suppressive? Carcinog. **31:**19-26.

52. **Roy, J., T. B. Fulton, and E. H. Blackburn.** 1998. Specific telomerase RNA residues distant from the template are essential for telomerase function. Genes Dev. **12:**3286-3300.

53. **Royle, N. J., A. Méndez-bermúdez, A. Gravani, C. Novo, J. Foxon, J. Williams, V. Cotton, and A. Hidalgo.** 2009. The role of recombination in telomere length maintenance. Biochem. Soc. Trans. **37:**589-595.

54. **Seto, A. G., A. J. Zaug, S. G. Sobel, S. L. Wolin, and T. R. Cech.** 1999. *Saccharomyces cerevisiae* telomerase is an Sm small nuclear ribonucleoprotein particle. Nature **401:**177-180.

55. **Singer, M. S., and D. E. Gottschling.** 1994. *TLC1*: template RNA component of *Saccharomyces cerevisiae* telomerase. Science **266:**404-409.

56. **Stewart, S. A., and R. A. Weinberg.** 2006. Telomeres: Cancer to Human Aging. Ann. Rev. Cell Dev. Biol. **22:**531-557.

57. **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:**8083-8093.

58. **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*. Prov. Natl. Acad. Sci. USA **102:**3348-3353.

59. **Tsai, Y. L., S. F. Tseng, S. H. Chang, C. C. Lin, and S. C. Teng.** 2002. Involvement of replicative polymerases, Tel1p, Mec1p, Cdc13p, and the Ku complex in telomere-telomere recombination. Mol. Cell. Biol. **22:**5679-5687.

60. **Tzfati, Y., T. B. Fulton, J. Roy, and E. H. Blackburn.** 2000. Template boundary in a yeast telomerase specified by RNA structure. Science **288:**863-867.

61. **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:**1779-1788.

62. **Ulaner, G. A., H. Huang, J. Otero, Z. Zhao, L. Ben-Porat, J. M. Satagopan, R. Gorlick, P. Meyers, J. H. Healey, A. G. Huvos, A. R. Hoffman, and M. Ladanyi.**

2003. Absence of a telomere maintenance mechanism as a favorable prognostic factor in patients with osteosarcoma. Cancer Res. **63:**1759-1763.

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:**369-384.

64. **Underwood, D. H., R. P. Zinzen, and M. J. McEachern.** 2004 Template requirements for telomerase translocation in *Kluyveromyces lactis.* Mol. Cell. Biol. **24:**912-923.

65. **Wang, Z., L. Guo, L. Chen, and M. J. McEachern.** 2009. Evidence for an additional base-pairing element between the telomeric repeat and the telomerase RNA template in *Kluyveromyces lactis* and other yeasts. Mol. Cell. Biol. **29:**5389-5398.

66. **Wellinger, R. J.** 2010. When the caps fall off: Responses to telomere uncapping in yeast. FEBS Letters **584:**3734-3740.

67. **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:**1111-1121.

68. **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:**4175-4179.

CHAPTER 4

Genetic analysis of recombinational telomere elongation and its postulated component steps in the yeast *Kluyveromyces lactis*

[4] Basenko, E. Y., M. Taylor Storey, G. O'Connell, R. Chaudhry, and M. J. McEachern. To be submitted to *Molecular and Cellular Biology*

Abstract

Yeast telomerase deletion post-senescence survivors elongate telomeres through recombinational telomere elongation (RTE). In *S. cerevisiae*, Type I and Type II RTE generate different types of elongated chromosome ends and occur via *RAD51*-dependent or *RAD50/RAD59*-dependent pathways, respectively. *K. lactis* telomerase deletion mutants only generate Type II survivors. Here we show that both of these pathways contribute to survivor formation in *K. lactis* and that neither is individually essential. Inactivation of *RAD51* in the absence of telomerase causes longer telomeres in survivors. We also examined the genetics of component steps of the roll-and-spread model for RTE: telomeric-circle (t-circle) formation, t-circle utilization, and subtelomerically initiated break-induced replication (BIR) events. We found that *RAD50* but not *RAD51* or *RAD59* is required for the maintenance of long uncapped telomeres and the formation of t-circles in two long telomere mutants. The ability of transformed t-circles to extend telomeres was eliminated by a *rad52* mutation but not by *rad50*, *rad51*, or *rad59* mutations. Finally, the subtelomeric BIR events were exclusively dependent on *RAD51*. We propose that both HR pathways work together to produce post-senescence survivors in *K. lactis*.

Introduction

Some human cancers rely on recombination rather than telomerase to maintain their telomeres. This Alternative Lengthening of Telomeres (ALT) pathway is associated with telomere instability, long and highly heterogeneous telomeres, abundant t-circles, and the accumulation of sub-cellular ALT-associated promyelocytic leukemia bodies (APBs) containing repair and telomere-binding proteins and circular and linear telomeric DNA (11, 19). Telomeres in ALT cells appear to be maintained through homologous recombination. This is supported by findings of elevated rates of intra- and inter-telomeric recombination events, with the latter potentially contributing to t-circle production (6, 20).

Recombinational Telomere Elongation (RTE) has been extensively studied in the yeasts *S. cerevisiae* and *K. lactis*. In the absence of telomerase, shortening of telomeres induces senescence characterized by irregular colonies, poor growth and eventually gross cellular mortality (36, 44, 56). Rare post-senescence survivors emerge with improved colony morphology and telomeres lengthened through RTE. *S. cerevisiae* forms two types of post-senescence survivors. Slow growing Type I survivors primarily amplify subtelomeric Y'-elements with short interstitial and terminal telomeric tracts (30). Type II survivors in contrast lack Y'-amplification, exhibit long telomeric tracts, and overgrow Type I survivors in liquid cultures (13, 30). Rad52, a protein essential for almost all forms of homologous recombination (HR) is required for both types of RTE (30, 36, 56). Type I RTE also employs Rad51, Rad54, Rad55, and Rad57, factors belonging to the canonical mitotic homologous recombination (HR) pathway (13, 30, 55). Rad51 is a RecA homolog that initiates strand invasion of homologous donor DNA (54). In contrast, the formation of Type II survivors is independent of Rad51 but requires Rad50, Rad59, and Sgs1 (2, 14, 23, 30). The Rad50 and Rad59 proteins are involved in

an alternative HR pathway able to utilize shorter regions of homology than the Rad51-dependent pathway (54). Rad50, along with Mre11 and Xrs2 make up the MRX complex which plays a variety of roles related to DNA ends including 5' end DNA resectioning, DNA bridging, and checkpoint signaling (51, 54). Rad59 has homology to the N-terminus of Rad52 and is important for repair by the single-strand DNA annealing (SSA) reaction (3, 52). Sgs1 is a helicase that works together with mismatch repair machinery to prevent formation of homeologous heteroduplexes during recombination (35). It also participates in 5' end resectioning with the nuclease Dna2 (41). Its role in Type II RTE might involve the resolution of secondary structures formed by G-rich telomeric sequences during recombination (2, 14).

Unlike *S. cerevisiae*, which has highly heterogeneous telomeric repeats present both at telomeric ends and in subtelomeric locations, the milk yeast *K. lactis* has uniform 25 bp telomeric repeats that are present solely at the very ends of chromosomes (38). 11 out of 12 *K. lactis* chromosome ends share subtelomeric homology known as the R element extending up to several kilobases from the telomere (46). *K. lactis* mutants lacking telomerase or with very weak telomerase form only Type II survivors with lengthened telomeric repeat tracts (37, 44). During this Type II RTE, adjacent subtelomeric sequences often undergo a degree of homogenization between chromosome ends but are not otherwise amplified (40, 58). Telomere elongation in telomerase RNA gene deletion mutants (*ter1*-Δ) is thought to occur through a roll-and-spread mechanism where an uncapped telomere produces a small t-circle which is then used in a rolling circle replication event to produce a lengthened telomere (44). Subsequently, this long telomere is thought to be a preferential template for BIR-like events that copy the lengthened telomeric tract onto other short telomeres in the cell. Considerable evidence now supports this model. Firstly, single- and double-stranded t-circles of various sizes are abundant in long telomere *K.*

lactis mutants with telomere capping defects (4, 12, 18). Secondly, when *ter1*-Δ cells were constructed with basal wild type and terminal mutationally-tagged Bcl repeats), post-senescence survivors emerged containing repeating patterns of the two repeat types (44). These patterns are consistent with being derived from a t-circle consisting of wild-type and Bcl telomeric repeats. Thirdly, Topcu et al. (58) demonstrated that the elongated telomeres of post-senescence survivors are derived from a single telomere. Spreading of telomere sequence from one telomere to others occurs through a BIR-like mechanism and can originate within subtelomeric sequences (45, 58). Such subtelomeric BIR events are also very common in mutants with stably short telomeres that do not undergo RTE (40).

In this study we demonstrate that telomerase deletion mutants in *K. lactis*, in contrast to those in *S. cerevisiae*, utilize both the canonical Rad51-dependent pathway and the alternative Rad50-dependent pathway to generate post-senescence Type II survivors. We have also investigated the genetic requirements of t-circle formation, t-circle utilization, and subtelomeric BIR, processes thought to be components of the roll-and-spread mechanism of RTE.

Materials and Methods

Yeast strains and growth conditions.

The complete genotypes of parental strains used in this study are listed in Table 4.1. Most yeast strains were maintained on YPD (yeast extract/peptone/dextrose) media, while ZT3 and ZT5 were grown on synthetic defined (SD) media lacking uracil or histidine, respectively. The ZT3 and ZT5 strains are derivatives of the haploid wild type *K. lactis* strain 7B520 (64) that had the *TER1* gene deleted through the plasmid loop-in/loop-out procedure described previously

(37, 50) and were subsequently complemented with the plasmids carrying *TER1* and either *ScURA3* or Sc*HIS* on plasmid pTER1-WT-URA3 or pTER1-WT-HIS3, respectively (50, 58). The *ter1-28C(Taq)* strain, here referred to as *ter1-Taq,* was previously described (40). The mutations in *RAD50, RAD51* and *RAD59* were described previously (8, 27, 62).

To combine these mutations with *ter1*-Δ we utilized *K. lactis* mating and sporulation techniques (59). First, we mated *rad50*-Δ (SAY557), *rad51*-Δ (SAY516) and *rad59*-Δ (Klrad59 1888) to GG1958. Haploid spores dissected from these diploids were then checked for having the expected *rad*-Δ mutation. G418 resistance of spores with *rad50*-Δ and *rad59*-Δ mutations were confirmed by growing yeast on YPD plates containing G418 (1mg/mL) while *rad51*-Δ spores were identified by Southern blotting. Multiple spores carrying the rad mutation and one or more appropriate auxotropic markers were then checked for the ability to mate to the ZT3 strain. The *ter1*-Δ *rad50*-Δ *rad51*-Δ mutants were constructed by mating a *ter1*-Δ *rad50*-Δ pTER1-WT-URA3 haploid strain to a *rad51*-Δ spore generated from *rad51*-Δ (SAY516) x GG1958 mating. The *ter1*-Δ *rad59*-Δ *rad51*-Δ was produced in a similar fashion by mating of a *ter1*-Δ *rad59*-Δ *pTER1-URA3* haploid to *rad51*-Δ spore generated from *rad51*-Δ (SAY516) x GG1958 mating. The loss of *pTER1-WT-URA3* from diploids was induced by growing diploids on YPD supplemented with 5-FOA (1 mg/mL). Diploids were then sporulated and spores were dissected using a Zeiss Axiolab E microscope equipped with a micromanipulator (Carl Zeiss Inc, Thornwood, NY). Strains carrying the *ter1*-Δ allele were identified by their rough colony morphology while other mutations were identified as described above.

To construct knockout alleles of *SGS1*, DNA sequences mapped to *Kluyveromyces lactis* chromosome D were obtained from Génolevures Consortium website and EMBL EBI databases. A 6222-kb fragment containing the *SGS1* promoter was generated by PCR from genomic DNA

of 7B520. Primers with *Bam*HI and *Kpn*I polylinkers (Forward primer: 5'TTCCTTCTTAACCGCATTCTT3'; Reverse primer: 5'CTGTGCCACATTTAAAGGCTA3') amplified region of DNA spanning from -1128 bp to +5535 bp. The cloned fragment was ligated into the pBlueScript plasmid pre-digested with *Bam*HI and *Kpn*I resulting in p*SGS1*. Further digestion of p*SGS1* with *Bgl*II created a 4572 bp deletion spanning from -996 bp to +3576 bp. The linearized plasmid fragment was then incubated with 1096bp *Bgl*II fragment of Sc*URA3*, excised from pJX3, in a ligation reaction and the resulting p*sgs1::URA3* plasmid was transformed into competent *E. coli* strain XL-10 gold. Digestion of p*sgs1::URA3* plasmid with *Pvu*II, *Kpn*I, and *Bam*HI released a 3.3 kb *Pvu*II *Bam*HI *sgs1::URA3* fragment. The *Pvu*II-*Bam*HI fragment was then gel extracted and subsequently transformed into 7B520 and ZT5 using Lithium acetate yeast transformation (40) to create EB17B (*sgs1::URA3 TER1*) and EB1D (*sgs1::URA3 ter1*-Δ *pTER1-HIS3*) strains. 7B520 transformants were plated on SD plates lacking uracil to select for *sgs1::URA3* allele incorporation, while ZT5 transformants were plated on SD plates lacking uracil and histidine to additionally retain p*TER1-HIS3*. The -996 bp to +3576 bp deletion of *SGS1* also removed the upstream regulatory sequence of *LAC9*, a gene required for lactose metabolism. Disruption of *LAC9* function does not have effect on colony morphology, growth or telomere phenotype. When plated on YPD+X-gal plates, both EB17B and EB1D can cleave X-gal but at a significantly lower lever as indicated by a lighter blue colony color when compared to wild type control.

To construct the *ter1*-Δ *rad50*-Δ *sgs1*-Δ strain, we transformed the EB17B strain with a ~3.4 kb *rad50::KANMX* construct obtained through PCR of the genomic DNA of *rad50*-Δ (SAY557) strain (Forward primer: 5'AATTTGTGAGTCGGAGGACACT3', Reverse primer:

5’GTATTGGACATGATGGTGAGCTATT3’). Correct transformants were identified by *Afl*II and *Xho*I digests of genomic DNA and Southern blotting.

Table 1. Yeast strains used in this study

***K. lactis* strains**	**Genotype**	**Reference**
7B520	*TER1 his2-2 trp1 uraA1*	(64)
ZT3	*ter1-Δ his2-2 trp1 pTER1-WT-ScURA3*	(39)
ZT5	*ter1-Δ trp1 uraA1 pTER1-WT-ScHIS3*	(39)
ter1-Taq	*ter1-Taq his2-2 trp1 uraA1*	(40)
SAY45	*TER1 leu2 lysA1 metA1 trp1 uraA1*	A gift from S. Åström
SAY557	*TER1 rad50::KANMX leu2 lysA1 metA1 trp1 uraA1*	(27)
SAY516	*TER1 rad51::ScLEU2 leu2 lysA1 metA1 trp1 uraA1*	(27)
Kl 1888	*TER1 rad59::KANMX ade2-202*	(62)
GG1958	*TER1 ade2-202*	a gift from B. Zonneveld
ter1-16T	*ter1-16T trp1 uraA1*	(61)
ter1-Acc	*ter1-Acc trp1 uraA1*	(37)
EB17B	*TER1 his2-2 trp1 sgs1::URA3*	This paper
EB1D	*ter1-Δ his2-2 trp1 sgs1::URA3 pTER1-WT-ScHIS3*	This paper
EB50T	*ter1-16T rad50::KANMX trp1 uraA1*	This paper
EB50A	*ter1-Acc rad50::KANMX his2-2 trp1 uraA1*	This paper
EB1T	*ter-16T sgs1::URA3 trp1 uraA1*	This paper
EB1A	*ter1-Acc sgs1::URA3 his2-2 trp1 uraA1*	This paper

The double mutants of *ter1-16T* and *ter1-Acc* with *rad50*-Δ or *sgs1*-Δ (*ter1-16T rad50*-Δ, *ter1-16T sgs1*-Δ, *ter1-Acc rad50*-Δ, and *ter1-Acc sgs1*-Δ) were created through transformation generating strains EB50T, EB1T, EB50A and EB1A, respectively. Replacement of *RAD50* was confirmed by *Afl*II and *Xho*I restriction digest and Southern blotting, while replacement of *SGS1* replacement was confirmed by *Fsp*I and *Msc*I digest of genomic DNA of transformants followed by Southern blotting. The *rad51*-Δ and *rad59*-Δ mutations in each of the long telomere mutants were introduced through mating and sporulation. The *rad59* mutation was confirmed using

selective plating on YPD+G418 and *rad51* mutation was confirmed by *Nci*I digest of genomic DNA and Southern blotting.

K. lactis stn1-M1 and *stn1-M1 ter1-Δ* strains were described previously (26). The *stn1-M1 ter1-Δ rad50-*Δ strain was constructed by transformation of ~3.4 kb *rad50::KANMX* into *stn1-M1 ter1-Δ* cells complemented by p*STN1-TER1-URA3*. The replacement of native *RAD50* allele by the *rad50::KANMX* disruption cassette occurred through homologous recombination and was confirmed by Southern blotting. The transformants carrying the correct replacement of *RAD50* allele were plated on medium containing 5-FOA to select for the loss of p*STN1-TER1-URA3* and to generate the *stn1-M1 ter1-Δ rad50-*Δclones. The *stn1-M1 rad51-*Δ strain was generated by mating the *stn1-M1* strain with the *rad51-*Δ strain (SAY516) followed by random spore analysis. The *rad51* mutation was identified as described above.

Senescence assay and recovery of survivors.

Senescence and survivor formation were observed by streaking 8-10 independent clones of each mutant on YPD for 5-6 consecutive streaks. The *ter1-*Δ *rad50-*Δ, *ter1-*Δ *rad51-*Δ, *ter1-*Δ *rad59-*Δ, *ter1-*Δ *rad59-*Δ *rad51*Δ, and *ter1-*Δ *rad50-*Δ *rad51-*Δ strains were streaked from spore colonies on YPD plates, while *ter1-*Δ *sgs1-*Δ strain was generated by plating *ter1-*Δ *sgs1-*Δ p*TER1-HIS3* on solid YPD medium and identifying colonies with rough colony morphology indicative of telomerase loss. Therefore, all strains lost telomerase at the point of spore cell generation except for *ter1-*Δ *sgs1-*Δ cells which lost telomerase during the streak on YPD. For this reason, the timing of senescence of *ter1-*Δ *sgs1-*Δ mutants was expected to be slightly delayed relative to other *ter1-*Δ mutants. Colony morphology was observed at every streak for each mutant clone. To control for a bias in colony selection, a mutant of an average colony

phenotype was chosen for subsequent streaking during the senescence phase. Formation of post-senescence survivors was identified by the presence of better growing colonies. Pictures were taken using Zeiss Stemi SV11 stereo microscope and a Leica DFC 320 camera (Diagnostic Instruments Inc., Sterling Heights, MI).

Assaying telomere length when cells were passaged non-clonally in liquid culture.

This assay used serial dilutions in liquid YPD to determine telomere length profiles in a non-clonal population. Eight to ten clones were isolated from senescing *ter1*-Δ *rad50*-Δ, *ter1*-Δ *rad51*-Δ, *ter1*-Δ *rad59*-Δ, *ter1*-Δ *rad59*-Δ *rad51*Δ, and *ter1*-Δ *rad50*-Δ *rad51*-Δ, and *ter1*-Δ *sgs1*-Δ mutants and grown in 1.5 ml of liquid YPD. The *ter1-Δ rad50-Δ, ter1-Δ rad51-Δ, ter1-Δ rad59-Δ*, and *ter1-Δ rad51-Δ rad50-Δ* spores were transferred into liquid YPD shortly after spore dissection. The *ter1-Δ* p*TER1-HIS3* and *ter1-Δ sgs1-Δ* p*TER1-HIS3* were first streaked on YPD to identify clones which lost p*TER1-HIS3* and then colonies with rough colony edges were transferred into liquid YPD. The cultures were diluted daily at a 1:1000 ratio for each of 20 days. DNA was extracted at passage 20 and digested with *Tsp*509I. *Tsp*509I is a 4 bp cutter that cuts from 19 to 425bp away from the first telomeric repeat.

Gel electrophoresis and Southern blotting.

Restriction digests of *K. lactis* genomic DNA were run on 0.8% agarose gels using 0.5X Tris Borate buffer. For Southern blotting, DNA from agarose gels was transferred onto Hybond N+ or Hybond XL (Amersham Biosciences, Piscataway, N.J.) membranes using 0.4 M NaOH. DNA was fixed to the membrane by ultraviolet light in an electronic ultraviolet crosslinker (Ultra Lum, Inc. Paramount, CA). For 2D gel analysis of low molecular weight DNA, uncut, RNase-

treated genomic DNA was separated in a 4% NuSieve 3:1 agarose gels (Lonza, Rockland, ME) (9). For 2D gel electrophoresis of high molecular weight DNA, *Eco*RI-digested DNA was separated as previously described (10).

Telomeres were visualized by hybridization to a telomeric G-strand probe (Klac1-25 ACGGATTTGATTAGGTATGTGGTGT) or C-strand telomeric probe (ACACCACATACCTAATCAAATCCGT) labeled with γ-^{32}P ATP (Perkin Elmer, MT, USA). Hybridizations with this probe were carried out overnight at 48° in 7% SDS, 0.5 M EDTA, and 0.5 M Na_2HPO_4. Membranes were washed three times for 5 min in buffer containing 100 mM Na_2HPO_4 and 2% SDS.

Clones carrying the *rad51*-Δ or *rad50*-Δ mutations were identified by whole *RAD51* or *RAD50* as a probe. *SGS1* fragment isolated from pSGS1 by *Kpn*I and *Bam*HI digest. The subtelomeric probe was generated from ~590-bp *Eco*RI-*Xba*I from the plasmid KL-11B (44). The *URA3* probe used was a *Hind*III fragment from pMH3 containing the *S. cerevisiae URA3* gene (21). DNA fragments described above were labeled with α-^{32}P dATP (Perkin Elmer, MT, USA) using a NEBlot® Kit (New England Biolabs, Ipswich, MA). Membranes were hybridized overnight at 65°C and washed three times for 20 minutes each in buffer containing 50 mM Na_2HPO_4 and 2% SDS. All Southern blots were visualized using a Storm Phosphoimager (Amersham Biosciences, Piscataway, N.J.), PhosphoImager QL software.

In-gel hybridization.

In-gel hybridizations were performed using a variation of method previously described (16). Genomic DNA was resolved on 0.7% agarose gel at 1V/cm. Gels were soaked in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 30 min and then blotted using

Whatman paper. Thin gels of a few mm thick were hybridized in 10× SSC and with C-strand telomeric probe at 23°C overnight. The gels were washed in 0.25× SSC three times, 1.5 hr each wash.

1.6 kb circle construction and t-circle utilization assay.

The 1.6 kb *URA3*-telomere circle was constructed by circularizing a *Bam*HI-*Bgl*II fragment from plasmid pMH3-1Tel (44). Approximately 85 μg of t-circle containing a Sc*URA3* marker gene, ~11.5 telomeric repeats and ~120 bp of subtelomeric sequence was transformed into yeast using yeast electroporation. In addition, 10 μg of an ARS plasmid pIB3 was used as control in all strains (64). T-circles were transformed into control strains (wild type 7B520 and *ter1-Taq*) and also *ter1-Taq rad50-Δ, ter1-Taq rad51-Δ, ter1-Taq rad52-Δ,* and *ter1-Taq rad59-Δ. ter1-Taq rad50-Δ* was constructed by *ter1-Taq* transformation with *rad50::KANMX*. *ter1-Taq rad51-Δ* and *ter1-Taq rad59-Δ* were constructed by mating *ter1-Taq* to *rad51-Δ* and *rad59-Δ* spores obtained from *rad51-Δ* x GG1958 and *rad59-Δ* x GG1958 crosses, respectively. *ter1-Taq rad52-Δ* was constructed by mating *ter1-1Taq* to previously generated *rad52-Δ* and diploid sporulation. The *rad50, rad51, rad52,* and *sgs1* mutations were identified by Southern blotting as described above, while *rad59* mutation was identified by plating on YPD+G418.

All transformants were grown on SD selective plates lacking uracil for 3 days at 30°C. The number of true transformants was calculated for 25 mm^2 areas at four different locations, then colony number average was calculated and adjusted to the standard Petri plate measurement of 5800 mm^2 . The genomic DNA was extracted from transformants and examined by Southern blotting for t-circle sequence incorporation using G-strand telomeric, *URA3*, and subtelomeric probes.

Subtelomeric gene conversion assay.

The assay for measuring subtelomeric gene conversion rates has been previously described (40). This assay utilizes STU (subtelomeric URA3) strains where a *URA3* gene is present ~120 bp from a telomere in the subtelomeric R element that is present at 11 of 12 telomeres in the wild type 7B520. To create *TER1* STU *rad-Δ* strains we mated a *TER1* STU strain (STU3) generated and described previously (40) to *rad51-Δ* and *rad59-Δ* mutants. The STU3 strain was also mated to the *ter1-Taq* strain. Diploids were sporulated and spores carrying *URA3* telomere the *ter1-Taq* mutation were identified by Southern blotting. Further, several *ter1-Taq* STU strains were mated to *rad51-Δ* and *rad59-Δ*. Diploids were sporulated and *ter1-Taq rad51-Δ* STU and *ter1-Taq rad59-Δ* STU spores were identified using Southern blotting or selective plating. Serial dilutions of *TER1 rad51-Δ* STU, *TER1 rad59-Δ* STU, *ter1-Taq rad51-Δ* STU, *ter1-Taq rad59-Δ* STU, *TER1* STU, and *ter1-Taq* STU were plated on SD media containing 5-fluoroorotic acid (5-FOA) to select for cells that have lost *URA3* subtelomeric gene conversion. Colony counts from YPD and SD+5-FOA plates were recorded and the subtelomeric gene conversion rates were determined using the method of the median (31).

Results

Individually, *RAD51, RAD50, RAD59,* and *SGS1* are not required for survivor formation in a *K. lactis* telomerase deletion mutant.

Studies in yeasts have demonstrated that deletion of telomerase leads to gradual telomere shortening, growth senescence, and eventual formation of post-senescence survivors with telomeres elongated by either Type I or Type II RTE (36, 37, 56). Each of these survivor types

form via homologous recombination as indicated by their dependence on Rad52. Work in *S. cerevisiae* has shown that the two survivor types rely on distinct sets of genes. Type I RTE utilizes Rad51, Rad54, and Rad57, genes involved in the "default" pathway of mitotic DSB repair, while Type II survivors rely on Rad50, Rad59, and Sgs1 (2, 13, 14, 30, 55).

We chose to examine the genetics of RTE in the yeast *K. lactis* because it differs from *S. cerevisiae* in both subtelomeric structure and in having uniform rather than degenerate telomeric repeats (38). As homologous recombination is highly sensitive to mismatched DNA sequences (53), this difference could be imagined to affect the efficiency and perhaps even the mechanism of RTE. To carry out our studies, we combined *K. lactis* the *ter1*-Δ mutation with deletions of the *RAD51, RAD50, RAD59,* and *SGS1* genes. The double mutants generated were then examined for their ability to produce post-senescence survivors.

Clones of the *ter1*-Δ control strain showed the expected gradual growth senescence over the first few streaks. By the 4th streak (after ~100 cell divisions) this was followed by the production of post-senescence survivors with improved growth and often wild type-like colony morphology (Figure 4.1A). These are similar to results reported previously (37). Next, we followed eight to ten independent clones of the *ter1-Δ rad51-Δ, ter1-Δ rad50-Δ, ter1-Δ rad59-Δ,* and *ter1-Δ sgs1-Δ* mutants. In all cases, these clones exhibited growth senescence during the serial streaking that was at least as rapid as that of *ter1-Δ* clones. Additionally, all clones of each of the tested mutants went on to produce better growing survivors within 4-5 streaks (~100-125 cell divisions) (Figure 4.1B-E). These results indicated that none of the four genes examined is individually essential for post-senescence survivor formation in *K. lactis*. However, in some cases, the timing of survivor formation and colony morphology phenotype varied among strains. For example, the senescence phenotype was more pronounced in the *ter1-Δ rad51-Δ* mutants

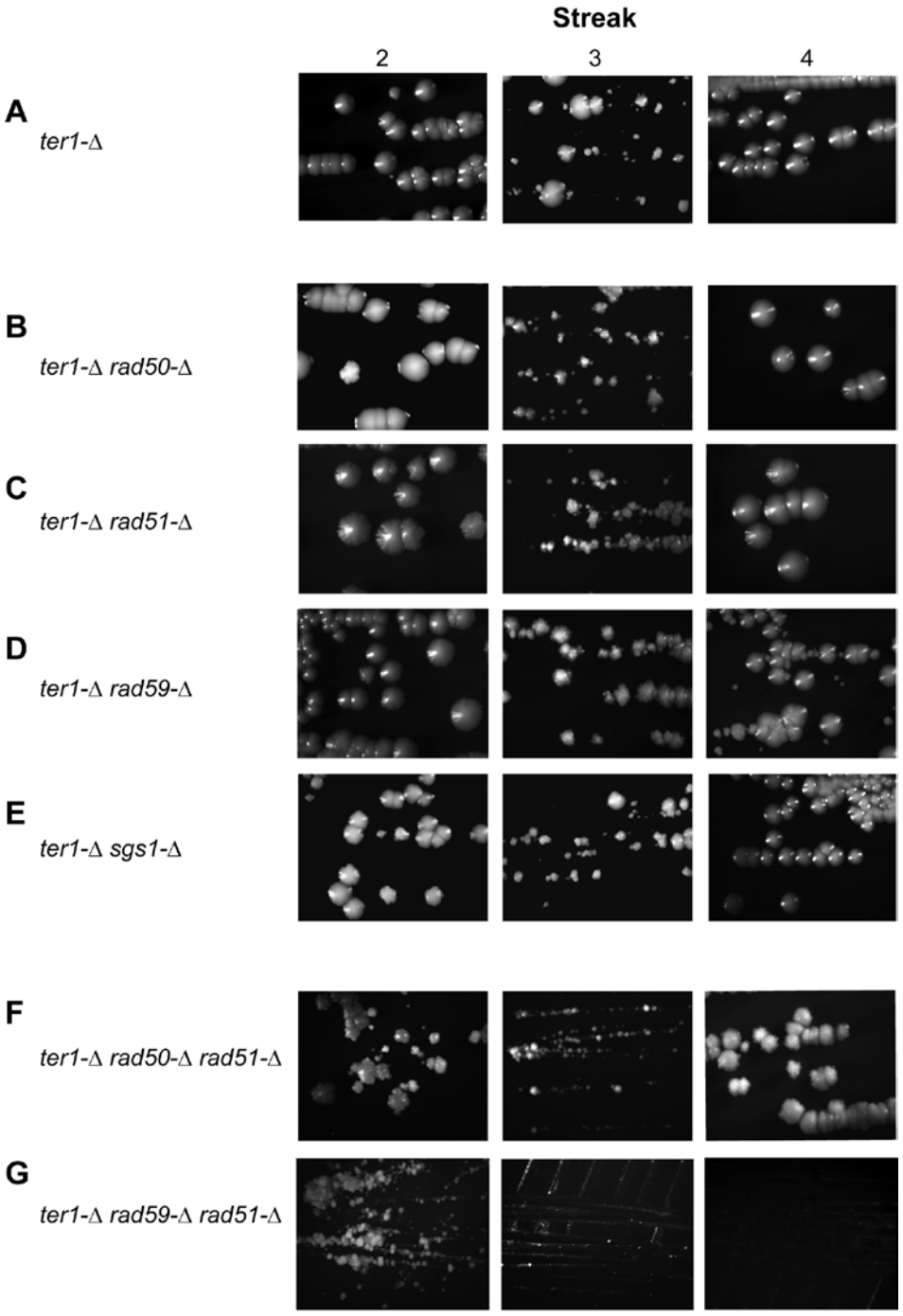

Figure 4.1. Individual deletions of *RAD50*, *RAD51*, *RAD59*, and *SGS1* do not abolish survivor formation in *K. lactis*. Photographs of clonal passaging of *ter1-Δ* (A), *ter1-Δ rad50-Δ* (B), *ter1-Δ rad51-Δ* (C), *ter1-Δ rad59-Δ* (D), *ter1-Δ sgs1-Δ* (E), *ter1-Δ rad50-Δ rad51-Δ* (F), and *ter1-Δ rad59-Δ rad51-Δ* (G) mutants on solid YPD media. Senescence of mutants shown in A-F was characterized by worsening colony morphology with eventual appearance of better growing survivors.

where senescent colonies generally showed a greater degree of colony roughness and more uniform roughness then did clones of *ter1-Δ* or the other mutants examined. Additionally, unlike other mutants examined, the *ter1-Δ sgs1-Δ* mutant clones rarely produced survivors with a smooth wild type-like colony morphology. These results suggested that Rad51 and Sgs1 may play roles in production of post-senescence survivors in *K. lactis* and that these roles are not identical.

To further address the possible relationships of the tested genes to RTE in *K. lactis* we constructed *ter1-Δ rad50-Δ rad51-Δ* and *ter1-Δ rad59-Δ rad51-Δ* triple mutants. Five to ten spores of independently generated triple mutants of each genotype were followed by serial streaking as described above. Results from this analysis showed that the *ter1-Δ rad50-Δ rad51-Δ* mutants (Figure 4.1F) exhibited a distinctly more severe senescence phenotype when compared to the *ter1-Δ rad50-Δ*, *ter1-Δ rad51-Δ,* or *ter1-Δ* mutants. This was characterized by faster senescence, more uniform colonies of much smaller size and rougher colony appearance than seen in *ter1-Δ* single mutant at equivalent streaks. On average, survivors in the *ter1-Δ rad50-Δ rad51-Δ* mutants were formed at the 4^{th} streak, but were significantly decreased in their frequency relative to the *ter1-Δ* control. Three out of eight clones examined did not produce survivors at all (data not shown). The post-senescence survivors that were recovered retained a senescent rough colony phenotype which did not improve with subsequent streaking. These results demonstrated that both Rad50 and Rad51 contribute to post-senescence survivor formation in *K. lactis* and that the simultaneous absence of both clearly impairs the RTE process.

The five independent clones of the *ter1-Δ rad59-Δ rad51-Δ* mutant that were studied all senesced at an increased rate when compared to the *ter1-Δ rad50-Δ rad51-Δ* or other mutants (Figure 4.1G). At the second streak, all colonies were very small and rough and by the third

streak growth essentially ceased. No survivors were observed in any of the five clones examined in each of three repetitions. This result indicated that Rad59 also contributes to post-senescence survivor formation in *K. lactis* and that it becomes essential to survivor formation if Rad51 is also missing.

***ter1*-Δ *rad51* post-senescent survivors have longer telomeres than other mutants.**

We next examined the telomeres of the post-senescence survivors from the different mutants using *Eco*RI digests and Southern blotting. The post-senescence survivors from *ter1-Δ* and double mutants of *ter1-Δ* (Figure 4.2A-E) were generally similar, exhibiting telomeric bands of various sizes including some substantially shorter and some substantially longer than those of wild type telomeres. In all mutants, the length of telomeres in the survivors was typically longer than those of cells at senescence (data not shown). The smear of telomeric signal visible in many lanes (e. g., clones 1 and 3 of *ter1*-Δ) most likely was due to some telomeres being variable in size between different cells in the population examined. Some survivors in the *ter1-Δ rad50-Δ*, *ter1-Δ rad51-Δ* and *ter1-Δ rad59-Δ* mutants had some telomeric signal that remained in the wells (Figure 4.2B-D). This telomeric DNA conceivably could be unresolved recombination intermediates with structures unable to migrate into gels. Overall our results here confirmed that individual deletions of *RAD50*, *RAD51, RAD59*, and *SGS1* did not block RTE in a *K. lactis* telomerase deletion mutant.

The telomeric patterns in the survivors from the various double mutants generally appeared similar. A notable exception was the *ter1-Δ rad51-Δ* mutant which had telomeres that were clearly a longer size than those seen in the other mutants (Figure 4.2C). This was particularly noticeable in that survivors in this mutant largely lacked telomeric *Eco*RI fragments

of below ~1 kb in size. The longer telomere sizes in *ter1-Δ rad51-Δ* survivors were confirmed by *Bsr*BI digests that removed all but 3 bp of subtelomeric DNA from 10 of 12 telomeres and in liquid-passaged cultures that each contained multiple independent survivors (data not shown).

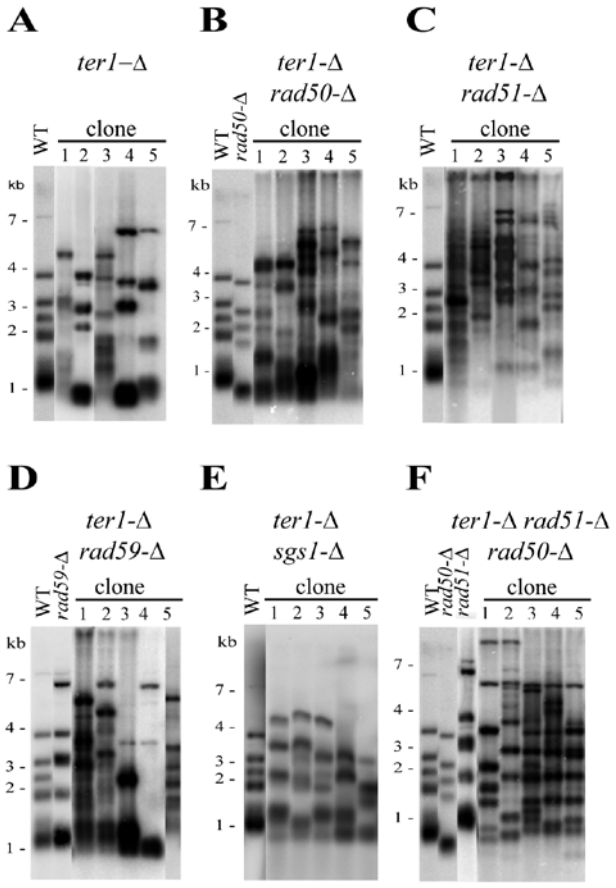

Figure 4.2. *ter1*-Δ *rad51* post-senescent survivors emerge with telomeres longer than other mutants. Shown is a Southern blot of *Eco*RI-digested genomic DNA hybridized to a telomeric probe. Five independent survivor clones from the *ter1-Δ* (A), *ter1-Δ rad50-Δ* (B), *ter1-Δ rad51-Δ* (C), *ter1-Δ rad59-Δ* (D), *ter1-Δ sgs1-Δ* (E), *ter1-Δ* rad50-*Δ rad51-Δ* (F) mutants were examined. Telomeres of control strains including the wild type (WT) are shown to the left of each panel.

We next examined telomeres in the *ter1-Δ rad50-Δ rad51-Δ* survivors (Figure 4.2F). Seven tested spores of the *ter1-Δ rad50-Δ rad51-Δ* genotype produced Type II survivors similarly to single and double mutants of *ter1-Δ* examined. All *ter1-Δ rad50-Δ rad51-Δ* survivors exhibited telomeric bands that appeared to be elongated. This result indicated that simultaneous

deletion of *RAD50* and *RAD51* still allowed formation of Type II survivors. Interestingly, the extent of elongation appeared to be less than that seen in *ter1-Δ rad51-Δ* survivors. Additionally, most telomeric bands appeared generally to be more sharply defined than the bands seen in survivors from other mutants.

To confirm that a *rad51*-Δ mutation affected telomere lengths in the post-senescent survivors, we grew the various mutants by non-clonal culturing in liquid medium. Five to ten spores from each of six types of mutants were serially passaged 20 times in liquid YPD cultures and then examined by Southern blotting. Unlike the clonal passaging on plates, which experience a bottleneck of a single cell with every streak, the liquid cultures are more likely to contain multiple independent lineages of survivors and therefore display a broader range of telomere lengths. Figure 4.3 shows telomeric fragments from DNAs from the 20th passage of each clone that were digested with *Tsp*509I. This enzyme cuts 19-425 bp from the most basal telomeric repeat at all telomeres and therefore provides a simpler means for estimating telomere lengths compared to *Eco*RI digests. In general, the double mutants produced smeary telomeric fragment profiles similar to those seen with a *ter1-Δ* mutant with much signal below the length exhibited by a wild type *TER1* control. Notably, each of five *ter1-Δ rad51-Δ* populations had greater average telomere lengths and reduced levels of very short telomeres than were seen in any other mutant. This confirms that *ter1-Δ rad51-Δ* cells have longer telomeres on average than is seen in the other mutants examined. Interestingly, the *ter1-Δ rad51-Δ rad50-Δ* clones exhibited less lengthening than *ter1-Δ rad51-Δ* cultures, suggesting that *RAD50* contributes to the lengthened telomeres in *ter1-Δ rad51-Δ* cells. Our results here confirmed that on average telomere lengths in telomerase deletion mutants increased with the deletion of *RAD51*.

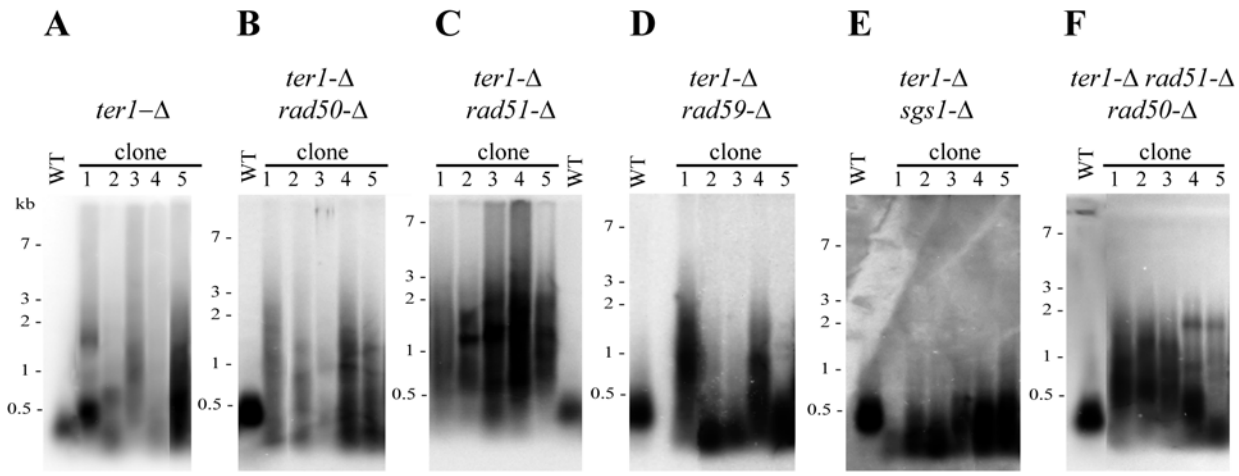

Figure 4.3. All mutants undergo RTE when passaged non-clonally with *ter1-Δ rad51-Δ* clones having longer telomeres than other mutants. Five clones of the *ter1-Δ* control (A) and the double mutants *ter1-Δ rad50-Δ* (B), *ter1-Δ rad51-Δ* (C), *ter1-Δ rad59-Δ* (D), *ter1-Δ sgs1-Δ* (E), and *ter1-Δ rad51-Δ rad50-Δ* (F) were passaged non-clonally for 20 serial dilutions in liquid YPD . Shown are Southern blotting of genomic DNA isolated at the 20th passage and digested with *Tsp*509I and subsequently hybridized to a G-strand telomeric probe.

The effect of *RAD50, RAD51, RAD59* and *SGS1* deletions on the long telomere phenotypes in *K. lactis* mutants that produce abundant t-circles.

To study the genetics of t-circle formation, we combined *rad50-Δ*, *rad51-Δ*, *rad59-Δ* and *sgs1-Δ* mutations with the *ter1-16T* and *ter1-Acc* mutations which produce highly elongated telomeres and abundant t-circles (5, 12, 18, 61). The *ter1-Acc* mutation produces immediate telomere lengthening, while the *ter1-16T* allele has a milder phenotype that develops highly elongated telomeres gradually (39, 61). Three independent clones of *ter1-16T rad50-Δ* and *ter1-Acc rad50-Δ* mutants were each followed for 14 consecutive streaks (Figure 4.4A-B). All clones of each of these double mutants rapidly exhibited a different phenotype than the *ter1-Acc RAD50* and *ter1-16T RAD50* controls. Specifically, they displayed distinct sharp telomeric bands that ran ahead of the high molecular weight telomeric signal, while also showing significant reduction in the smear of low molecular weight telomeric signal that extended to well below 1kb.

With subsequent streaks, the distinct telomeric bands became gradually shorter and the smear of low molecular weight extrachromosomal telomeric DNA became less distinct or disappeared. Interestingly, the average rate of gradual shortening was greater in the *ter1-Acc rad50-Δ* clones. By the 14th streak, some telomeric fragments in clones of this mutant had been reduced to sizes shorter than the shortest telomeric fragments in wild type cells. We also observed some sudden changes in length that shortened telomeric fragments up to several hundred bp (examples are indicated by the white arrows). By the 4th or 5th streak, the colony morphology of the *ter1-16T rad50-Δ* and *ter1-Acc rad50-Δ* mutants improved from rough and irregular (the normal colony morphology of the long telomere mutants) to smooth and round (the characteristic of the wild type strains). Along with the general trend of telomere shortening, a few instances of bands apparently becoming longer were also observed. Whether these represent telomere lengthening from recombination or telomerase is not clear. Altogether, our results here indicated that *RAD50* is required for the long, unstable telomeric phenotype of the *ter1-16T* and *ter1-Acc* mutants. This result was probably not surprising as Rad50 is known to be important not just for RTE but also for telomerase-mediated telomere maintenance (8, 60).

Next, we investigated the effect of the *rad51-Δ, rad59-Δ,* and *sgs1-Δ* mutations on the telomere phenotypes of the *ter1-16T* and *ter1-Acc* mutants. Sibling spores produced from the dissected tetrads of *ter1-16T* and *ter1-Acc* double mutants with *rad51-Δ* and *rad59-Δ* were followed for 10 consecutive streaks and examined on Southern blots (Figure 4.5). Each spore initially had a mix of long and short telomeres inherited from their respective long and short telomere parental strains. Those spores carrying the wild type *TER1* rapidly exhibited signs of gradual shortening of its long telomeric sequences (data not shown). On the other hand, spores with the telomerase mutations were found to have restored all telomeres to very long sizes by the

10th streak (Figure 4.5A-F). These results indicate that, unlike the *rad50-Δ* mutation, the *rad51-Δ, rad59-Δ* and *sgs1-Δ* mutations did not block the formation and maintenance of long telomeres in the *ter1-16T* and *ter1-Acc* mutants.

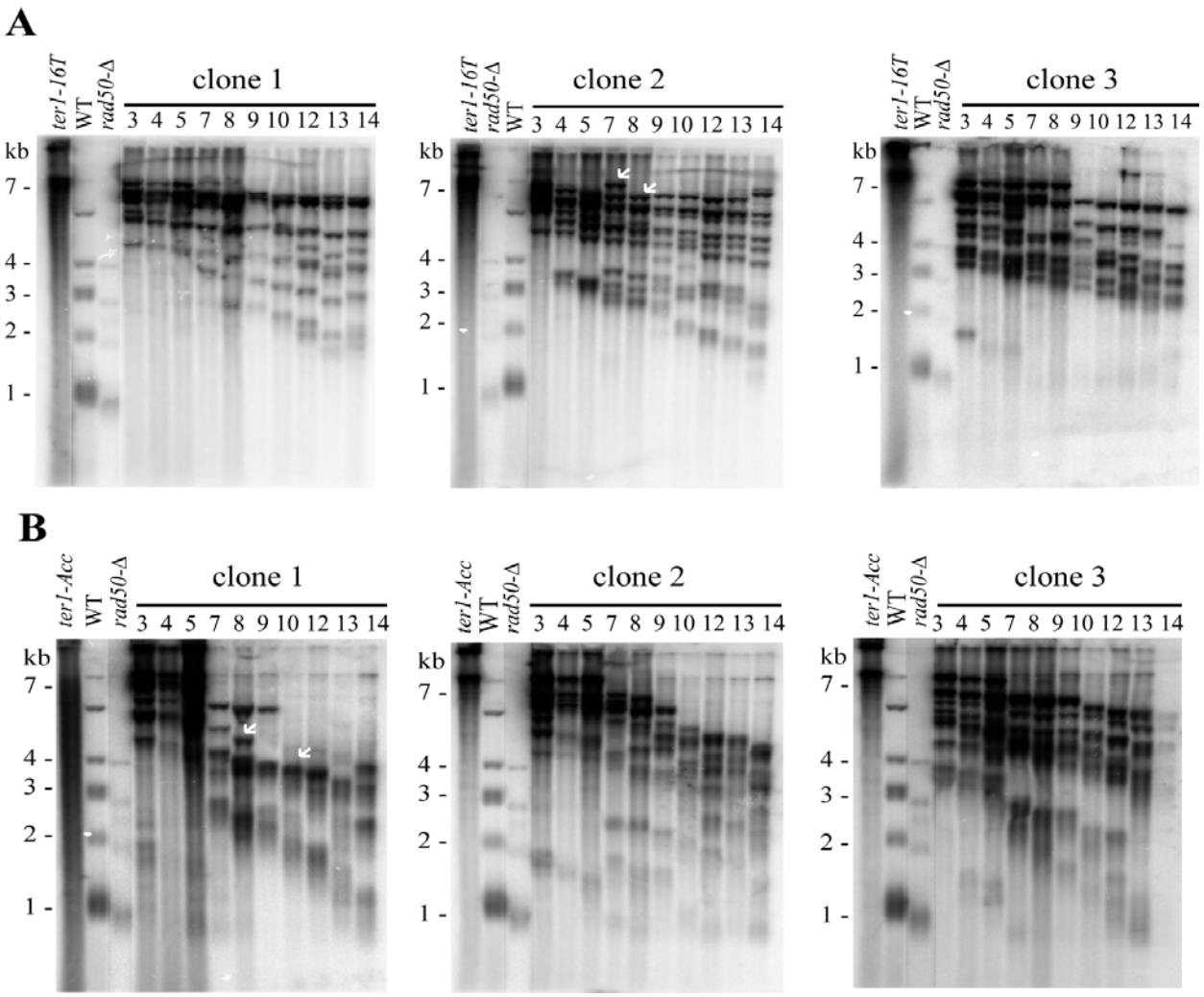

Figure 4.4. Effect of *RAD50* deletion on telomeres in the *ter1-16T* and *ter1-Acc* mutants. Southern blotting of genomic DNA isolated from three independent clones of *ter1-16T* (A) and *ter1-Acc* (B) followed for 14 consecutive streaks. DNA was digested with *Eco*RI. The untransformed parental strains of *ter1-16T* and *ter1-Acc*, and *rad50-Δ* and WT are shown on the left of each panel. Telomere shortening events included deletions of several hundred base pairs (two examples are indicated by pairs of white arrows) with the *ter1-Acc* mutant experiencing a greater rate of telomere shortening than the *ter1-16T* mutant.

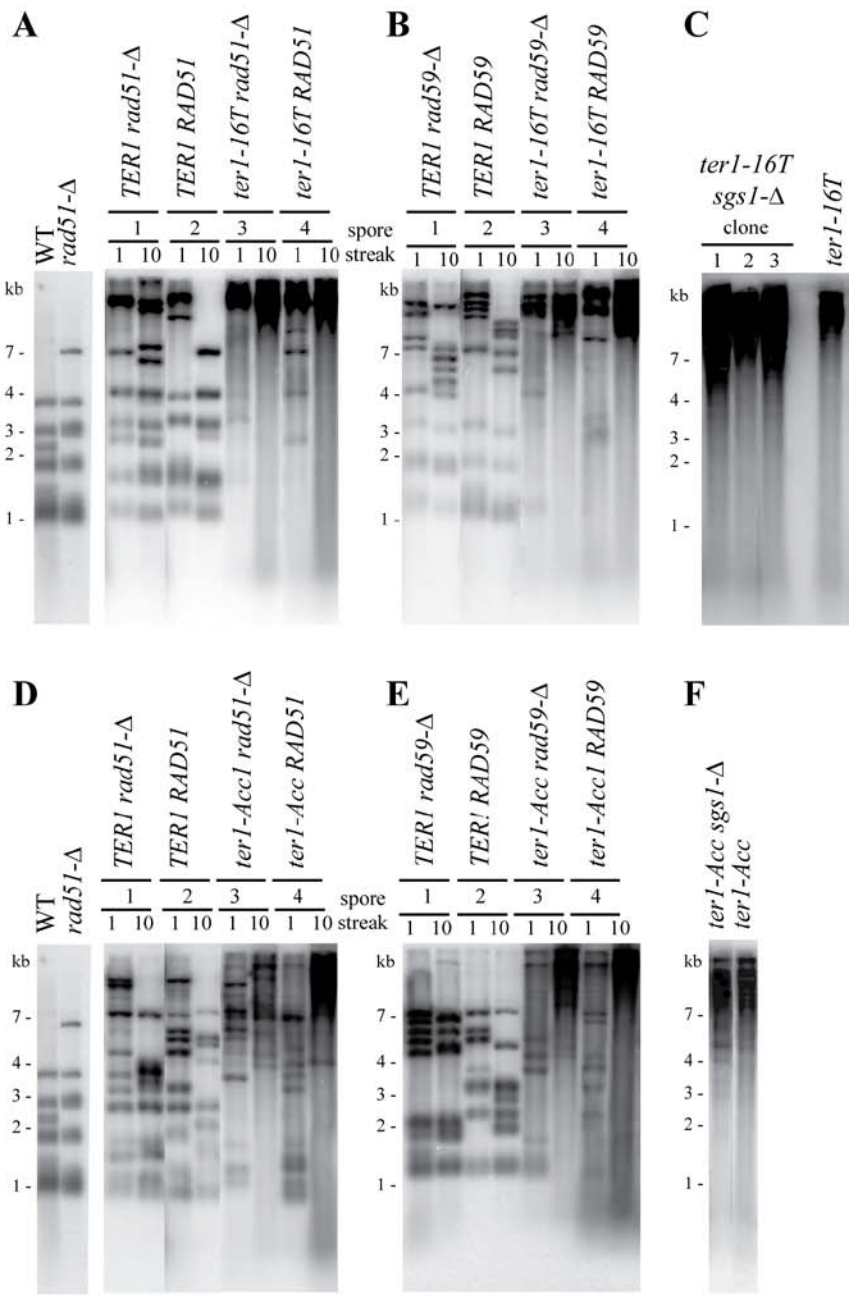

Figure 4.5. Effect of *RAD51, RAD59* and *SGS1* deletions on telomeres in the *ter1-16T* and *ter1-Acc* mutants. Shown is a Southern blot of genomic DNA, digested with *Eco*RI and hybridized to a G-strand telomeric probe. A set of spores obtained from sporulation of diploid strains of *ter-16T* or *ter1-Acc* also carrying either *rad51-Δ* or *rad59-Δ* mutations is shown in A and B, C and D, respectively. Three independent clones of *ter1-16T sgs1-Δ* and one clone of *ter1-Acc sgs1-Δ* are shown in C and F, respectively. Each spore clone was passaged for 10 consecutive streaks on solid rich medium (YPD).

Absence of *RAD50* greatly reduces the amount of small and large t-circles present in the *ter1-16T* and *ter1-Acc* mutants.

Previous work has shown that the smear of low molecular weight telomeric signal in *ter1-16T* mutants was primarily due to small t-circles (18). To investigate the role of *RAD50, RAD51, RAD59* and *SGS1* in the formation of small t-circles, we examined the genomic DNA from newly made double mutants using two-dimensional electrophoresis with 4% agarose gels containing chloroquine. In most mutants, hybridization of DNA to a G-strand telomeric probe produced a strong diagonal signal. This diagonal of small double-stranded t-circles was shown previously to be absent in *ter1-16T* mutants that lacked *RAD52* (18). While both long telomere *ter1* mutants retained the diagonal of small t-circles when combined with the *rad51*, *rad59,* or *sgs1* mutations, the *ter1-16T rad50-Δ* and *ter1-Acc rad50-Δ* mutants notably lacked the small t-circles (Figure 4.6A, C). A wild type control, as expected, also did not produce a diagonal of small t-circles in this assay (4). The same filters were then stripped and probed with a C-strand telomeric probe (Figure 4.6B, D). These hybridizations detected the same diagonal pattern and also an additional set of spots that sometimes ran slightly below and further than the double stranded track. This signal was shown previously to correspond to small single stranded t-circles (18). For reasons that are not clear, most of our experiments here were not able to achieve the degree of separation of double-stranded and single-stranded spots as was seen previously with the *ter1-16T* mutant (18). Our results indicated that *RAD50* is important for small t-circle formation but that *RAD51*, *RAD59,* and *SGS1* are not.

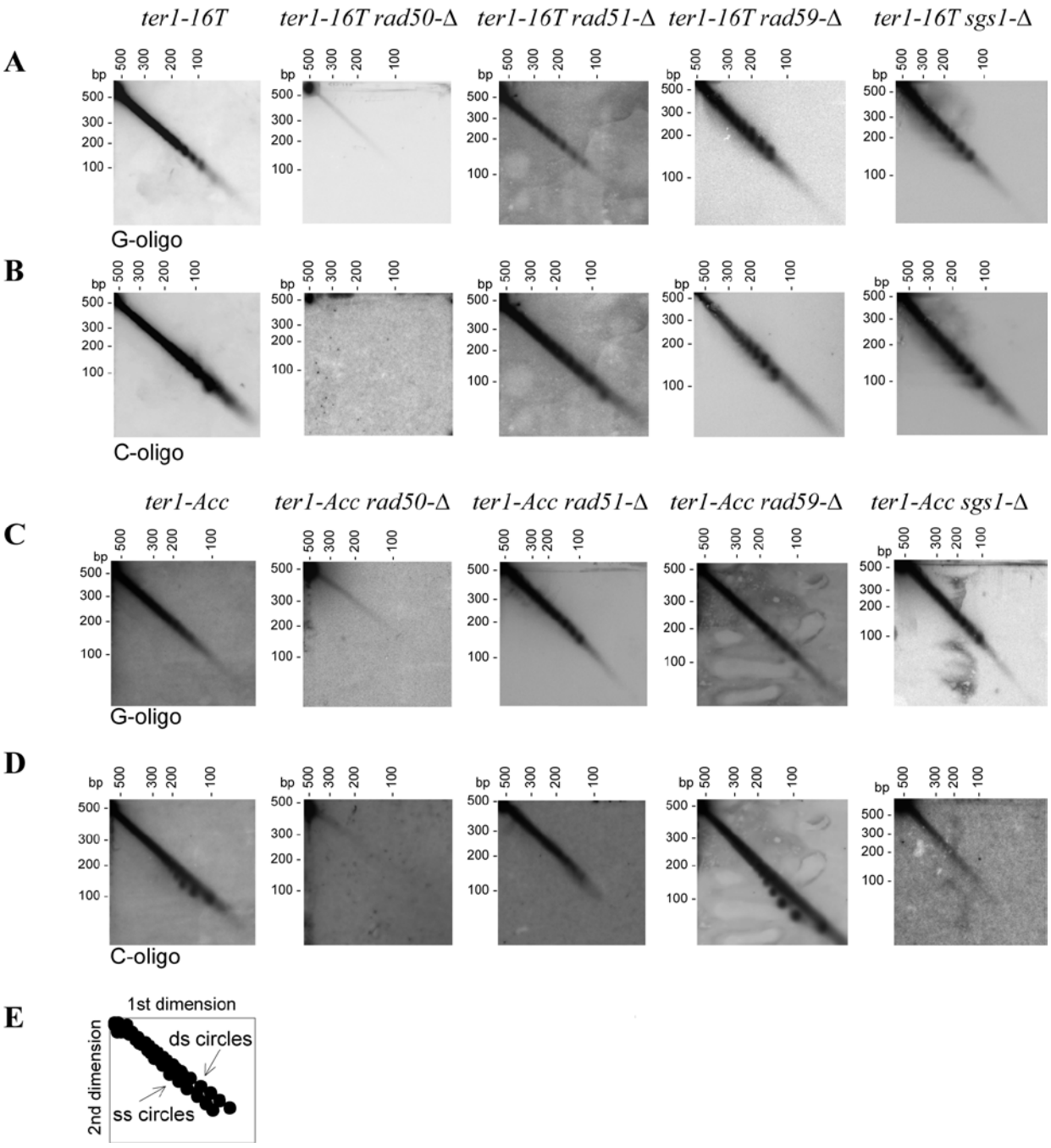

Figure 4.6. *RAD50* is important for small t-circle formation in the *ter1-16T* and *ter1-Acc* mutants. Low molecular weight telomeric DNA from single and double mutants of *ter1-16T* and *ter1-Acc* was examined by two dimensional (2D) agarose gel electrophoresis. Shown is Southern blotting of 4% 2D gels where filters with DNA from each mutant (as designated above the filters) was hybridized to G-strand (panels 4.6A,C) and C-strand (panels 4.6B,D) telomeric probe. The wild type parental strain 7B520 did not produce visible t-circles. A diagram showing positions of single-stranded and double-stranded circles is shown in E.

Next, we separated *Eco*RI-digested genomic DNA from *ter1-16T* and *ter1-Acc* and their double mutants on low percentage agarose 2D gels in order to determine the effect of the mutations on production of large t-circles. The wild type strain produced an arc of spots corresponding to telomere fragments visualized in 1D gels (Figure 4.7C). In contrast, the *ter1-16T* and *ter1-Acc* strains produced two arcs; a lower arc corresponding to linear telomeric DNA and an upper arc corresponding to circular telomeric DNA (Figure 4.7C). Results shown in Figure 4.7A and B indicate that the *rad51*, *rad59,* and *sgs1* mutations had little or no effect on the level of the circular telomeric DNA arc. In contrast, the *rad50* mutation greatly reduced but did not eliminate the circular telomeric DNA arc in both the *ter1-16T* and *ter1-Acc* mutants. A *rad52*-Δ mutation also greatly decreased the presence of large t-circles in *ter1-16T.* We were not able to recover *ter1-Acc rad52* spores. Our results demonstrated that both *RAD52* and *RAD50* significantly contribute to the formation of large t-circles and that one or more minor pathway of t-circle formation exists in their absence.

RAD50 is important for the generation of single-stranded G-rich overhangs in _ter1-16T and ter1-Acc_.

The 3' G-rich overhang plays a critical role in telomere function. Excessive processing leading to the generation of long 3' overhangs is a common feature of telomere uncapping (5, 26). To determine if the mutations studied here influenced the generation of 3' overhangs, we resolved *Eco*RI-digested genomic DNA of *ter1-16T* and *ter1-Acc* and their double mutants using non-denaturing in-gel hybridizations. Our results showed that all three clones of the *ter1-16T rad50-Δ* mutant and both tested clones of the *ter1-Acc rad50-Δ* mutant had significantly decreased G-strand overhangs relative to *RAD50* controls (Figure 4.8A and B) This result was

consistent with *RAD50* being involved in DNA resection at uncapped telomeric ends in these mutants. The in-gel telomeric signal also appeared reduced in *ter1-Acc rad51-Δ* clones when compared to *ter1-Acc rad59-Δ* or *ter1-Acc* (Figure 4.8D). This correlated with the general decrease of telomeric smear below 4 kb in the denatured gel.

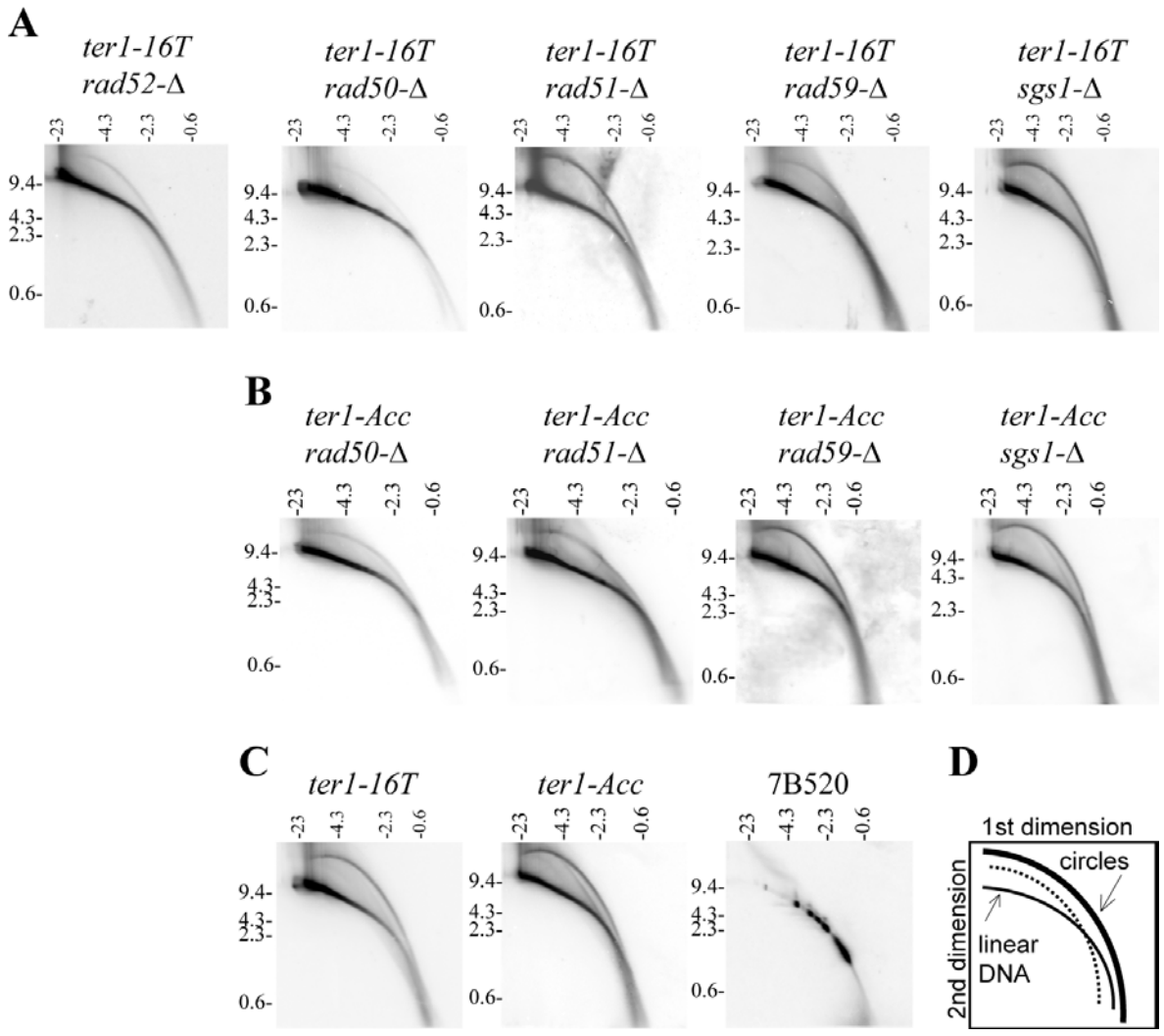

Figure 4.7. *RAD52* and *RAD50* significantly contribute to the formation of large t-circles. 8 µg of *Eco*RI-digested DNA from *ter1-16T* and its double mutants (A), *ter1-Acc* and its double mutants (B), and wild type (7B520) and parental strains (C) was resolved on low percent 2D agarose gels. High molecular weight linear telomeric DNA corresponds with the lower arc while the top arc represents circular telomeric DNA. The wild type strain produced a distinct set of bands corresponding to telomeric fragments previously visualized in 1D gels and did not produce visible t-circles. A diagram identifying the arcs of telomeric material is shown in D. Single-stranded G-rich telomeric DNA is depicted by perforated line.

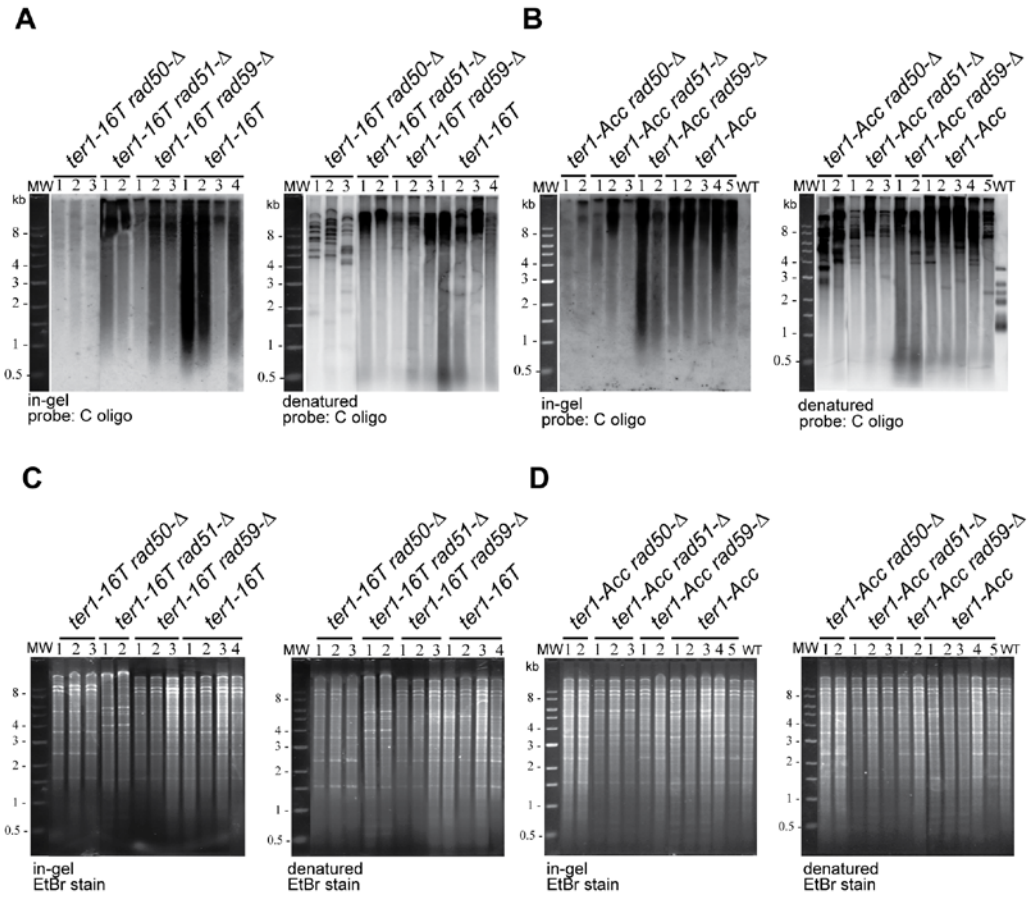

Figure 4.8. *RAD50* is important for the generation single stranded G-rich overhangs in *ter1-16T and ter1-Acc*. Mutants with long telomeres often show large amounts of single-stranded telomeric G-strand DNA. *Eco*RI-cut genomic DNAs from *ter1-16T* (A) and *ter1-Acc* (B) single and double mutants were run on a 0.7% gel. Half of the digestion reaction was used in an in-gel hybridization to visualize the single-stranded telomeric DNA (left panel in A and B). The other half was run similarly and blotted using standard denaturing protocol for Southern blotting (right panel in A and B). Both filters were hybridized to C-strand telomeric probe. The EtBr- stained gels are shown in C and D.

The utilization of t-circles to elongate telomeres occurs in the absence of *RAD50, RAD51,* and *RAD59*.

When DNA circles containing telomeric repeats and a *URA3* gene are transformed into *K. lactis* cells, their sequence becomes incorporated at telomeres as long tandem arrays that are thought to be the result of a rolling circle copying mechanism (43, 44). In *TER1* cells, typically

only a single telomere acquired a tandem array while in mutants with short recombinogenic telomeres, the arrays were present at many telomeres. Mixing experiments with two slightly different forms of the *URA3*-telomere circle demonstrated that all integrated copies were derived from a single original t-circle molecule (43, 44).

To determine if this t-circle-dependent telomere elongation requires the genes studied here, we performed transformations of a 1.6 kb *URA3*-telomere circle into the various recombination-defective mutants that had been engineered to also contain the *ter1-Taq* mutation. Like the *ter1-Δ* mutant, the *ter1-Taq* mutant has short telomeres that are prone to high levels of subtelomeric recombination and efficient incorporation of t-circle sequences at multiple telomeres (40, 43). However, the *ter1-Taq* mutant has stable telomere lengths (at ~1/4 normal length) and consistent recombination phenotypes rather than the continuously changing phenotypes (produced by continuously changing telomere lengths) seen in *ter1-Δ* cells. This makes the *ter1-Taq* allele much better suited for controlled comparisons between strains. The parental *ter1-Taq* strain normally exhibits slightly irregular colony morphology when grown on rich YPD media. The additional deletion of *RAD50*, *RAD51*, *RAD52,* but not *RAD59* significantly worsened the growth phenotype of the *ter1-Taq* mutant (data not shown). Colonies of all these double mutants had rough, senescent-like colony morphologies. These results indicate that *RAD50*, *RAD51,* and *RAD52*, but not *RAD59*, aid *ter1-Taq* cells' ability to grow.

The four double mutant strains as well as wild type and the *ter1-Taq* single mutant controls and were transformed separately with a 1.6 kb *URA3*-telomere circle and an ARS plasmid control. The *URA3*-telomere circle used in these experiments was identical to one used previously (44) that was made by circularization of a restriction fragment containing the *S. cerevisiae URA3* gene and an array of ~11.5 *K. lactis* telomeric repeats. Results from this

transformation showed that deletions of *RAD50*, *RAD51,* or *RAD59* did not appreciably affect the ability to obtain transformants with either the ARS plasmid or the *URA3*-telomere circle (data not shown). In contrast, the *rad52* mutation resulted in a large general defect in transformation as indicated by the ARS plasmid transformation rate being ~10-fold less than in a *ter1-Taq RAD52* mutant. The *rad52* mutation reduced the number of transformants from the *URA3*-telomere circle by an even greater amount. This suggested that *RAD52* is probably required for most *URA3*-telomere transformants in *ter1-Taq* cells. The basis of the poor transformability of the *ter1-Taq rad52* mutant is not known. However, two independent clones of that genotype behaved similarly (data not shown).

To visualize the telomeric structure of the transformants, genomic DNA was collected from 10-12 transformants from each mutant and digested with *Eco*RI. Representative results from these transformants are shown in Figure 4.9. When DNA from wild type transformants were hybridized to a subtelomeric probe that detects 11 out of 12 telomeres, all eight clones had new, larger bands that also hybridized to telomeric and *URA3* probes (Figure 4.9A). In five transformants, these new bands migrated at >10 kb while in the other three (clones 4, 6 and 8), they migrated at 4-6 kb. As demonstrated previously (44), this outcome was due to incorporation of tandem arrays of the t-circle sequence at a telomere. The different sizes were due to which telomeric fragment the tandem array had integrated in as well as to the number of *URA3*-telomere units in the array. Consistent with this interpretation, *Eco*RV, which cleaves once in the *URA3*-telomere circle's sequence, cleaved the bulk of the *URA3* signal in the clones down to a single band of ~1.6 kb (Figure 4.10F, G). One transformant (#2) had an *Eco*RI fragment that was clearly slightly shorter than the fragments in other transformants. This outcome was seen previously

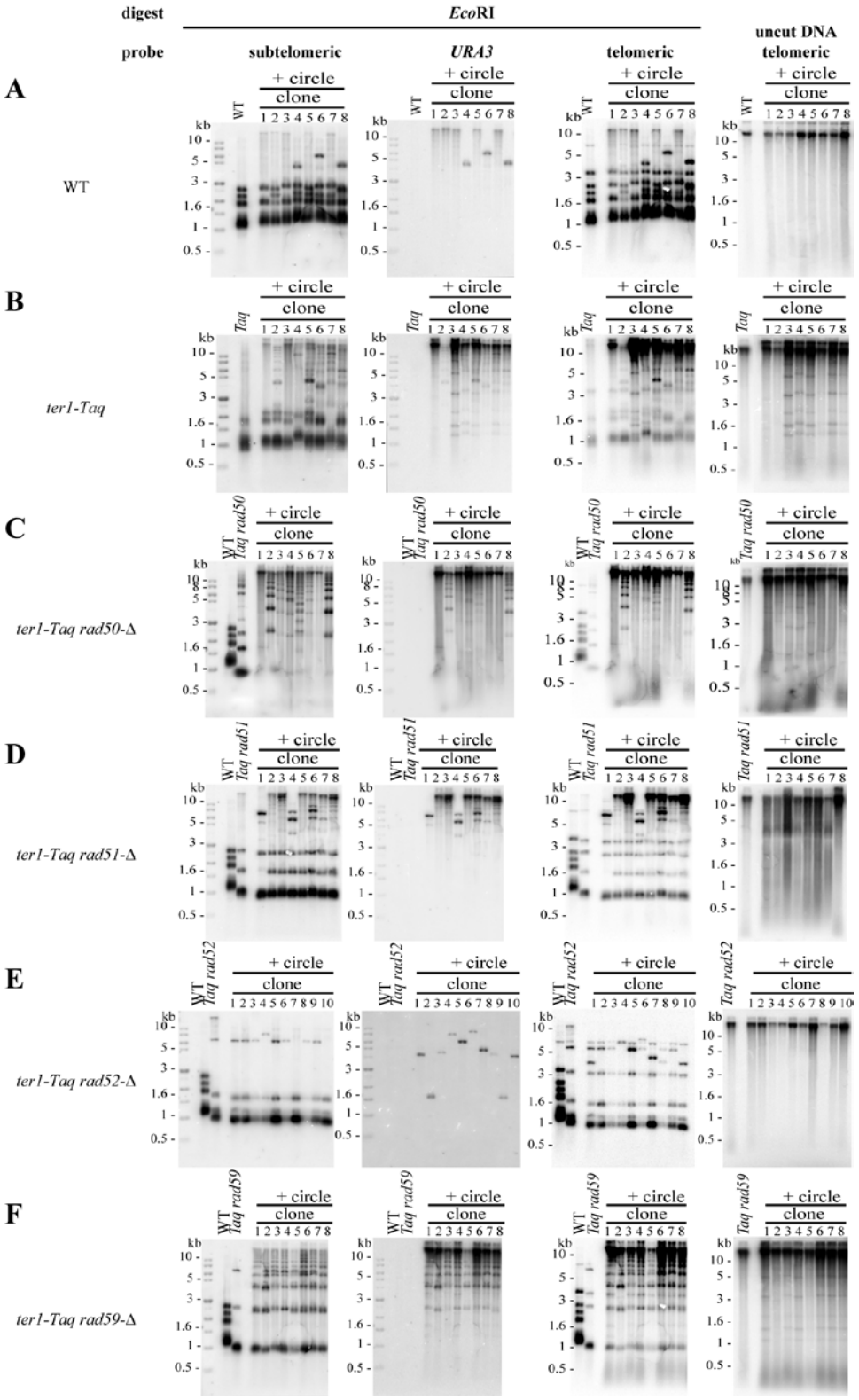

Figure 4.9. *RAD50, RAD51* and *RAD59* were not individually essential for the *URA3*-telomere circle utilization. The ability to utilize a 1.6 kb circle was determined in the short

telomere mutant *ter1-Taq*. The control strains *ter1-Taq* and WT as well as *ter1-Taq rad50-Δ*, *ter1-Taq rad51-Δ*, *ter1-Taq rad52-Δ,* and *ter1-Taq rad59-Δ* mutants were transformed with a 1.6 kb *URA3*-telomere circle. Southern blotting of genomic DNA from independent transformants was digested with *Eco*RI and the same filter was hybridized firstly to a *URA3* probe, then to subtelomeric, and telomeric G-strand probes. The untransformed WT and *ter1-Taq* mutant strains are shown to the left in each panel.

with transformants of the 1.6 kb *URA3*-telomere circle and was found to be due to recombination that deleted some telomeric repeats in the *URA3*-telomere circle, apparently prior to the sequence of the circle becoming copied onto telomeric ends (43). Also as seen previously, most of the WT transformants had slightly elongated telomeres, a result suggested to be due to the titration of telomere binding protein Rap1 by the greatly elongated telomeres (44).

Transformants of *ter1-Taq* mutants also behaved as described before (43). All of them had one or more telomeres that were significantly elongated as indicated by fragments running near limit mobility that hybridized to a subtelomeric probe and hybridized intensely to telomeric and *URA3* probes in *Eco*RI digested DNA (Figure 4.9B). The presence of multiple heterogeneously sized bands within each sample was indicative of these telomeric arrays being unstable and generating telomeres with a variable number of copies of the circle sequence. Further evidence of this instability was seen when uncut DNA from *ter1-Taq* transformants was examined with a telomeric probe. This experiment revealed a ladder of bands consisting of extrachromosomal linear and/or circular telomeric DNA. Similar ladders were seen previously in transformants of the *URA3*-telomere circle into *ter1-Δ* mutants where they are thought to be due to frequent recombination events that have excised one or more tandem *URA3*-telomere units from the *URA3*-telomere arrays (44).

With the exception of *ter1-Taq rad52* cells, all examined transformants from each of the tested double mutants showed clear evidence of having generated *URA3*-telomere arrays at their

telomeres that were overall quite similar to those seen in *ter1-Taq* single mutants (Figure 4. 9C, D, F). Most or all transformants of these mutants had *Eco*RI fragments that ran near limit mobility that hybridized to subtelomeric, telomeric, and *URA3* probes (Fig. 4.9 A-C). These bands were also all largely cleaved by *Eco*RV to produce a major band of ~1.6 kb (Fig. 4.10A, B, D-F). These results indicate that *RAD50*, *RAD51,* and *RAD59* were not individually essential for *ter1-Taq* cells to utilize the *URA3*-telomere circle for greatly extending their telomeres. Some differences between these mutants were evident though. While all transformants of all *ter1-Taq* mutants showed some *URA3* signal running at limit mobility, 4 of 12 *ter1-Taq rad51* mutant transformants, including two shown in Fig. 4.9D, had their largest *URA3*-hybridizing band between ~5-8 kb. The *URA3* signal in the transformants of this mutant also showed fewer signs of heterogeneity compared to other mutants with tandem arrays. These results suggested that the *rad51* mutation likely had a negative effect on the ability of the *URA3*-telomere circle to generate long tandem arrays and/or the ability to rearrange those arrays after their formation. Another interesting observation was made in the t-circle transformants of the *ter1-Taq rad50-Δ* mutant. Subtelomeric hybridization in these clones showed that a much higher percentage of the total group of telomeric fragments had become elongated compared to the other mutants with tandem *URA3*-telomere arrays (Figure 4.9C). This suggested that *rad50* mutation led to a greater degree of spreading of arrays to multiple telomeres. Most transformants of the *ter1-Taq rad50-Δ* mutants also showed high levels of apparently extrachromosomal telomeric DNA that ran below ~500 bp (Figure 4.9C, see telomeric probes of uncut and *Eco*RI-digested DNA). Evidence of elevated levels of this small extrachromosomal telomeric DNA was also apparent in the *ter1-Taq rad59-Δ* transformants (Figure 4.9F). 5 of 8 *ter1-Taq rad50-Δ* transformants had suffered deletions within the telomeric repeat blocks within the *URA3*-telomere arrays as judged by the

presence of shorter fragments in the *Eco*RV digests (Figure 4.10A). These deletions were previously reported to be more common in the short telomere *ter1-Taq* mutant than in wild type cells (43). As the *ter1-Taq rad50-Δ* mutant has even shorter telomeres than *ter1-Taq RAD50* (Figure 4.9; compare WT/ *ter1-Taq rad50-Δ* in C to WT/mutant pairs in D-F), this might account for the different behavior of *URA3*-telomere integration events in these strains. The extra short telomeres in *ter1-Taq rad50-Δ* cells might also account for the poorer growth of this strain relative to *ter1-Taq RAD50* cells.

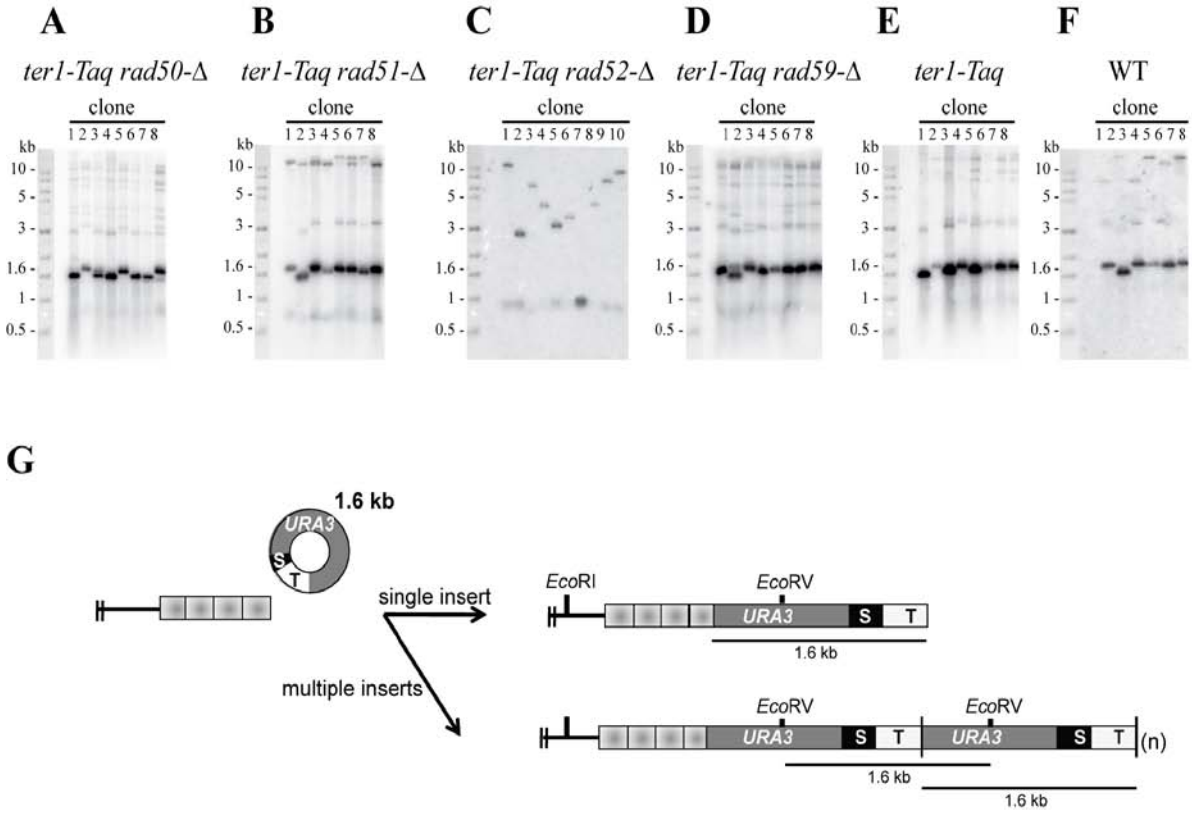

Figure 4.10. Transformants suffer deletions within the telomeric repeat blocks within the *URA3*-telomere arrays. (A-F) Genomic DNA from *URA3*-telomere circle transformants visualized in Figure 4.9 is digested with *Eco*RV which releases a 1.6kb fragment copied from the transformed circle. As judged by the presence of shorter fragments in the *Eco*RV digests, the excision most likely occurred within telomeric sequences and is consistent with previous observations (43). (G) Diagram of the expected structure of *URA3*-telomere circle transformants

is shown. Not drawn to scale. The location of the restriction enzymes cleavage sites used to ascertain the structure of telomeres in t-circle transformants are marked.

In contrast to the other mutants, the *ter1-Taq* rad52-*Δ* transformants examined (Figure 4.9E and Fig. 4.10C) completely lacked evidence of long telomeric arrays formed by the *URA3*-telomere circle. Instead, these transformants showed weak hybridization signal from *URA3* that seldom also hybridized to a subtelomeric probe and did not form a 1.6 kb band in *Eco*RV digests. This suggested that these transformants were likely single inserts into non-telomeric DNA. The mechanism of this sequence incorporation is not clear. One possibility is that the occasional linearization of the *URA3*-telomere circle led to NHEJ-dependent integration.

Subtelomeric BIR events are dependent on *RAD51*.

RTE in senescing *K. lactis* cells often utilize BIR events originating within subtelomere to spread both subtelomeric and elongated telomeric sequences to other chromosome ends (58). To determine the genetic requirements for subtelomeric BIR events we utilized cells containing a single telomere with a *URA3* gene inserted ~120 bp from the telomere in subtelomeric sequence common to 11 of 12 *K. lactis* telomeres. By plating on medium containing 5-FOA, cells that have lost the subtelomeric *URA3* gene were selected and quantified. Previous work showed that these subtelomeric BIR events were dependent upon *RAD52* and independent of *RAD50* (8, 40). To investigate whether *RAD51* and *RAD59* were required for subtelomeric BIR events, we first created *rad51-Δ* and *rad59-Δ* mutations in *ter1-Taq* and *TER1* backgrounds. The *ter1-Taq* mutation has a subtelomeric BIR rate that is elevated 17-56 fold due to telomeres that are about one quarter the length of those in *TER1* cells (Table 4.2). This is consistent with previous results (40). Our results also showed that the *rad59*-Δ mutation did not significantly affect subtelomeric BIR in WT or *ter1-Taq* (Table 4.2). The *rad51*-Δ mutation, on the other hand, reduced the

subtelomeric BIR events by ~30 fold in *ter1-Taq* cells and by ~5 fold in *TER1* cells. The greater reduction seen in *ter1-Taq* cells compared to *TER1* cells was also been seen in *rad52* mutants (40). These results indicate that subtelomeric BIR events caused by shortened telomeres in *K. lactis* are due to the canonical Rad51-dependent pathway of homologous recombination and are independent of Rad59.

Table 4.2. The subtelomeric gene conversion rates in strains with wild type (*TER1)* or mutant *ter1-Taq* telomerase*.* The standard error was calculated as the standard deviation divided by the square root of the number of colonies assayed for each strain (n). The relative rate was estimated taking the gene conversion frequency in wild type strain equal 1.

Strain	Rate of subtelomeric gene conversion	Standard error	Number of clones assayed (n)	Relative rate
TER1 RAD51	6.26E-06	1.26E-06	31	1.00
TER1 rad51-Δ	1.34E-06	2.11E-07	48	0.21
TER1 RAD59	5.82E-06	8.03E-05	39	0.93
TER1 rad59-Δ	5.98E-06	1.91E-05	34	0.96
ter1-Taq RAD51	1.09E-04	1.91E-04	16	17.48
ter1-Taq rad51-Δ	3.74E-06	6.33E-07	35	0.60
ter1-Taq RAD59	3.52E-04	3.06E-04	20	56.27
ter1-Taq rad59-Δ	2.52E-04	7.94E-05	23	40.35

Discussion

Our work here studied the genetics of RTE and its proposed component steps in *K. lactis.* Our findings demonstrated that, in contrast to *S. cerevisiae*, both the *RAD51*- and *RAD50/RAD59* -dependent pathways of homologous recombination contribute to the emergence of Type II survivors in telomerase deletion mutants of *K. lactis*. Individual mutations in either pathway did not significantly disrupt survivor formation, but double mutants affecting both pathways simultaneously diminished (in the case of *rad50 rad51* mutants) or eliminated (in the case of

rad59 rad51 mutants) survivor formation, suggesting that all three of these proteins play important roles in type II RTE in *K. lactis*.

In non-telomeric mitotic recombination in *S. cerevisiae*, the Rad50/59-dependent pathway of HR is able to function with as little as 30 bp of homologous sequence while the Rad51-dependent pathway requires ~100 bp or more (25). This could account for why the very short telomeres of senescing telomerase deletion mutants (which are generally <100 bp in highly *ter1*-Δ senescent cells (37) can utilize the Rad50/59 pathway. Consistent with this, Type II RTE has been seen to require *RAD51* in *S. cerevisiae* mutants with certain telomere capping defects postulated to lead to 3' overhangs with longer telomeric sequences than would occur in telomerase deletion mutants (17). Another reason that *S. cerevisiae* Type II telomerase deletion survivors may be more dependent upon the Rad50/59 pathway compared to *K. lactis* is that *S. cerevisiae* telomeric repeats are highly heterogeneous in size and sequence while those of *K. lactis* are uniform. The *RAD51*-dependent HR pathway is more strongly inhibited by homeologous DNA than is the *RAD50/59* dependent HR pathway (53). Also, a defect in mismatch repair increases the rate of survivor formation more strongly in *S. cerevisiae* than in *K. lactis* (49). Telomeric sequences in *K. lactis* harboring a single base change in each telomeric repeat (that is otherwise phenotypically silent) have been shown to be less able to be copied by HR onto other telomeres during RTE in the *stn1-M1* mutant in a mismatch repair-dependent fashion (Xu and McEachern, submitted).

Still another reason the *RAD51* pathway has not been linked to type II survivors in *S. cerevisiae* telomerase deletion mutants is likely due to differences between what subtelomeric HR can accomplish in the two species. In *S. cerevisiae,* because subtelomeric blocks of telomeric repeats are present next to Y' elements, *RAD51*-dependent subtelomeric HR rapidly gives rise to

the amplification of these subtelomeric elements in type I RTE (13, 36). Because *K. lactis* lacks subtelomeric blocks of telomeric repeats, it does not normally carry out any equivalent to Type I RTE and any *RAD51*-dependent subtelomeric HR can instead contribute to Type II RTE in the form of subtelomeric BIR events.

The formation of post-senescent survivors in both *S. cerevisiae* and *K. lactis* is strongly dependent upon Rad52. This protein is the most broadly essential recombination protein in yeast with roles in both strand invasion and strand annealing. Previous work showed that Rad52 was essential to both subtelomeric BIR events and the formation of small t-circles in *ter1-16T* (18, 40). Our work here indicates that Rad52 is important for the formation of large t-circles in *ter1-16T* cells. We also found that Rad52 was important but not completely essential for copying a transformed *URA3*-telomere circle into tandem arrays at short telomeric ends. The first observation was unexpected and suggests that there could be an HR-independent pathway of large t-circle formation.

Rad51 is a homologue of RecA and a DNA recombinase forming a protein filament on single-stranded DNA (48, 54). Our results showed that subtelomeric BIR events depend on the Rad51 pathway and are entirely independent of Rad50 and Rad59. The requirement for Rad51 was particularly strong for BIR events occurring in a short telomere background. Approximately 50% of the telomere lengthening events that generate *K. lactis ter1*-Δ survivors appear to be BIR events initiated in subtelomeric sequences (58). These events are therefore likely to be a major reason why Rad51 is involved in survivor formation in *ter1*-Δ cells. HR events that copy telomeric sequence from one telomere onto other telomeres without copying subtelomeric sequence have also been shown to be common during *ter1*-Δ survivor formation (58). Whether these events depend upon Rad51 remains to be determined.

Other results of ours indicated that absence of Rad51 has at most a minor effect on the formation of t-circles in long telomere mutants. However, we found that the acquisition of sequence of a transformed *URA3*-telomere circle onto telomeric ends is partially inhibited in the absence of Rad51. We suggest that Rad51 probably plays a role in the initial copying of *URA3*-telomere circle, which is likely a rolling circle copying event that otherwise resembles BIR. However, the sometimes shorter size and apparently greater stability of *URA3*-telomere arrays against sequence spreading in *rad51* mutants suggest that *RAD51* probably also plays a role in the events that copy these arrays onto other telomeres and that potentially generate fresh *URA3*-telomere circles from them.

The t-circles inferred to drive RTE in *ter1*-Δ mutants are thought to be in the area of 100 bp in size (44). Not only is the 1.6 kb total size of the *URA3*-telomere circle used in our transformation assay much larger than this, but the ~285 bp of telomeric DNA (11+ telomeric repeats) and the ~120 bp of subtelomeric DNA present in the *URA3*-telomere circle mean that the extent of potential homology available to recombine with a *K. lactis* telomere is also appreciably larger. This could mean that the role of Rad51 in utilization of small t-circles in Type II RTE is less than our results would suggest. The *URA3*-telomere DNA circle used in our studies produced long arrays with alternating *URA3* and telomeric sequences that are similar in general structure to the long arrays of alternating Y' and telomeric sequences present in *S. cerevisiae* Type I survivors. It may therefore not be surprising that *RAD51* contributes to the formation of both. The lesser frequency of spreading and therefore the higher frequency of retention of non-extended telomeres in the absence of *RAD51* is consistent with the notion that spreading of arrays containing non-telomeric sequences is more heavily dependent of *RAD51*.

Interestingly, *K. lactis ter1-*Δ *rad51*Δ mutants produced survivors with longer telomeres (on average by 1 kb) than those generated in other double mutants. This suggests that Rad51 in some way interferes with telomere lengthening by RTE in *K. lactis*. We speculate the *RAD51*-dependent telomeric HR might be most likely to precisely copy an existing telomere onto another chromosome end, as would be expected for BIR events initiated subtelomerically. This might act to interfere with *RAD50*-dependent telomeric HR, which conceivably could be more likely to generate lengthened telomeres by unequal telomeric recombination or via t-circle formation. One way that exceptionally long telomeres can be generated by RTE is through telomere capping defects that permit recombination at any telomere size. Such runaway RTE has been observed in *K. lactis stn1-M1* mutant and human ALT cancers (11, 26). The roll and spread model also predicts that the extent of DNA synthesis achieved by the initial rolling circle copying of a t-circle size would determine the length that other telomeres could acquire. As 100 nt t-circles generate far shorter telomeric tandem arrays than the 1.6 kb t-circles used here (43), t-circle size could be predicted to be a critical determinant of telomere length in survivors in *ter1-*Δ cells. It is possible that slightly bigger t-circles can be generated in *RAD50*-dependent HR which in turn might lead to longer telomere in survivors.

Rad50 is a member of the structural maintenance of chromosomes protein family that functions in the MRX complex with the Mre11 and Xrs2 proteins (22). This multifaceted complex binds DNA ends and is involved not only in HR but also in the proper functioning of telomerase and in some NHEJ reactions. It can bridge two DNA ends and is involved in the initial phase of 5' end resectioning (51) possibly due its overlapping functions with other 5' DNA end nucleases (7, 42). We showed here that deletion of *RAD50* in the long telomere *ter1-Acc* and *ter1-16T* mutants leads to smaller telomeric 3' overhangs and the loss of small t-circles

and most large t-circles. This suggests that telomere resectioning by Rad50 is an important factor in forming extended overhangs and t-circles in these mutants. However, the deletion of *RAD50* also results in a gradual loss of the long telomeres in the *ter1-Acc* and *ter1-16T* mutants. We suggest that the immediate consequence of loss of *RAD50* in these mutants is decreased 5' resectioning and that the reduced resectioning leads to better capped telomeres that have a diminished ability to be either lengthened by telomerase or to be acted upon by HR to produce t-circles. It could also be that shorter telomeres, even in the absence of altered capping, might be less prone to generating t-circles as has been shown in human cells (47). The Acc mutant telomeric repeats (which are made by the *ter1-Acc* telomerase) are known to be defective at binding Rap1 (28), and this is believed to be the reason they incur greater 5' resectioning (5).

We showed previously that *RAD50* is not required for subtelomeric BIR (8). However, it remains to be tested whether the *RAD50/59* pathway contributes to BIR events initiated within telomeric repeats. Transformation of the *URA3*-telomeric circle into *rad50*-Δ mutants demonstrated that Rad50 is not required for this t-circle's ability to lengthen telomeres. The spreading of tandem arrays to multiple telomeres was in fact enhanced in this mutant. This apparently increased recombination might result from the particularly short telomeres caused by the *rad50*-Δ mutation. Alternatively, the absence of the *RAD50/59* pathway might allow greater access for the *RAD51*-dependent BIR pathway. The nature of the small extrachromosomal DNA seen in *rad50-Δ* and *rad59-Δ* transformants is not known but it is possible that absence of *RAD50* or *RAD59* somehow contributes to instability of the modified telomeric tracts.

The functioning of the Rad59 protein in HR is not well understood. It is known to interact with Rad52 and can facilitate strand annealing (29, 48). It also interacts with RSC and aids in recombination between sister chromatids (34). The role of *RAD59* in *K. lactis* survivor formation

remains a mystery. Although *RAD59* is clearly important to survivor formation, our assays showed that it is not required for t-circle formation, t-circle utilization, or subtelomeric BIR. It remains possible that it is required for t-circle formation or utilization with substrates or conditions different from those used in our assays. Several circumstances can be imagined where strand annealing could occur at recombining telomeres. Excised linear pieces of telomeric DNA with both 5' ends resected could be annealed into circles or annealed back onto another telomere. In mammals, the telomeric protein TRF2 is able to locally unwind a short DNA region adjacent to its binding site that can in turn permit annealing of single-stranded telomeric DNA (1). This has been shown to allow t-loop formation. Although yeast lack TRF2, conceivably some similar telomeric unwinding process might occur at yeast telomeres that could facilitate telomeric recombination in some circumstances.

Sgs1, a RecQ helicase, has been previously shown to be required for Type II survivor formation in *S. cerevisiae* (14, 23, 32). Its role in survivor formation is unclear, but it may involve resolution of G-quadruplex structures that form at 3' overhangs (24). In *K. lactis,* we have shown that *SGS1* is not required for survivor formation or t-circle production. However, the worse colony phenotype and apparently decreased ability to generate longer telomeres in post-senescence survivors of *ter1-Δ sgs1* cells relative to *ter1-Δ SGS1* cells suggests that Sgs1 probably does have a role in *K. lactis* Type II RTE. Unfortunately, technical difficulties prevented us from obtaining *ter1-Δ sgs1 rad51* mutants which might have permitted a more conclusive statement regarding the role of *SGS1* in survivor formation.

Our results here indicate that both *RAD51*-dependent and *RAD50/59*-dependent enzymatic pathways work together to generate Type II survivors in *K. lactis*. Our results further argue that certain processes involved in Type II RTE, such as subtelomeric BIR, may be strictly

limited to a single HR pathway while other processes such as t-circle formation can go through more than one pathway. Further evidence that t-circles can form via more than one HR pathway comes from other work. For example, the ALT-like *stn1-M1* mutant of *K. lactis* produces very long telomeres through recombination and abundant t-circles (26). However t-circles in this mutant are still present in the absence of *RAD50* (Xu and McEachern, submitted). Additionally, in human cells, t-circles have been found to require the Rad51 paralog XRCC3 in some situations with abnormal telomere function (15) but not in others (33, 63). It is likely that the specifics of the steps and pathways of RTE can vary considerably according to many variables such as the nature of the telomere capping defect, the sizes of telomeres, and the heterogeneity of telomeric and subtelomeric sequences. It could also be predicted that mutations affecting DNA repair pathways would contribute to variation in the mechanisms of RTE or ALT. Many cancers are known to have defects affecting proteins with roles in DNA repair and recombination. Some evidence also exists that ALT may occur through more than one mechanism (57).

Acknowledgments

This work was supported a grant to M.J.M. from the National Institutes of Health (RO1GM061645). The authors would also like to thank Dr. Cindy Groff-Vindman for construction of p*SGS1* and p*SGS1::URA3*; Shilpa Iyer and her undergraduate students for initial observations of survivor formation in the *ter1*-Δ *rad59*-Δ mutant; and Ondrej Sprusansky for his helpful suggestions and expertise.

References

1. **Amiard, S., M. Doudeau, S. Pinte, A. Poulet, C. Lenain, C. Faivre-Moskalenko, D. Angelov, N. Hug, A. Vindigni, P. Bouvet, J. Paoletti, E. Gilson, and M. Giraud-Panis.** 2007. A topological mechanism for TRF2-enhanced strand invasion. Nat. Struct. Mol. Biol. **14:**147-154.

2. **Azam, M., J. Y. Lee, V. Abraham, R. Chanoux, K. A. Schoenly, and F. B. Johnson.** 2006. Evidence that the *S. cerevisiae* Sgs1 protein facilitates recombinational repair of telomeres during senescence. Nucleic Acids Res. **34:**506–516.

3. **Bai, Y., and L. S. Symington.** 1996 A Rad52 homolog is required for *RAD51*-independent mitotic recombination in *Saccharomyces cerevisiae.* Genes Dev. **10:**2025-2037.

4. **Basenko, E. Y., A. J. Cesare, S. Iyer, J. D. Griffith, and M. J. McEachern.** 2010. Telomeric circles are abundant in the *stn1-M1* mutant that maintains its telomeres through recombination. Nucleic Acids Res. **38:**182-189.

5. **Bechard, L. H., B. D. Butuner, G. J. Peterson, W. McRae, Z. Topcu, and M. J. McEachern.** 2009. Mutant telomeric repeats in yeast can disrupt the negative regulation of recombination-mediated telomere maintenance and create an alternative lengthening of telomeres-like phenotype. Mol. Cell. Biol. **29:**626-639.

6. **Blagoev, K. B., and E. H. Goodwin.** 2008. Telomere exchange and asymmetric segregation of chromosomes can account for the unlimited proliferative potential of ALT cell populations. DNA Repair **7:**199-204.

7. **Bonetti, D., M. Martina, M. Clerici, G. Lucchini, and M. Longhese.** 2009. Multiple pathways regulate 3' overhang generation at *S. cerevisiae* telomeres. Mol. Cell **35:**70-81.

8. **Carter, S. D., S. Iyer, J. Xu, M. J. McEachern, and S. U. Astrom.** 2007. The role of nonhomologous end-joining components in telomere metabolism in *Kluyveromyces lactis*. Genetics **175:**1035-1045.

9. **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:**9948-9957.

10. **Cesare, A. J., C. Groff-Vindman, S. A. Compton, M. J. McEachern, and J. D. Griffith.** 2008. Telomere loops and homologous recombination-dependent telomeric circles in a *Kluyveromyces lactis* telomere mutant strain. Mol. Cell Biol. **28:**20-29.

11. **Cesare, A. J., and R. R. Reddel.** 2010. Alternative lengthening of telomeres: models, mechanisms and implications. Nat. Rev. Genet. **11:**319-330.

12. **Cesare, A. J., and R. R. Reddel.** 2008. Telomere uncapping and alternative lengthening of telomeres. Mech. Ageing Dev. **129:**99-108.

13. **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:**1819-1827.

14. **Cohen, H., and D. A. Sinclair.** 2001. Recombination-mediated lengthening of terminal telomeric repeats requires the Sgs1 DNA helicase. Proc. Natl. Acad. Sci. U.S.A. **98:**3174-3179.

15. **Compton, S. A., J. H. Choi, A. J. Cesare, S. Ozgür, and J. D. Griffith.** 2007. Xrcc3 and Nbs1 are required for the production of extrachromosomal telomeric circles in human alternative lengthening of telomere cells. Cancer Res. **67:**1513-1519.

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:**13902–13907.

17. **Grandin, N., and M. Charbonneau.** 2003. The Rad51 pathway of telomerase-independent maintenance of telomeres can amplify TG_{1-3} sequences in *yku* and *cdc13* mutants of *Saccharomyces cerevisiae.* Mol. Cell Biol. **23:**3721-3734.

18. **Groff-Vindman, C., S. Natarajan, A. Cesare, J. D. Griffith, and M. J. McEachern.** 2005. Recombination at dysfunctional long telomeres forms tiny double- and single-stranded t-circles. Mol. Cell Biol. **25:**4406-4412.

19. **Henson, J. D., Y. Cao, L. I. Huschtscha, A. C. Chang, A. Y. M. Au, H. A. Pickett, and R. R. Reddel.** 2009. DNA C-circles are specific and quantifiable markers of alternative-lengthening-of-telomeres activity. Nat. Biotech. **27:**1181-1185.

20. **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.

21. **Hollingsworth, N. M., and Byers, B.** 1989. *HOP1*: a yeast meiotic pairing gene. Genetics **121:**445-462.

22. **Hopfner, K., L. Craig, G. Moncalian, R. A. Zinkel, T. Usui, B. A. L. Owen, A. Karcher, B. Henderson, J. Bodmer, C. T. McMurray, J. P. Carney, J. H. J. Petrini, and J. A. Tainer.** 2002. The Rad50 zinc-hook is a structure joining Mre11 complexes in DNA recombination and repair. Nature **418:**562-566.

23. **Huang, P., F. E. Pryde, D. Lester, R. L. Maddison, R. H. Borts, I. D. Hickson, and E. J. Louis.** 2001. *SGS1* is required for telomere elongation in the absence of telomerase. Curr. Biol. **11:**125-129.

24. **Huber, M. D., D. C. Lee, and N. Maizels.** 2002. G4 DNA unwinding by BLM and Sgs1p: substrate specificity and substrate-specific inhibition. Nucleic Acids Res. **30:**3954-3961.

25. **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:**6384-6392.

26. **Iyer, S., A. 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:**8064-8073.

27. **Kegel, A., P. Martinez, S. D. Carter, and S. U. Åström.** 2006. Genome wide distribution of illegitimate recombination events in *Kluyveromyces lactis.* Nucleic Acids Res. **34:**1633-1645.

28. **Krauskopf, A., and E. H. Blackburn.** 1996. Control of telomere growth by interactions of RAP1 with the most distal telomeric repeats. Nature **383:**354-357.

29. **Krogh, B. O., and L. S. Symington.** 2004. Recombination proteins in yeast. Ann. Rev. Genet. **38:**233-271.

30. **Le, S., J. K. Moore, J. E. Haber, and C. W. Greider.** 1999. *RAD50* and *RAD51* define two pathways that collaborate to maintain telomeres in the absence of telomerase. Genetics **152:**143-152.

31. **Lea, D., and C. Coulson.** 1948. The distribution of the numbers of mutants in bacterial populations. . J. Genet. **49:**226-284.

32. **Lee, J. Y., J. L. Mogen, A. Chavez, and F. B. Johnson.** 2008. Sgs1 RecQ helicase inhibits survival of *Saccharomyces cerevisiae* cells lacking telomerase and homologous recombination. J. Biol. Chem. **283:**29847-29858.

33. **Li, B., S. Jog, S. Reddy, and L. Comai.** 2008. WRN controls formation of extrachromosomal telomeric circles and is required for TRF2ΔB-mediated telomere shortening. Mol. Cell. Biol. **28:**1892-1904.

34. **Liefshitz, B., and M. Kupiec.** 2011. Roles of RSC, Rad59, and Cohesin in double-strand break repair. Mol. Cell Biol. **31:**3921-3923.

35. **Lo, Y., K. S. Paffett, O. Amit, J. A. Clikeman, R. Sterk, M. A. Brenneman, and J. A. Nickoloff.** 2006. Sgs1 regulates gene conversion tract lengths and crossovers independently of its helicase activity. Mol. Cell Biol. **26:**4086-4094.

36. **Lundblad, V., and E. H. Blackburn.** 1993. An alternative pathway for yeast telomere maintenance rescues *est1*- senescence. Cell **73:**347-360.

37. **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:**1822-1834.

38. **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:**3453-3457.

39. **McEachern, M. J., and E. H. Blackburn.** 1995. Runaway telomere elongation caused by telomerase RNA gene mutations. Nature **376:**403-409.

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

41. **Mimitou, E., and L. Symington.** 2008. Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. Nature **455:**770-774.

42. **Mimitou, E. P., and L. S. Symington.** 2009. DNA end resection: many nucleases make light work. DNA Repair **8:**983-995.

43. **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:**1115-1127.

44. **Natarajan, S., and M. J. McEachern.** 2002. Recombinational telomere elongation promoted by DNA circles. Mol. Cell Biol. **22:**4512-4521.

45. **Natarajan, S., K. Nickles, and M. J. McEachern.** 2006. Screening for telomeric recombination in wild-type *Kluyveromyces lactis.* FEMS Yeast Res. **6:**442-448.

46. **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:**813-830.

47. **Pickett, H. A., A. J. Cesare, R. L. Johnston, A. A. Neumann, and R. R. Reddel.** 2009. Control of telomere length by a trimming mechanism that involves generation of t-circles. EMBO J. **28:**799-809.

48. **Prado, F., F. Cortés-Ledesma, P. Huertas, and A. Aguilera.** 2003. Mitotic recombination in *Saccharomyces cerevisiae*. Curr. Genet. **42:**185-198.

49. **Rizki, A., and V. Lundblad.** 2001. Defects in mismatch repair promote telomerase-independent proliferation. Nature **411:**713-716.

50. **Roy, J., T. B. Fulton, and E. H. Blackburn.** 1998. Specific telomerase RNA residues distant from the template are essential for telomerase function. Genes Dev. **12:**3286-3300.

51. **Rupnik, A., N. Lowndes, and M. Grenon.** 2010. MRN and the race to the break. Chromosoma **119:**115-135.

52. **Signon, L., A. Malkova, M. L. Naylor, H. Klein, and J. E. Haber.** 2001. Genetic requirements for *RAD51*- and *RAD54*-independent break-induced replication repair of a chromosomal double-strand break. Mol. Cell Biol. **21:**2048-2056.

53. **Spell, R. M., and S. Jinks-Robertson.** 2003. Role of mismatch repair in the fidelity of *RAD51*- and *RAD59*-dependent recombination in *Saccharomyces cerevisiae.* Genetics **165:**1733–1744.

54. **Symington, L. S.** 2002. Role of *RAD52* epistasis group genes in homologous recombination and double-strand break repair. Microbiol. Mol. Biol. Rev. **66:**630-670.

55. **Teng, S., J. Chang, B. McCowan, and V. A. Zakian.** 2000. Telomerase-independent lengthening of yeast telomeres occurs by an abrupt Rad50p-dependent, Rif-inhibited recombinational process. Mol. Cell **6:**947-952.

56. **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:**8083-8093.

57. **Tomaska, L., J. Nosek, J. Kramara, and J. D. Griffith.** 2009. Telomeric circles: universal players in telomere maintenance? Nat. Struct. Mol. Biol. **16:**1010-1015.

58. **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:**3348-3353.

59. **Treco, D. A., and F. Winston.** 2001. Growth and Manipulation of Yeast., Current Protocols in Molecular Biology. John Wiley & Sons, Inc.

60. **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:**1328-1335.

61. **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:**369-384.

62. **van den Bosch, M., J. B. Zonneveld, P. H. Lohman, and A. Pastink.** 2001. Isolation and characterization of the *RAD59* homologue of *Kluyveromyces lactis.* Curr. Genet. **39:**305-310.

63. **Wang, R. C., A. Smogorzewska, and T. de Lange.** 2004. Homologous recombination generates T-loop-sized deletions at human telomeres. Cell **119:**355-368.

64. **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:**1111-1121.

CHAPTER 5

Perspectives

Telomere elongation is an important component of cancer cell immortalization mechanism and this mechanism is vigorously studied in human cells and many model organisms (3-5). Human ALT cancers exhibit multiple characteristics of chromosomal and telomere instability. Some ALT cancers exhibit a number of hallmarks such as specialized compartments containing DNA repair and DNA recombination proteins, and linear and circular telomeric DNA. Telomeres can be elongated via sister chromatid recombination or t-circle rolling circle replication. T-circles are abundant in ALT cancer cells and are thought to be formed by aberrant recombination and resolution of t-loops (2).

The work described here addresses the mechanism of telomere maintenance and elongation through recombination in *Kluyveromyces lactis*. In particular, it addresses the genetic requirements for survival in the absence of telomerase and looks deeper into the genetics of the steps postulated to be involved in the roll-and-spread model of RTE.

Long telomere mutants *ter1-16T* and *ter1-Acc* and an ALT-like *K. lactis* mutant *stn1-M1* are particularly important models of RTE. These mutants mimic the telomeric instability of human ALT cancers and produce abundant extrachromosomal telomeric DNA. I have shown that the *stn1-M1* mutant produces extrachromosomal telomeric DNA consisting of linear and circular DNA. t-circles in the *stn1-M1* mutant can range from a few hundred bp up to 23 kb (1). Although all mutants exhibit similar long and heterogeneous telomeres, the deletion of *RAD50* led to two different outcomes. In the *ter1-16T* and *ter1-Acc* mutants, absence of *RAD50* prevented

maintenance of long telomeres and led to gradual telomere shortening and sharp decrease in the production of extrachromosomal DNA (linear and circular). Alternatively, the same mutation had very little effect on overall telomere length or t-circle formation in the *stn1-M1* mutant (J. Xu and M. McEachern, unpublished data). These observations suggest that t-circle formation, and perhaps telomere lengthening by RTE itself, might be modified by telomere capping mutations. This notion further poses the question of whether the differences observed between ALT cancers stem from differences in the mechanism of their emergence. If so, further understanding of telomere dysfunctions is absolutely necessary for successful anti-cancer drug discovery programs. Furthermore, it would be interesting to determine if alterations in human capping proteins lead to the emergence of ALT and whether certain mutations are associated with more aggressive cancer behavior.

Results reported here demonstrated that *K. lactis*, an organism with uniform telomeric repeats, can utilize Rad51- and Rad50-dependent mechanisms of RTE simultaneously in a survivor formation pathway. This novel finding allows us to hypothesize that the genetics of telomere maintenance may be affected by the uniformity of telomeric repeats. Interestingly, the recombination factor Rad59 has not been assigned a clear role in RTE. This suggests either that it contributes at many stages of RTE, possibly as an enhancer of recombination, or that the current model of RTE may have additional steps yet to be identified. It would be interesting to determine the impact of several additional mutations on RTE in the background of a telomerase deletion mutation (*ter1*-Δ). It is possible that triple mutants of *ter1*-Δ with *rad50*-Δ and *rad59*-Δ, *rad50*-Δ and *sgs1*-Δ, and *rad51*-Δ and *sgs1*-Δ will further demonstrate the epistatic relationship of the recombinational factors in the roll-and-spread model of RTE.

While I have shown that the steps of the roll-and-spread model of RTE have different genetic requirements, further delineation of the specific details of these processes is needed for a comprehensive understanding of the RTE. There are several limitations to the assays used in this study. One concerns the t-circle utilization assay which utilizes a *URA3*-marked circle. While it is the only technique allowing selection circle transformants, it may skew the interpretations of the genetic requirements for t-circle utilization. A possible, although labor-intensive, alternative would be a transformation of *rad* and *sgs1 ter1-Taq* double mutants with an unmarked small t-circle (~100 bp) containing mutationally tagged telomeric repeats. Such transformation has been achieved previously by doing a co-transformation of the 100 nt circle with an ARS plasmid into a short telomere mutant (to better permit integration of the t-circle). The transformation approach proposed would address the genetics of t-circle utilization in a manner that would more closely mimic the physiological conditions of the *ter1*-Δ mutants.

Sequence copied from a t-circle onto a telomere is thought to be spread to other short telomeres in surviving *ter1*-Δ cells. In this study we have address the genetics of BIR initiated within subtelomeric sequences. We have postulated that telomeric BIR events (i.e., BIR events initiated within telomeric rather than subtelomeric sequence) may have different genetic requirements than subtelomeric BIR. It would be interesting to follow up on this. To determine if telomeric spreading events utilize a *RAD51*-dependent or a *RAD51*-independent mechanism of BIR one could conduct an experiment as previously described by Topcu et al. (8). In this experiment, a telomere marked with a subtelomeric *URA3* and phenotypically silent Bcl mutant telomeric repeats is placed in mutants also lacking *RAD50, RAD51, RAD59*, or *SGS1*. The affects of genetic mutations on the spreading of *URA3* and Bcl telomeric repeats could then be assayed using Southern blotting.

S. cerevisiae Type I survivors, when compared to Type II survivors, experience more frequent DNA damage signaling as indicated by telomere aggregation at the nuclear pore complex (6) and telomere movement across the bud neck (7). I would be interesting to observe a response at telomeres to the defects in repair machinery and to further elucidate reasons for some phenotypic differences in senescing phenotypes of the *ter1*-Δ mutants. A simple experiment to determine if mutants undergo more prolonged cell cycle arrest or experience trouble with replication would be observation of the number of population doublings and DNA content in senescing mutants and post-senescence survivors. The mutants that have difficulties with repair or DNA replication would be expected to lag in cell division and DNA content. These defects should be also detectable in survivors if they are further propagated.

One of the main conclusions of this work is that *K. lactis*, as a representative of species with uniform telomeric repeats, utilizes both Rad50 and Rad51 recombination pathways in one single pathway of survivor formation. It is possible that human cancers similarly utilize all recombination factors in telomere elongation. However, the variance of ALT phenotype may be dependent on the interactions between specific mutations with telomere capping complex and recombination machinery. It would be interesting to determine if this hypothesis holds true in other model organisms with uniform telomeric repeats.

References

1. **Basenko, E. Y., A. J. Cesare, S. Iyer, J. D. Griffith, and M. J. McEachern.** 2010. Telomeric circles are abundant in the *stn1-M1* mutant that maintains its telomeres through recombination. Nucleic Acids Res. **38:**182-189.

2. **Cesare, A. J., and R. R. Reddel.** 2010. Alternative lengthening of telomeres: models, mechanisms and implications. Nat. Rev. Genet. **11:**319-330.

3. **Chang, M., J. C. Dittmar, and R. Rothstein.** 2011. Long telomeres are preferentially extended during recombination-mediated telomere maintenance. Nat. Struct. Mol. Biol. **18:**451-456.

4. **Donate, L. E., and M. A. Blasco.** 2011. Telomeres in cancer and ageing. Philos. Trans. R. Soc. B Biol. Sci. **366:**76-84.

5. **Grandin, N., and M. Charbonneau.** 2008. Protection against chromosome degradation at the telomeres. Biochimie **90:**41-59.

6. **Khadaroo, B., M. T. Teixeira, P. Luciano, N. Eckert-Boulet, S. M. Germann, M. N. Simon, I. Gallina, P. Abdallah, E. Gilson, V. Geli, and M. Lisby.** 2009. The DNA damage response at eroded telomeres and tethering to the nuclear pore complex. Nat. Cell Biol. **11:**980-987.

7. **Straatman, K., and E. Louis.** 2007. Localization of telomeres and telomere-associated proteins in telomerase-negative *Saccharomyces cerevisiae.* Chromosome Res. **15:**1033-1050.

8. **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:**3348-3353.