The Role Of NHEJ-Components In Telomere Metabolism In *Kluyveromyces lactis*

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Running Title: NHEJ and telomeres in *K. lactis*

Key Words: [telomere, fusions, NHEJ, Nej1, DNA damage]

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ABSTRACT

The relationship between telomeres and nonhomologous end-joining (NHEJ) is paradoxical, as NHEJ-proteins are part of the telomere cap, which serves to differentiate telomeres from DNA double-strand breaks. We explored these contradictory functions for NHEJ proteins by investigating their role in *Kluyveromyces lactis* telomere metabolism. The *ter1-4LBsr* allele of the *TER1* gene resulted in the introduction of sequence altered telomeric repeats and subsequent telomere-telomere fusions (T-Tfs). In this background, Lig4 and Ku80 were necessary for T-Tfs to form. Nej1, essential for NHEJ at internal positions, was not. Hence, T-Tf formation was mediated by an unusual NHEJ mechanism. *Rad50* and *mre11* strains exhibited stable short telomeres, suggesting that Rad50 and Mre11 were required for telomerase recruitment. Introduction of the *ter1-4LBsr* allele into these strains failed to result in telomere elongation as normally observed with the *ter1-4LBsr* allele. Thus, the role of Rad50 and Mre11 in the formation of T-Tfs was unclear. Furthermore, *rad50* and *mre11* mutants had highly increased subtelomeric recombination rates, while *ku80* and *lig4* mutants displayed moderate increases. *Ku80* mutant strains also contained extended single-stranded 3’ telomeric overhangs. We concluded that NHEJ proteins have multiple roles at telomeres, mediating fusions of mutant telomeres and ensuring end protection of normal telomeres.
INTRODUCTION

Telomeres are specialized protein-DNA complexes at the ends of linear chromosomes. Telomeric DNA in most eukaryotes consists of tandem repeats of short G-rich sequences. In *Saccharomyces cerevisiae* and *Kluyveromyces lactis* telomeric repeats are bound by Rap1 (Repressor activator protein 1) (CONRAD et al. 1990; KRAUSKOPF and BLACKBURN 1996), a sequence specific DNA-binding protein containing two Myb-domains. Myb-domain proteins bind to telomeres in other organisms as well, for example Taz1 in fission yeast (COOPER et al. 1997), and Trf1/Trf2 in mammals (BROCCOLI et al. 1997).

Telomeres are synthesized by a specialized enzyme called telomerase (CHAN and BLACKBURN 2004). In both *S. cerevisiae* and *K. lactis*, the catalytic core of this enzyme is composed of a reverse transcriptase, Est2, and an internal RNA component (*TLC1* in *S. cerevisiae* and *TER1* in *K. lactis*) that serves as template during DNA synthesis. Complete loss of telomerase causes shortening of telomeres and leads to replicative senescence, due to the inability of the traditional DNA replication machinery to fully synthesize linear DNA ends (LUNDBLAD and SZOSTAK 1989; MCEACHERN and BLACKBURN 1996).

Natural chromosome termini are protected from fusing with other DNA ends, indicating that telomeres have properties that differentiate them from DNA double strand breaks (DSBs) elsewhere in the genome. This protective feature of telomeres, first observed by McClintock (MCCLINTOCK 1941) and Muller (MULLER 1938), is often referred to as the telomere cap-function. One result of a compromised telomere cap is the
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recognition of chromosome termini as DSBs and subsequent attempts by the cell to repair these breaks. One pathway for DSB repair is the homologous recombination (HR) pathway, dependent on the RAD52 group of genes (SYMINGTON 2002). The hallmark of the HR pathway is the use of a sister chromatid or homologous chromosome as a template during a mostly error-free repair process. In contrast, the nonhomologous end-joining (NHEJ) pathway for DSB repair (DALEY et al. 2005) requires little or no homology and simply fuses two free DNA ends, often generating small deletions and insertions in the process. Normally, these ends are the result of DSBs that arise at internal chromosome positions. The Ku70/Ku80 heterodimer (MILNE et al. 1996) is required for NHEJ and is thought to protect and align the DNA ends for subsequent end-processing and ligation (FELDMANN et al. 2000). The DNA ligase, Lig4, (Dnl4 or Ligase IV) (SCHÄR et al. 1997; TEO and JACKSON 1997; WILSON et al. 1997) and Lif1 (Ligase four interacting factor 1) (HERRMANN et al. 1998) provide the ligase activity during NHEJ. Lif1 is the yeast functional homologue of mammalian Xrcc4 and is required for the stability and full activity of Lig4 (TEO and JACKSON 2000). In addition, the MRX complex (Mre11, Rad50, and Xrs2), is also required for efficient NHEJ in S. cerevisiae and K. lactis (BOULTON and JACKSON 1998; KEGEL et al. 2006; MOORE and HABER 1996). Mre11 is an endo/exo nuclease, but mutant forms of Mre11 lacking detectable nuclease activity still support NHEJ (LEWIS et al. 2004). Cell-type regulates NHEJ in S. cerevisiae, such that haploid $\text{MAT}^a$ or $\text{MAT}^\alpha$ strains perform NHEJ efficiently, while diploid $\text{MAT}^a/\text{MAT}^\alpha$ strains perform NHEJ inefficiently (ÅSTRÖM et al. 1999; LEE et al. 1999). This regulation of NHEJ is the result of transcriptional repression of the $\text{NEJ1}$ gene in diploid cells. $\text{NEJ1}$ encodes a protein that interacts with Lif1, and is essential for
NHEJ by all standard assays (FRANK-VAILLANT and MARCAND 2001; KEGEL et al. 2001; OOI et al. 2001; VALENCIA et al. 2001). The K. lactis NEJ1 ortholog is also essential for NHEJ, but its transcription is not regulated by cell-type, and K. lactis diploid cells perform NHEJ with an efficiency similar to either haploid cell-type (KEGEL et al. 2006). In humans, the recently identified XLF/Cernunnos gene was suggested to be the ortholog of yeast NEJ1 (CALLEBAUT et al. 2006).

It is known that telomeres with a compromised cap can engage in both HR- and NHEJ-repair (LUNDBLAD and BLACKBURN 1993; MCEACHERN and BLACKBURN 1996). HR-repair can result in telomerase independent telomere elongation or shortening. In contrast, NHEJ-repair of telomeres results in telomere-telomere fusions (T-Tfs). This is illustrated in telomerase deficient fission yeast in which the majority of cells die as a result of gradual telomere attrition. In most surviving cells chromosomes lacking detectable telomeric sequence circularize as a result of T-Tfs, while a smaller fraction of survivors appear to maintain their telomeres by recombination (NAKAMURA et al. 1998). In addition, in S. cerevisiae and fission yeast, both telomerase and Tel1, a member of the ATM family of protein kinases, protect telomeres from fusions (CHAN and BLACKBURN 2003; MIECZKOWSKI et al. 2003; NAKAMURA et al. 1998).

Other mutations related to telomere function can also compromise the telomere cap. For example, overexpression of a dominant-negative form of the human telomere protein hTrf2 resulted in T-Tfs in which telomere sequences were still present (VAN STEENSEL et al. 1998). Fission yeast Taz1 (FERREIRA and COOPER 2001) and S. cerevisiae Rap1 (PARDO and MARCAND 2005) also protect telomeres from fusing, suggesting that these Myb-domain proteins are part of the DNA-protein complex.
constituting the cap. In *K. lactis*, T-Tfs are observed in strains with specific mutations in the template region of the telomerase RNA. These mutations are predicted to abolish or decrease Rap1 binding to the telomere (McEachern *et al.* 2000). T-Tfs containing telomeric repeats appear to arise by a NHEJ-dependent mechanism since their formation requires Lig4 in both yeasts and mammals (Ferreira and Cooper 2004; Liti and Louis 2003; Mieczkowski *et al.* 2003; Smogorzewska *et al.* 2002). The role of individual NHEJ components in generating T-Tfs is not clear, however. *S. cerevisiae* Ku has been reported to be both required (Pardo and Marcand 2005) and dispensable (Mieczkowski *et al.* 2003) for the formation of T-Tfs. Paradoxically, knock-down alterations of mammalian Ku86 expression promote chromosome fusions involving telomeric sequences (Hsu *et al.* 2000; Samper *et al.* 2000) suggesting that Ku acts to prevent formation of T-Tfs. In addition, *S. cerevisiae* Nej1 has been reported to prevent T-Tfs, despite being required for promoting NHEJ (Liti and Louis 2003).

In this study, we explored the role of NHEJ proteins in telomere metabolism in *K. lactis*. We found that Ku80 and Lig4 were required for the formation of T-Tfs and that subtelomeric gene conversion rates were dramatically increased in strains lacking Mre11 or Rad50. Surprisingly, Nej1 was not required for T-Tf formation.

**MATERIALS AND METHODS**

**Yeast strains:** The strains used in this study are listed in Table 1. Strains lacking *LIG4* (AKY124), *NEJ1* (SAY572), *KU80* (SAY573), *MRE11* (SAY559) and *RAD50* (SAY557) were generated previously (Kegel *et al.* 2006). The double mutant strains *sir4*
lig4 (SAY703), sir4 nej1 (SAY701), sir4 ku80 (AKY116) and sir4 mre11 (SAY704) were generated by crossing a sir4 strain (SAY100) with the respective nonhomologous end-joining mutant strain followed by tetrad analysis. For T-Tf assays, a strain containing a duplicated TER1 locus containing both a wild type and the mutant ter1-4L-Bsr allele (generated by plasmid loop-in) was crossed to strains carrying null mutations in LIG4, NEJ1, KU80, MRE11 and RAD50. Tetrad analysis resulted in the isolation of TER1::URA3-ter1-4L-Bsr ku80 (SAY600), TER1::URA3-ter1-4L-Bsr mre11 (SAY579), TER1::URA3-ter1-4L-Bsr rad50 (SAY604), TER1::URA3-ter1-4L-Bsr nej1 (SAY562), and TER1::URA3-ter1-4L-Bsr lig4 (SAY563) double mutant strains. The TER1::URA3-
ter1-4LBsr nej1 lig4 triple mutant strain (SAY564) was obtained through the same procedure following a cross between 4LBsr and a nej1 lig4 double mutant strain (SAY545). Additionally, TER1::URA3-ter1-4L-Bsr single mutant strains were isolated from each of the above mentioned crosses to serve as controls. Each strain was subsequently plated on 5-FOA medium (BOEKE et al. 1987) and clones that had lost the wild type TER1 gene but retained the mutant ter1-4L-Bsr allele were identified. The resulting strains were maintained by serial re-streaks on rich medium (YEPD) for 48 to 72 h at 30°.

Random spore analysis: The ter1-AccSna strain used in this experiment is a derivative of the wild type haploid strain 7B520 (ura3-1 his2-2 trp1) (WRAY et al. 1987). Analysis was performed on the spores generated by mating ter1-AccSna strain with GG1958 (ade2). Diploids cells were plated on sporulation media for four days. Cells were then incubated in 200μl of Zymolyase (0.17 mg/ml) in 1M Sorbitol at 37° for 10 min to digest the asci. Subsequently, they were subjected to heat treatment at 55° for 10 min to kill all
vegetative cells. The resulting spores were serially diluted in TE (10mM Tris and 1mM EDTA) and plated on YPD plates.

**Hybridizations:** DNA blots were hybridized with the previously described wild type *K. lactis* telomeric probe (Klac1-25) at 50° following a standard protocol (MCEACHERN and BLACKBURN 1995). In-gel hybridization experiments were performed as previously described (DIONNE and WELLINGER 1996), using oligonucleotides probes complementary to either the 5’ telomeric strand (G-probe) (Klac1-25), or the 3’ telomeric strand (C-probe). Approximately 3 μg of digested DNA was electrophoresed through a 0.7% agarose gel and analyzed using the conditions described in (UNDERWOOD et al. 2004).

**Subtelomere gene conversion assay:** The gene conversion assay was performed according to the protocols described previously (MCEACHERN and IYER 2001). In brief, a native telomere in *rad50, mre11, ku80, lig4* and wild type strains was replaced by transformation with an ~2.0-kb *Eco*RI and *Sac*II “STU” (subtelomeric *URA3*) fragment containing the *URA3* gene from *S. cerevisiae*. The marker was inserted ~120 bp from the junction of the cloned telomere and subtelomere fragments of *K. lactis*. Serially diluted cells of the clones containing a single-copy “STU” fragment were plated on synthetic complete (SC) medium, SC + 5-FOA and YPD. Measurement of the loss of the *URA3* gene was performed by counting colonies grown on 5-FOA compared to the total number of colonies grown on YPD and SC.

**Analysis of telomere-telomere fusions:** The strains used for analysis of T-Tfs were *ter1*-
4LBsr strains SAY605 and SAY561 following twenty-five and five serial re-streaks, respectively. Genomic DNA was prepared using MasterPure Yeast DNA Purification Kit (EPICENTRE, Madison, WI). One primer (P1 5’-CAGGGCGGGTAACATGAC-3’) contained a sequence unique to *K. lactis* chromosome 2L and corresponded to 268-285 nucleotides upstream of the telomeric repeat sequence start (Nickles and McEachern 2004). The second primer (P2-11 5’ GAAAGAGGAAAATCCGTTTCG-3’) contained a sequence common to subtelomeric region of at least nine chromosome ends other than 2L. Potential annealing sites for this primer ranged between 167-254 nucleotides from the telomeric repeat sequence start, depending upon the chromosome end (Nickles and McEachern 2004). PCR reactions (50µl) contained 0.2ng genomic DNA, 1X PCR reaction buffer (10mM Tris-HCl, 1.5mM MgCl2, 50mM KCl), dNTP 0.1mM each, primers 0.5µM each and 1U Taq DNA polymerase. Reaction conditions were as follow: 95° 1min; 35 cycles of 95° 30s, 55° 30s, 72° 2min; followed by 72° 10min. The products were separated on a 1% agarose gel and visualized by ethidium bromide staining. Portions of the resulting DNA smear were cut from the gel and the DNA products were recovered. Isolated products were then cloned into the Promega pGEM T-Easy vector using the suggested protocol and transformed into *Escherichia coli* strain DH5α cells. Approximately 1µg of plasmid DNA was digested with *Eco*R1 or doubly digested with *Eco*R1 and *Bsr*GI. The mixtures were separated on a 2% agarose gel and visualized by ethidium bromide staining to determine fragment sizes. Clones were submitted for DNA sequencing by Macrogen (South Korea).
RESULTS

Telomere length in strain lacking NHEJ-components: To explore if NHEJ proteins were required for maintaining normal telomere length in *K. lactis*, we determined telomere lengths in strains lacking individual NHEJ proteins. The 12 telomeres in *K. lactis* give rise to a characteristic pattern of 4 to 5 bands on DNA blots after digestion of the genomic DNA with EcoRI (Figure 1A). Comparing the pattern using DNA from strains with null alleles in NHEJ genes revealed that *lig4*, *ku80* and *nej1* mutant strains had normal telomere lengths, but telomeres were shorter in *rad50* and *mre11* mutants. The shortened telomeres in strains lacking the MRX complex were consistent with previous results obtained in *S. cerevisiae* (TSUKAMOTO et al. 2001). The observation that a *ku80* mutant strain had normal length telomeres was surprising since the corresponding mutation leads to telomere shortening in *S. cerevisiae*.

Next we investigated if we could observe a telomere length defect in the absence of NHEJ proteins in a *sir4* background. It has previously been shown that strains lacking Sir4 in *K. lactis* have longer telomeres than wild type strains (ÅSTRÖM and RINE 1998). We hypothesized that using a *sir4* mutant background might sensitize the assay. The telomere lengths of a *sir4* single and a *sir4 ku80* double mutant strain indeed differed. The double mutant had telomeres longer than the wild type strain, but shorter than the *sir4* single mutant strain (Figure 1B). In contrast, telomere lengths in the *nej1 sir4* and *lig4 sir4* double mutant strains were similar to the *sir4* single mutant strain, confirming that NHEJ function was not required for maintaining normal telomere length (Figure 1B). Telomere length in the *mre11 sir4* double mutant strain was shorter than those of wild
type cells, but longer than those of the mre11 single mutant, indicating that Mre11 and Sir4 affected telomere length homeostasis by different and opposing mechanisms.

**Single-stranded 3’ overhangs were formed in the ku80 mutant:** One manifestation of telomere capping loss is the generation of extended single-stranded 3’ telomeric overhangs. We therefore employed non-denaturing in-gel hybridization, a method that detects relative amounts of 3’ overhangs, to analyze ku80, lig4, rad50 and mre11 mutant strains (DIONNE and WELLINGER 1996). Using a probe complementary to the 3’ strand (C-probe), a strong signal was visible in the ku80 strain relative to wild type, while only weak signals were detected in the lig4, mre11 and rad50 mutant strains (Figure 2A). No signal was observed using a probe complementary to the 5’ telomeric DNA (G-probe) (data not shown). EtBr staining of the gel prior to hybridization confirmed that similar amounts of DNA were loaded in all lanes (Figure 2B). These results showed that extended 3’ overhangs were present in the ku80 strain, and are consistent with Ku80 contributing to telomere end protection. The precise length of these overhangs and whether they were cell cycle dependent remains unclear.

**Increased subtelomeric recombination in rad50, mre11, lig4 and ku80 mutants:** Both shorter telomere length and elongated 3’ telomere overhangs have been associated with increased recombination in and near telomeres (IYER et al. 2005; MCEACHERN and IYER 2001; UNDERWOOD et al. 2004). We therefore hypothesized that ku80, mre11, and rad50 mutant strains would exhibit elevated levels of subtelomeric recombination. To test this idea, we performed assays on ku80, mre11, rad50, lig4 and wild type strains to measure
the rates of subtelomeric gene conversion as previously described (MCEACHERN and IYER 2001). This assay measures the loss of a URA3 marker gene, which was artificially positioned close to a telomere in each of the different strains (see MATERIALS AND METHODS). Relative to the wild type control strain, we observed dramatic increases in URA3 loss (Table 2). The ~250 to 1000-fold increased gene conversion rates in rad50 and mre11 indicate that these mutants were particularly compromised in their ability to protect telomeric and subtelomeric regions against recombination. An interesting observation is the modest increase in subtelomeric recombination in the absence of Lig4. This is the first observation that Lig4 might have a role in telomere end protection. Our results indicate that despite possessing telomeres of normal length, ku80 and lig4 mutants have a telomere capping defect.

**Telomere-telomere fusions were produced by an atypical NHEJ mechanism:**

Mutations in the template region of telomerase RNA, encoded by the *K. lactis TER1* gene, lead to the introduction of the corresponding changes in newly synthesized telomeric repeats (MCEACHERN and BLACKBURN 1995; UNDERWOOD et al. 2004). Specific mutations in the *TER1* gene, predicted to abolish Rap1 binding to the newly synthesized repeats, can lead to the formation of T-Tfs (MCEACHERN et al. 2000). We used strains containing a combination of a particular *TER1* allele, termed *ter1-4LBsr*, (abbreviated *ter1-4L*) with mutations in NHEJ components and asked if T-Tfs were formed.

The *ter1-4L* single mutant strains used as controls showed the expected pattern of immediate severe telomere elongation and degradation, as indicated by a smeared DNA
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blot signal. After prolonged passaging the smeared signal was resolved into a series of sharp, discrete bands (Figure 3B, 4L early versus late). These bands were previously shown to be indicative of T-Tfs (MCEACHERN et al. 2000). Two independent isolates of ter1-4L strains lacking Lig4 or Ku80 showed no signs of T-Tfs even after 10 and 25 streaks, respectively (Figure 3A, B). In contrast, strains lacking Nej1 formed T-Tfs at a rate similar to those of a wild type strain (Figure 3A and data not shown). We concluded that K. lactis T-Tfs induced by the ter1-4L allele appeared to arise by an atypical NHEJ mechanism; a mechanism which required Lig4 and Ku80, but not Nej1.

Combining the ter1-4L mutation with rad50 or mre11 mutations revealed a surprising result with respect to the initial telomere elongation phenotype. The ter1-4L rad50 and the ter1-4L mre11 double mutant strains did not show a loss of telomere length regulation and the telomeres remained stably short even after 25 streaks (Figure 3C, and data not shown). In addition, we observed no signs of T-Tfs in either strain. A useful feature of this particular TER1 mutant allele is that it introduces a BsrGI restriction site into each newly synthesized telomeric repeat. Digestion with BsrGI showed that in spite of failing to undergo telomere elongation, a few mutant repeats had indeed been incorporated in the rad50 and mre11 mutant strains (Figure 3D, and data not shown). Hence, these results were consistent with a role for Mre11 and Rad50 in telomerase recruitment, but the contribution of these proteins to the formation of T-Tfs remained unclear.

Molecular characterization of telomere-telomere fusions: The fusions between telomeres were further characterized using a PCR-based assay similar to a previously
described strategy in *S. cerevisiae* (MIECZKOWSKI et al. 2003; PARDO and MARCAND 2005). Taking advantage of recent sequence data (NICKLES and MCEACHERN 2004), we designed four PCR primers that annealed within the subtelomeric regions of *K. lactis* telomeres. We tested the specificity of these primers using chromosomal DNA from non-fused and post-fusion strains as template. One primer pair specifically and consistently amplified DNA from several post-fusion strains (Figure 4A), resulting in a smeared signal. This pair corresponded to a primer complementary to a sequence unique to the left end of chromosome II (P1) and a primer that was complementary to at least nine subtelomeric regions (P2-11) (See MATERIALS AND METHODS for details). PCR with only primer P2-11 failed to generate a product. This result did not rule out fusions between telomeres not involving telomere 2L, since such fusions probably resists PCR amplification being almost perfect palindromes.

The PCR products from two post-fusion strains were cloned and amplified in *E. coli*. Digestion with *BsrGI* enabled us to make estimations of the number of telomeric repeats containing *BsrGI* sites. In addition, DNA sequencing made it possible to count most wild type repeats. The template DNA used originated from independently isolated *ter1-4L* single mutant strains from late (25) and early (5) streaks. Given the smeary nature of the signal three or four independent clones were sequenced. In all cases the anticipated subtelomeric sequences as well as basal telomeric repeats were identified, but the T-Tfs could not be sequenced through the fusion point. The three clones originating from the late streak (Figure 4B Fusion 1-3) had very few *BsrGI* containing repeats, while the four clones originating from the early streak contained numerous mutant repeats [4 to 19]. In all of the fusions sequenced, the number of wild type repeats on each end was
very limited [0 to 5].

We assume that the PCR-based assay for T-Tfs was much more sensitive than the DNA-blots used previously. Therefore, we investigated if T-Tfs could be detected in strains with mutations in NHEJ components using the PCR-based assay. The results showed evidence of T-Tfs in \textit{ter1-4L nej1} mutant strains, but not in \textit{ter1-4L} strains lacking Ku80, Lig4, Mre11, or Rad50 (data not shown). The two assays used for detection of T-Tfs generated the same results, and were thus confirmatory.

\textbf{Multiple T-Tfs are highly detrimental to \textit{K. lactis} meiosis:} The \textit{ter1} template mutants that cause fusions between most or all telomeres have been postulated to select for derivatives that have circularized each of their six individual chromosomes (MCEACHERN \textit{et al.} 2000). Mutant cells with no detectable free telomeres and six telomere fusions displayed only moderately slow mitotic growth. Nevertheless, such cells were expected to incur difficulties while undergoing meiosis. This is because crossovers involving a circular chromosome would dimerize a homologous chromosomal pair and interfere with their disjunction at meiosis I. To test this notion, we used a \textit{ter1-AccSna} mutant for conducting mating and sporulation analysis. This mutant strain, which contains a single base substitution in both the left half and right half of the Rap1 binding site (Figure 5), was slower to undergo fusions than the \textit{ter1-4L} mutant strain. However, the T-Tfs formed in this strain were more stable, presumably because of the smaller average size of the fused telomere sequences.

A \textit{ter1-AccSna} fusion strain showed no defect in the ability to mate with GG1958, a strain with normal telomeres, when compared to an isogenic wild type control.
(7B520). However, when diploids generated by crossing wild type 7B520 with GG1958 and ter1-AccSna with GG1958 were sporulated, a dramatic difference in successful tetrad production was observed. Microscopic observation showed that tetrads were abundant among cells of the control strain but seemingly absent from the diploids derived from the telomere fusion strain. To further characterize this meiotic defect, random spore analysis was performed on the sporulating cultures of the same diploid strains. Recovery of viable spores, following heat treatment at 55°, was consistently reduced by ~100 fold for cells derived from the ter1-AccSna diploids relative to the control. Those cells that did survive exhibited one or more phenotypes (Ade-, Ura-, His-), which suggested that they were derivatives from the same haploid parental strains that were used to set up the initial cross. We next examined the structure of the telomeres in sixteen of these segregants from the ter1-AccSna fusion diploids by DNA blots. The results confirmed that each of the clones contained mixtures of fused and unfused telomeres (Figure 5). The unfused telomeres were either all of normal length or all shorter than normal, consistent with the presence of either wild type TER1 or ter1-AccSna (which produces short telomeres prior to fusion formation). In most cases, the fusion bands were identical in size with those of the ter1-AccSna haploid parent, consistent with them being unaltered by meiosis. In at least two instances (Figure 5, lanes 14 and 15) novel sharp bands were also visible in ter1-AccSna haploids.
We examined the role of several NHEJ genes in telomere metabolism in *K. lactis*. The impetus behind our work is the seemingly contradictory roles of NHEJ proteins at telomeres versus other genomic locations. At telomeres, several NHEJ proteins have been implicated as integral components of the protective telomere cap. In this capacity, NHEJ proteins contribute to the prevention of the very same processes that they promote within the genome. We explored several aspects of telomere maintenance including length homeostasis, the extent of 3’ overhangs, subtelomeric gene conversion rates and formation of T-Tfs. In addition, we examined the ability of strains containing T-Tfs to undergo meiosis. Taken together, this study shows that NHEJ proteins have multiple and complex roles in imparting normal telomere metabolism.

With respect to telomere length, the short telomeres of the *mre11* and *rad50* strains were similar to results seen in *S. cerevisiae*, where unusually short but stable telomeres have been seen upon deletion of certain genes in DNA repair pathways, including the MRX-complex and the Tel1 kinase (BOULTON and JACKSON 1998; KIRONMAI and MUNIYAPPA 1997). Our data argue that the *K. lactis* MRX-complex functions in maintenance of telomere length in much the same way that it does at *S. cerevisiae* telomeres. In contrast, both the *ku80* and *sir4* mutant strains showed telomere length phenotypes different from the corresponding *S. cerevisiae* mutant strains. The modest telomere elongation observed in a *K. lactis* *sir4* mutant differs from the result observed in *S. cerevisiae*, where both *sir3* and *sir4* mutations both produce modest telomere shortening (PALLADINO et al. 1993). However, our results are consistent with
the Sir4 protein being present at \textit{K. lactis} telomeres and contributing in a minor way to telomere length regulation.

The Ku70/Ku80 heterodimer has been shown to be involved in both telomere length regulation and telomere capping in \textit{S. cerevisiae} \cite{BERTUCH2003a,BERTUCH2003b,BOULTON1998,FLEATON1999,GRANDIN2000,GRAVEL1998,STELLWAGEN2003}. Genetic analysis has shown that MRX and Tel1 function in the same pathway of telomere maintenance but in a different pathway than Ku \cite{RITCHIE2000}. Loss of either Ku70 or Ku80 caused a large decrease in telomere length and an increase in the length of 3’ overhangs present at telomeres \cite{BERTUCH2003a} due to degradation of the 5’ strand of telomeric DNA \cite{GRAVEL1998,MARINGELE2002,POLOTNIANKA1998}. These two roles are genetically separable \cite{BERTUCH2003b}. In \textit{S. cerevisiae}, Ku’s effect on telomere length appears to result from its ability to bind directly to a particular stem loop region of the telomerase RNA, therefore assisting in telomerase recruitment \cite{MIYOSHI2003,PETERSON2001,STELLWAGEN2003}. In this study we found that loss of the \textit{K. lactis} \text{KU80} gene does not appreciably alter telomere length. A possible explanation for this result is that the binding site for Ku at the stem loop region is absent in the telomerase RNA of \textit{K. lactis} \cite{TZFATI2003}. As a consequence, the \textit{K. lactis} Ku protein may not have a major role in recruitment of telomerase to telomeres. However, the observation that a \text{ku80} \text{sir4} double mutant exhibited slightly shorter telomeres than a \text{sir4} single mutant is consistent with Ku contributing to telomere length regulation to some degree. Although a Ku deficiency leads to telomere shortening in many organisms studied to date \cite{FISHER1998}
and Zakian 2005), there are exceptions to this rule. For example, loss of Ku in Arabidopsis results in considerable telomerase dependent telomere elongation (Riha and Shippen 2003). An increased length of telomere 3’ overhangs was shared between S. cerevisiae and K. lactis ku80 mutants, indicating that the role of Ku in protecting telomeres from extensive degradation is conserved.

Large increases in subtelomeric gene conversion were previously seen in K. lactis telomerase RNA gene (TER1) mutants with stably shortened telomeres (Mceachern and Iyer 2001). These events may represent break-induced replication (BIR) as subtelomeric loss of URA3 is accompanied by replacement of tagged telomeric repeats (Natarajan et al. 2006). The increased subtelomeric BIR rates seen in mre11 and rad50 mutants may therefore be an indirect consequence of shorter telomere length rather than an additional separate defect in telomere capping. A previous report found that S. cerevisiae mre11 and rad50 mutants did not display a large increase in the rate of telomeric recombination despite having very short telomeres (Dubois et al. 2002). However, this study employed a telomere capture assay that required recombination with a telomeric repeat tract that was only ~80bp in length. Subsequent work has shown that recombination involving homologous sequences of less than 100bp can require RAD50 for function (Ira and Haber 2002). We hypothesize that a Rad51 filament formed on a single stranded 3’ telomeric end could invade another telomere with the invasion extending into subtelomeric sequences. Certain outcomes of this strand invasion complex could result in loss of the URA3 gene and replacement of the original telomeric repeats with a sequence copied from the donor molecule. As the subtelomeric sequence shared between at least 11 of 12 K. lactis telomeres extends for well over 1 kb, there would be ample homology
available for Rad51-dependent recombination to occur, even if the adjoining telomeric repeat tract was very short. Additionally, Ku deficiency has been reported to trigger increased recombination (Baumann et al. 2002; Dubois et al. 2002; Miyoshi et al. 2003; Polotnianka et al. 1998). Our results are consistent with these observations as we show that loss of ku80 results in a detectable, though moderate, increase in subtelomeric recombination.

Lig4 is an essential part of the NHEJ pathway along with Ku80, but no role has yet been assigned to Lig4 in telomere metabolism. Therefore, the 13-fold increase in recombination near telomeres in a K. lactis lig4 mutant observed in this study provides the first suggestion of Lig4 contributing to chromosome end protection at the telomeres. Alternatively, HR and NHEJ compete for repairing DSBs in subtelomeric regions and absence of Lig4 shuttles more repair events into the HR pathway. In support of this idea, we have observed competition between HR and NHEJ for an ectopic DSB induced ~25kb from a telomere in K. lactis (P. Martinez and S.U. Åström, unpublished observation). It will be worthwhile to conduct further experiments to ascertain if Lig4 plays a role in telomere maintenance in the absence of telomerase.

On rare occasions, chromosome circularization has been observed in the genomes of yeasts and mammals as a result of a compromised telomere cap (Iyer et al. 2005; Nakamura et al. 1998). Several lines of evidence have pointed out that the genetic requirements for the formation of T-Tfs can differ between species. In addition to the concomitant loss of all telomeric sequence, the circularized chromosomes of telomerase deficient fission yeast have been shown to form independently of Ku80 and Lig IV (Fisher and Zakian 2005; Nakamura et al. 1998). In contrast, the T-Tfs of S. pombe
mutants lacking Taz1 formed between long telomeres and were dependent on Ku80 and Lig IV (Ferreira and Cooper 2001). In this study, the essential requirement of Ku80 and Lig4 provide the first evidence that fusions in the ter1-4L mutant were dependent on the NHEJ pathway. In addition, deleting the RAD50 and MRE11 genes, crucial for NHEJ at internal DSBs, led to very short telomeres and lack of any T-Tfs. The simplest explanation for this result was that Rad50 and Mre11 were required for both telomere length homeostasis and for the formation of T-Tfs. Another possibility could be that a certain minimum number of mutant telomeric repeats were required for T-Tf formation, and that this threshold was not reached in the mre11 and rad50 strains. This explanation was suggested for a T-Tf-free ter1 template mutant that contained two sets of base changes that, when present in cells individually did cause T-Tfs (MEachern et al. 2000).

A key finding from this body of work was that strains lacking Nej1, a protein necessary for NHEJ at internal DSBs, formed fusions with similar rates compared to the wild type. One possibility was that the function of Nej1 was replaced by another protein during the formation of T-Tfs. It was also possible that the nature of NHEJ at telomere termini differs from NHEJ at internal chromosome positions in a manner as to render Nej1 function dispensable. Given that the role of Nej1 during NHEJ is unknown, it is difficult to speculate about the nature of such a function. In any event, NHEJ-mediated T-Tfs had different molecular requirements than NHEJ at other parts of the genome. In addition, we showed that the fusions formed in a ter1-4L strain did in fact contain a variable number of mutant and/or wild type telomeric repeats. As observed with DNA-blots, the size of telomere fusions in the ter1-4L mutant strains appeared to vary between
individual isolates. This variation could contribute to the observed difference in mutant telomere repeat number between the assayed early and late streaks. However, it is possible that loss of one or several telomeric repeats occurred as a result of complications in cloning repetitive DNA sequences in *E. coli*. It is therefore difficult to draw extensive conclusions from the telomeric sequence data, or speculate about the total number of telomeric repeats present before cloning. Nevertheless, the sequencing data provides a verification of the presence of mutant telomeric repeats, which suggests that a reduced number of Rap1 molecules were bound to the telomeres prior to fusions. This presumably led to uncapping and a possibility for the NHEJ pathway to fuse telomere termini.

Others studies have shown that *S. pombe* strains containing T-Tfs displayed severe defects in their ability to undergo meiosis (NAITO *et al.* 1998). This defect was interpreted as either a problem inherent to circular chromosomes, or a consequence of a complete lack of telomeric repeats. Our results showed that *ter1-AccSna* cells containing T-Tfs also have severe problems going through meiosis, displaying 100-fold reduced sporulation efficiency. As the T-Tfs observed in this study retained telomeric repeats, this result suggests that the circular chromosomes themselves caused the observed meiotic defect. A possible explanation for the rare spores that did result from meiosis, is that an even number of crossover events took place between each homologous pair. This would prevent formation of dicentric chromosomes and enable chromosome disjunctions with fused telomeres, thus allowing the cells to proceed through meiosis.

This study provides further evidence for the complex interplay between DSB repair pathways and telomeres. It will be especially interesting to continue exploring the
mechanism underlying T-TF formation in *K. lactis*, as the mechanism appears different from NHEJ at internal DSBs.

This work was supported by a grant from the National Institute of Health (GM 61645-01) to M.J.M and by grants from the Swedish Research Council (621-2004-1924) and the Swedish Cancer Society (4592-B03-03XAB) to S. U. Å. We would like to thank Mohammed Rahman and Laura Harris for their technical assistance and Sidney Kushner, Mattias Mannervik and Christos Samakovlis for critical reading of the manuscript.


CALLEBAUT, I., L. MALIVERT, A. FISCHER, J. P. MORNON, P. REVY et al., 2006 Cernunnos interacts with the XRCC4 x DNA-ligase IV complex and is homologous to the yeast nonhomologous end-joining factor Nej1. J Biol Chem 281: 13857-13860.


<table>
<thead>
<tr>
<th>Yeast strains used in this study</th>
</tr>
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<tbody>
<tr>
<td><strong>K. lactis</strong></td>
</tr>
<tr>
<td>4LBsr</td>
</tr>
<tr>
<td>CK213-4C</td>
</tr>
<tr>
<td>AccSna</td>
</tr>
<tr>
<td>AKY116</td>
</tr>
<tr>
<td>AKY124</td>
</tr>
<tr>
<td>SAY100</td>
</tr>
<tr>
<td>SAY545</td>
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<tr>
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</tr>
<tr>
<td>SAY703</td>
</tr>
<tr>
<td>SAY704</td>
</tr>
<tr>
<td>K17B520</td>
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<td>GG1958</td>
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</table>
TABLE 2

Elevated levels of recombination near telomeres in rad50, mre11, ku80, lig4 mutant strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Gene Conversion Rates</th>
<th>Relative Rate</th>
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<tbody>
<tr>
<td></td>
<td>Mutation rate ± SE(n)</td>
<td></td>
</tr>
<tr>
<td>wild type</td>
<td>2.8 x 10^{-6} ± 1.5 x 10^{-6} (13)</td>
<td>1</td>
</tr>
<tr>
<td>ku80</td>
<td>2.3 x 10^{-5} ± 9.5 x 10^{-6} (15)</td>
<td>~8</td>
</tr>
<tr>
<td>lig4</td>
<td>3.5 x 10^{-5} ± 6.0 x 10^{-6} (10)</td>
<td>~13</td>
</tr>
<tr>
<td>rad50</td>
<td>6.5 x 10^{-4} ± 8.8 x 10^{-5} (18)</td>
<td>~230</td>
</tr>
<tr>
<td>mre11</td>
<td>2.8 x 10^{-3} ± 1.1 x 10^{-3} (15)</td>
<td>~1000</td>
</tr>
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</table>

The gene conversion rates of the mutants are based on measurement of several (3 to 4) independent transformants of the individual mutant strains, containing the URA3 gene inserted near one telomere (see MATERIALS AND METHODS). For each strain the number of samples, n, used for the assay has been written in brackets. The standard error (SE) was calculated as the standard deviation divided by the square root of n.
FIGURE LEGENDS

FIGURE 1. — Telomere length in strains compromised for NHEJ. DNA blot of EcoR1 digested chromosomal DNA hybridized with a probe specific to K. lactis telomeric repeats. a) Telomere lengths from strains CK214-4C (WT), AKY124 (lig4), SAY572 (nej1), SAY573 (ku80), SAY559 (mre11) and SAY557 (rad50). b) Telomere lengths of strains SAY100 (sir4), AKY116 (sir4 ku80), SAY703 (sir4 lig4), SAY701 (sir4 nej1), SAY704 (sir4 mre11) and SAY559 (mre11). Molecular weight markers are in kb.

FIGURE 2. — Long 3’ overhangs in ku80 cells. Non-denaturing in-gel hybridization of genomic DNA digested with Pst1 from WT, ku80, lig4, mre11 and rad50 strains. a) Shows hybridization to a G-strand telomeric probe. b) Shows the EtBr stained gel prior to hybridization. Molecular weight markers are in kb.

FIGURE 3. — T-TFs in NHEJ mutant strains. a) LIG4, but not NEJ1 was required for formation of T-TFs. Shown is a DNA blot using two independent isolates of ter1-4LBsr (ter1-4L), ter1-4L lig4, ter1-4L nej1 and one ter1-4L lig4 nej1 strain. Chromosomal DNA was prepared from the strains following 10 streaks. b) KU80 was required for formation of T-TFs. The blot is divided into early and late lanes. Chromosomal DNA from early lanes was isolated immediately following the generation of the mutant strains. Late lanes are of the same mutant strains following 24 serial re-streaks. Two independent isolates of ter1-4L ku80 and one isolate of a ter1-4L single mutant are shown. c) ter1-4L mre11 double mutant strains had short, stable telomeres. The blot is divided into early
and late lanes as described above. Shown are ter1-4L, mre11 and ter1-4L mre11 strains.

Wild types for all blots are TER1 strains recovered from the same tetrads used in the
generation of the respective mutant strains. d) A ter1-4L rad50 double mutant strain
incorporated mutant repeats. EcoRI and EcoRI/BsrGI digestions of genomic DNA
isolated from a ter1-4L rad50 mutant strain following 24 serial re-streaks. Molecular
weight markers are in kb.

FIGURE 4. — Molecular characterization of T-TFs. a) Agarose gel electrophoresis of
PCR amplifications using genomic DNA from pre- and post-fusion strains, as indicated
above the lanes, and subtelomeric specific primers. Results were from strains SAY561
and SAY605 following 5 and 25 serial re-streaks, respectively. Control lanes result from
PCR of genomic DNA isolated from the parental strains used in the generation of each
mutant strain prior to the TER1 loop-out procedure. Size markers indicated on the left. b)
The table shows K. lactis T-TFs analyzed by restriction digests and DNA sequencing.
Seven different T-TFs (first column) were analyzed, 1 to 3 originates from strain
SAY605 and 4 to 7 from strain SAY561. The second column shows an estimate of the
number of mutant repeats present as determined by BsrGI digestion of each cloned
fragment followed by agarose gel electrophoresis. The third column shows a schematic of
the sequencing results. Sequenced wild type repeats (black arrows) and mutant repeats
(blue arrows) are indicated. Red and yellow boxes represent subtelomeric sequences. The
numbers within brackets indicate the estimated difference, in base-pairs, between the
fragment submitted for sequencing and the actual sequence recovered from the analysis.
FIGURE 5. — Multiple T-TFs are detrimental to ter1-AccSna strain undergoing meiosis. Shown is a DNA blot of EcoRI digested genomic DNA from 16 haploid strains (lanes 1-16) derived from a cross between a ter1-AccSna strain with stable T-Tfs and the wild type strain GG1958. Lanes marked as 7B520, GG1958, ter1-AccSna represent the parental haploid controls used to set up the crosses. The sharp bands visible in lanes 1-16 that are identical in size to bands present in the ter1-AccSna parent represents T-Tfs passed unaltered through meiosis. The genomic DNA was hybridized to a G-strand telomere probe (Klac1-25). Molecular weight markers are in kb. Above the blot is a schematic representation of the ter1-AccSna allele, indicating the position of the introduced mutations.
FIGURE 2

A

B

C-probe

kb

wt  ku80  lig4  rad50  mre11

kb

wt  ku80  lig4  rad50  mre11
FIGURE 3

A

B

C

D

wt 4L 4L lig4 4L nei1 4L nei1 lig4

EcoRI  +  +
BsrGI  -  +
**A**

[Image of gel electrophoresis with bands labeled for Telomere fusion # and bp measurements.]

**B**

<table>
<thead>
<tr>
<th>Telomere fusion #</th>
<th>Estimated # of mutant repeats - BsrG1 digest</th>
<th>Sequenced repeats</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
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<td>5</td>
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<td>6</td>
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<tr>
<td>7</td>
<td>10</td>
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- **Sequenced repeats**
  - **wt**
  - **mutant**

- [30bp]
- [25bp]
- [0bp]
- [20bp]
- [280bp]
- [70bp]
- [10bp]
FIGURE 5

ter1 -AccSna

GATTAGGTATGTGGTGTACGGATTT

Random Spores

7B520 GG1958 ter1 -AccSna ter1-AccSna

kb 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

0.5 1 2 3 4