Long Telomeres Produced by Telomerase-Resistant Recombination Are Established from a Single Source and Are Subject to Extreme Sequence Scrambling

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Abstract
Considerable evidence now supports the idea that the moderate telomere lengthening produced by recombinational telomere elongation (RTE) in a Kluyveromyces lactis telomerase deletion mutant occurs through a roll-and-spread mechanism. However, it is unclear whether this mechanism can account for other forms of RTE that produce much longer telomeres such as are seen in human alternative lengthening of telomere (ALT) cells or in the telomerase-resistant type IIR “runaway” RTE such as occurs in the K. lactis stn1-M1 mutant. In this study we have used mutationally tagged telomeres to examine the mechanism of RTE in an stn1-M1 mutant both with and without telomerase. Our results suggest that the establishment stage of the mutant state in newly generated stn1-M1 ter1-A mutants surprisingly involves a first stage of sudden telomere shortening. Our data also show that, as predicted by the roll-and-spread mechanism, all lengthened telomeres in a newly established mutant cell commonly emerge from a single telomere source. However, in sharp contrast to the RTE of telomerase deletion survivors, we show that the RTE of stn1-M1 ter1-A cells produces telomeres whose sequences undergo continuous intense scrambling via recombination. While telomerase was not necessary for the long telomeres in stn1-M1 cells, its presence during their establishment was seen to interfere with the amplification of repeats via recombination, a result consistent with telomerase retaining its ability to add repeats during active RTE. Finally, we observed that the presence of active mismatch repair or telomerase had important influences on telomeric amplification and/or instability.

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Introduction
Recombination can maintain telomeres in many situations where telomerase is absent. Natural examples of this include the chromosomal telomeres in the mosquito Anopheles [1–2] and the mitochondrial telomeres in certain ciliates and yeasts [3–5]. Of particular importance are the 5–10% of human cancer cells where telomerase activity is undetectable and telomeres are maintained by a mechanism termed Alternative Lengthening of Telomeres (ALT) [for a review, see [6]]. ALT cells are characterized by long and heterogeneous telomeres [7–10] and the presence of ALT-associated PML bodies (APB) that contain telomeric DNA as well as telomeric and recombinational proteins [8,11–14].

Several lines of evidence suggest that recombination is involved in maintaining telomeres when the long and heterogeneous telomeres already exist in ALT cells. Plasmid tags introduced into a telomere can be duplicated to other telomeres or at the same telomere in ALT cells but not in telomerase positive cells [15–16]. Extrachromosomal telomeric circles (t-circles), likely products of intratelomeric recombination, are abundant in ALT cells [17–19]. Telomeric sister chromatid exchanges (t-SCE) occur at highly elevated rates in ALT cells [20–22]. However, the details of how recombination can establish these long and heterogeneous telomeres from normal-length telomeres in ALT cells are still unknown.

Recombinational telomere elongation (RTE) has been described in yeast mutants lacking telomerase in the species Saccharomyces cerevisiae [23], Kluyveromyces lactis [24], Candida albicans [25] and Schizosaccharomyces pombe [26]. The recombination in these cases is thought to be caused by the shortening telomeres eventually losing part or all of their protective capping function. These mutants commonly display gradual growth senescence when telomeres are gradually shortening that is followed by the formation of better growing post-senescent survivors with longer telomeres [25]. The recombination in these cases is thought to be caused by the shortening telomeres eventually losing part or all of their protective capping function. These mutants commonly display gradual growth senescence when telomeres are gradually shortening that is followed by the formation of better growing post-senescent survivors with longer telomeres [25]. Two types of RTE were initially described in telomerase deletion mutants of S. cerevisiae. Both depend upon RAD52, suggesting that they require homologous recombination (HR). Type I RTE is characterized by amplification of subtelomeric Y elements and short tracts of telomeric repeats, and is dependent upon the canonical mitotic HR pathway involving RAD51, RAD54, RAD55, and RAD57. Type II RTE is characterized by lengthened tracts of telomeric repeats and is dependent upon a different pathway involving RAD50, RAD59, and SGS1 [28–33]. Only type II RTE normally occurs in K. lactis telomerase deletion mutants (ter1-A) [24]. Studies, particularly in K. lactis, have suggested that type II RTE occurs through a roll-and-spread mechanism, where a t-circle is used as a template to lengthen one short telomere which in turn can be used as a template to lengthen other telomeres via break-induced replication.
Author Summary

Indefinite growth of tumor cells requires a mechanism to maintain telomeres. While most cancers use telomerase for this, some maintain long and heterogeneous telomeres using a recombination-dependent mechanism termed alternative lengthening of telomeres (ALT). What causes ALT and how their long and heterogeneous telomeres form and are maintained are not well understood. In this study, we use mutationally tagged telomeric repeats to probe the mechanisms by which highly elongated telomeres are generated by recombination in an ALT-like yeast mutant. Our data show that during senescence, most or all lengthened telomeres in a newly established mutant cell are commonly generated by amplifying sequence from a single telomere source. This is consistent with the roll-and-spread model, which proposes that a single circle of telomeric DNA can be the ultimate source of all newly amplified telomeres. Other evidence showed that the telomeres of the mutant are exceptionally dynamic. Rapid terminal deletions preceded telomere elongation at the establishment of the mutant state. Also, patterns of telomeric repeats present in long telomeres became rapidly scrambled. These findings may have implications for the establishment and maintenance of long telomeres in human ALT cells.

BIR events [34–37]. Consistent with this model, post-senescent survivors derived from cells with two kinds of telomeric repeats often contain repeating patterns in most or all lengthened telomeres [36]. Additionally, when a DNA circle containing telomeric repeats is transformed into a K. lactis telomerase deletion mutant, its sequence becomes efficiently amplified onto telomeric ends as long tandem arrays [36]. T-circles are also prevalent in yeast mutants with telomere dysfunction [34,38–39]. Furthermore, sequence from a single telomere is used as the source of all lengthened telomeres in K. lactis post-senescent survivors [37]. Type II RTE in S. cerevisiae has also been suggested to involve rolling circle copying of t-circles [39].

RTE can also be triggered by perturbation of telomeric capping proteins. For example, in S. cerevisiae, acdc13-1 yku70 mutant can generate type II survivors without gradual growth senescence [40]. In K. lactis, telomerase deletion mutants containing telomeric repeats with defects in Rap1 binding develop much longer telomeres than equivalent mutants with only wild type repeats [37,41]. Of particular interest is the stn1-M1 mutant of K. lactis [42]. Stn1 is a part of the Cdc13/Stn1/Ten1 (CST) complex that binds to the 3′ single-stranded telomeric overhang and protects the telomere from degradation and engagement in recombination (for a review see [43]). Stn1 also regulates telomerase recruitment and telomeric C-strand synthesis, the latter via its interaction with Pol32/primase [44–45]. In many ways, the stn1-M1 mutant displays more similarity to ALT than do tel1-A mutants. It shares with ALT a steady state of very long and highly heterogeneous telomeres that are produced by recombination as well as the immediate presence of chronic but slight growth defects instead of the gradual growth senescence and survivor formation seen in tel1-A mutants [8,42,46]. Both ALT cells and stn1-M1 cells show high levels of telomere instability including elevated telomere recombination, rapid telomere deletions, and abundant extrachromosomal telomeric DNA including t-circles [10,21,38,42,47–48]. Finally, the phenotypes of stn1-M1 and of most ALT cells, in sharp contrast to that of tel1-A mutants, are largely not affected by whether telomerase is present or not, a property that we refer to as telomerase-resistant [8,42]. While telomeric recombination in tel1-Δ cells appears repressed once telomeres are even moderately elongated, the telomere recombination in stn1-M1 cells is thought to occur at telomeres of all sizes. To distinguish the fundamental differences between telomere capping defects in the two mutants, the RTE in stn1-M1 mutant was termed type II RTE for its “runaway” lengthening characteristics [42]. Given its similarities with ALT cells, the stn1-M1 mutant may therefore be a useful model system to obtain more clues about mechanisms that establish long telomeres in ALT cells.

In this work, we utilize mutationally tagged telomeric repeats to study the mechanism of type II RTE in stn1-M1 mutant during the establishment stage where long telomeres are generated from much shorter telomeres. Our results are consistent with predictions of the roll-and-spread model in demonstrating that sequence from one telomere is commonly spread to most or all telomeres of newly formed tel1-M1 mutants. Our results also suggest that rapid telomere truncations routinely precede the generation of long telomeres and that the presence of active mismatch repair or telomerase can impact the outcomes observed.

Results

Generating stn1-M1 mutants from precursors with mutationally tagged telomeric termini

To study the type II RTE that forms highly elongated telomeres in the stn1-M1 mutant of K. lactis, we generated stn1-M1 mutants from two kinds of precursors with mutationally tagged telomeric repeats. Previously, similar approaches were informative in studying the type II RTE that forms the more modestly elongated telomeres in K. lactis telomerase deletion (tel1-A) mutants [36–37]. The experimental setup for generating stn1-M1 cells from the first kind of precursor is diagrammed in Figure 1A. An stn1-M1 tel1-A mutant was first transformed with a plasmid (pSTN1-TER1)-(ApaLI) containing both the STN1 and the TER1-20C(ApaLI) genes. The TER1-20C(ApaLI) gene forms a telomerase that adds mutated ApaLI repeats onto all telomeric termini. ApaLI repeats are phenotypically silent but contain a single base change that both forms an ApaLI site and eliminates the native Ral site [49] (Figure 1A). A transformant was then serially passaged for ten streaks to allow telomeres to shorten to near normal length and to incorporate ApaLI repeats at their termini. These passaged cells are referred to as the ApaLI precursor cells.

All telomeres in the ApaLI precursor cells had acquired ApaLI repeats at termini as indicated by their EcoRI-digested telomeric fragments being shorter after ApaLI digestion (Figure 1B and data not shown). In contrast, telomeric fragments in a wild type control were digested away with Ral but resistant to ApaLI (Figure 1B–1C). To estimate the number of ApaLI repeats at telomeric termini of ApaLI precursors, we digested their DNA by BsaBI+ApaLI and BsaBI+Ral (Figure 1C and data not shown). BsaBI cleaves 3 bp away from 10 of 12 telomeres in K. lactis (Figure 1A), and ApaLI and Ral specifically cleave ApaLI and wild type (WT) repeats, respectively. The size ranges of the telomeric signal in BsaBI+ApaLI and BsaBI+Ral digestions reflect the size ranges of internal WT repeats and terminal ApaLI repeats, respectively. From this, we estimated that the terminal 100–300 bp of the 350–600 bp total telomere length was composed of ApaLI repeats. This was verified by cloning and sequencing two telomeres from an ApaLI precursor. One cloned telomere contained 11 basal WT repeats and three terminal ApaLI repeats and the other contained ~9 basal WT repeats and five terminal ApaLI repeats (Figure S1A). The sequence of this 13 bp repeat suggested it arose when the terminal bases of a
Figure 1. Use of mutationally tagged repeats at telomeric termini to study RTE in stn1-M1 cells reveals evidence for repeating structures in telomeres of some newly generated stn1-M1 ter1-Δ mutants. (A) Experiment outline. After transformation of pSTN1-TER1(Apal) into stn1-M1 ter1-Δ strains, followed by serial passaging for 10 streaks, the ApaL precursor was generated that contains telomeres composed of terminal ApaL repeats (gray boxes) and internal WT repeats (white boxes). The sequences of a WT repeat and an ApaL repeat are shown. The

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Positions of ApaLI and RsaI restriction sites in the repeats and the subtelomeric BsrBI(B) site are indicated. Upon loss of pSTN1-TER1(Apal), newly generated stn1-M1 ter1-A mutants with long telomeres can be recovered. Four possible outcomes for the telomeres in these mutants are illustrated; 1. A uniformly repeating pattern of the two repeat types. 2. Randomly mixed repeats. 3. Pure ApaL repeats and 4. Pure WT repeats. (B) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’) and EcoRI+ApaL (indicated by ‘’+’’) digested DNA from stn1-M1 (M1), WT and ApaL precursor (Pr). (C) BsrBI, BsrBI+ApaL and BsrBI+RsaI-digested DNA from WT and ApaL precursor (Pr) separated on a 3% agarose gel electrophoresis and hybridized with the telomere probe. (D) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’) and EcoRI+ApaL (indicated by ‘’+’’) digested DNA from 10 newly generated stn1-M1 ter1-A mutants generated from the ApaL precursor. (E) Same filter as in panel D after stripping and rehybridization with a subtelomeric probe. (F) EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels. (H) Same filter as in panel D after stripping and rehybridization with a subtelomeric probe. (I) EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels. (J) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels. (K) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels. (L) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels. (M) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels. (N) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels. (O) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels. (P) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels. (Q) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels. (R) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels. (S) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels. (T) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels. (U) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels. (V) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels. (W) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels. (X) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels. (Y) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels. (Z) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by ‘’+’’), EcoRI+ApaL (indicated by ‘’+’’) lane, and EcoRI+RsaI (indicated by ‘’+’’) lane digested DNA from 10 newly generated stn1-M1 ter1-A mutants separated by electrophoreses on 0.8% (Upper panel) and 3% (Bottom panel) agarose gels.
fragments of the two mutants in *Apa*I digests, initially ~110–150 bp, became more variable at later streaks and tended to produce new fragments of larger sizes. This observation supports the idea that telomeres in *stn1-M1* mutants are extremely dynamic and are prone to high rates of recombination that can rapidly alter their structure.

The absence of mismatch repair leads to more heterogeneously structured telomeres in newly made *stn1-M1* mutants

Base mismatches in DNA can reduce the rate of homologous recombination [50]. Therefore, the base mismatch between WT and *Apa*I repeats is likely to interfere with the recombination between telomeres that contain them. To test this, we disrupted the *MSH2* gene (required for mismatch repair) in an *Apa*I precursor, and generated 17 *stn1-M1 ter1-A msh2-A* mutants by losing pSTN1-TER1(*Apa*I) as described above. Telomeric signals in 5 of these mutants showed little or no cleavage by *Apa*I (data not shown). However, telomeric signals in the other 12 mutants were cleaved into broad ladders of bands with ~25 bp steps by both *Apa*I and *Rsa*I (Figure 2A and data not shown). Unlike what was seen in mutants derived from a *MSH2* background, these mutants showed no obvious favored fragments that might have indicated the presence of a degraded pattern. The appreciably higher percentage of *stn1-M1* mutants made in the *msh2-A* background that had amplified *Apa*I repeats might also be related to mismatch repair. Alternatively, we cannot rule out the possibility that the increased incorporation of *Apa*I repeats occurring in the precursor during the additional cell divisions (equivalent to 2–3 streaks) needed to disrupt the *MSH2* gene altered the result.

We speculated that *stn1-M1 ter1-A* mutants made in a *msh2-A* background had sufficiently high levels of telomeric recombination to rapidly break down any repeating structure that might have been formed initially. To test this idea, we attempted to knock out the *MSH2* gene in the A1 clone of *stn1-M1 ter1-A* mutant that had highly favored small telomeric fragments in both *Apa*I and *Rsa*I digests (Figure 1D). Among 72 clones transformed with the knockout cassette, only one had the *MSH2* gene successfully disrupted. As homologous gene disruption rate, even with the fragment used in this experiment, is generally 10–40% in *STN1* cells, it is conceivable that very high levels of telomeric recombination might interfere with homologous recombination elsewhere in the genome of *stn1-M1* cells. The one *stn1-M1 msh2-A* clone we did recover was serially passaged on YPD plates for three streaks and its telomeres were examined after each streak by Southern blot (Figure 2B). The same procedure was carried out with a control transformant that still had the intact *MSH2* gene. Upon disruption of *MSH2*, the simple banding patterns of the A1 clone, particularly the larger fragment in *Apa*I digests, become distinctly heterogeneous as soon as the samples could be analyzed (Figure 2B). This heterogeneous pattern is similar to those from *stn1-M1 ter1-A* mutants generated directly in a *msh2-A* background shown in Figure 2A. In contrast, the control transformant having the intact *MSH2* exhibited a banding pattern that remained distinctly more stable. This result demonstrates that the elevated telomeric recombination in a *msh2-A* background is sufficient enough to rapidly break down a repeating structure that might have been formed initially in telomeres of a newly generated *stn1-M1 ter1-A* mutant. A diagram summarizing our experiments with *stn1-M1 ter1-A* mutants generated from *Apa*I precursors is shown in Figure 2C.

The sequence from a single telomere can be spread to all other telomeres during the establishment of the *stn1-M1 ter1-A* state

RTE in *ter1-A* mutants of *K. lactis* regularly involves the spreading of sequence from a single telomere to all other telomeres to generate modest elongated telomeres [37]. To test whether the *stn1-M1* mutant can also use this mechanism to generate highly elongated telomeres, we constructed telomeric DNA fragments consisting of the subtelomeric sequence, a *HIS3* marker and Bcl telomeric repeats (Figure 3A). The subtelomeric sequence is shared by 11 of 12 *K. lactis* telomeres allowing the transformed fragments to replace a native telomere through homologous recombination. The Bcl repeats each contain a phenotypically silent base change that generates a *Bul* site [51–53]. DNA fragments containing either ~11 or ~40 Bcl repeats were transformed into *stn1-M1 ter1-A* cells containing pSTN1-TER1 integrated at the *stn1-M1* locus (Figure 3A). While the shorter telomeric fragment generated a new telomere of normal length (Figure 3B), the long telomeric fragment generated a telomere substantially longer than those of wild type cells (Figure 3F). The resulting transformants are hereafter referred to as “normal length Bcl precursors” and “long Bcl precursors”, respectively.

Next, we generated 55 clones of *stn1-M1 ter1-A* mutants from four normal length Bcl precursors by plating the precursors on 5-FOA-containing medium that selected for the loss of the pSTN1-TER1 plasmid. Telomeric signals in 49 of these clones appear essentially identical in both *Eco*I and *Eco*RI+Bul digestion (Clones 1, 3, 4 and 5 in Figure 3B and data not shown) which indicated that telomeres in these clones were composed mostly or entirely of WT repeats with very few or no Bcl repeats. Other data were also consistent with this view. The weak signal observed in these clones with a probe specific to Bcl telomeric repeats was not sensitive to *Bul* digestion, suggesting that it was due to background hybridization to the heavily amplified wild type repeats (Figure 3C). Additionally, subtelomeric signals in these clones were largely resistant to *Bul* digestion, except for ~1.5 kb fragments generated in some clones (Clones 2, 4 and 5 in Figure 3D–3E) that also hybridized to *HIS3* probe, consistent with them being derived from the original integrated telomere. We classified these 49 clones as having no amplification or spreading of Bcl repeats (Figure 4, column 1). Our results suggest that Bcl repeats were actively avoided as a source of sequence to be amplified during establishing the long telomeres in the *stn1-M1 ter1-A* mutant.

The remaining 6 of 55 clones, including clone 2 in Figure 3B, showed amplification of Bcl repeats estimated to be 3–5 times that present in the single telomere of the precursor strain (Figure 4 and data not shown). At least two of these clones showed extra copies of the *HIS3* gene (data not shown), suggesting that the modest amplification of Bcl repeats occurred primarily by subtelomeric break-induced replication (BIR) events that produced extra copies of the tagged telomere but without the Bcl repeats otherwise becoming amplified.

Of the 55 total clones, 41 (including 38 of 49 clones with no amplification and spreading of Bcl repeats) showed no *HIS3* signal (Figure 3E and data not shown). This indicates that these mutants lost the *HIS3*-tagged telomere and probably all the Bcl repeats originally attached to that telomere. Such frequent loss of the subtelomeric *HIS3* was not entirely surprising as that *stn1-M1* cells showed very high rates of loss of a *URA3* gene inserted at the same subtelomeric location [42]. These deletions are likely BIR events that replace one telomere with sequence from another telomere. The same mechanism is likely responsible for occasions in some mutants where the *HIS3*-tagged telomere became duplicated.
Analysis of 76 newly generated clones of \textit{stn1-M1 ter1-\Delta} mutants from long Bcl precursors showed somewhat different results (Figure 4, column 2). While 53 clones (70\%) showed no amplification or spreading of Bcl repeats, the other 23 clones (30\% of the total) did show some degree of amplification and spreading of Bcl repeats. Most notably, telomeric signals in three of these clones, including clones 6 and 13 in Figure 3F, can be completely or nearly completely cleaved away by \textit{Bcl} digestion, suggesting that telomeres in these clones were composed mostly or entirely of Bcl repeats (Figure 4). Consistent with this interpretation, the Bcl repeat-specific signals of these three clones were intense in \textit{EcoRI} digests but were eliminated by \textit{Bcl} digestion (Figure 3G). Furthermore, the long smeared subtelomeric signal of these clones in \textit{EcoRI} digests was largely or entirely cleaved by \textit{Bcl} digestion.

Figure 2. The effect of mismatch repair deficiency on telomeric structure in \textit{stn1-M1} mutants derived from the ApaL precursor. (A) Southern blot, hybridized with a telomere probe, of \textit{EcoRI} (1\textsuperscript{st} lane of every clone), \textit{EcoRI}+\textit{ApaLI} (2\textsuperscript{nd} lane of every clone) and \textit{EcoRI}+\textit{RsaI} (3\textsuperscript{rd} lane of every clone) digested DNA from nine newly generated \textit{stn1-M1 ter1-\Delta} mutants in a \textit{msh2-\Delta} background separated on a 3\% agarose gel. (B) Southern blot, hybridized with a telomere probe, of \textit{EcoRI} (1\textsuperscript{st} lane of every clone), \textit{EcoRI}+\textit{ApaLI} (2\textsuperscript{nd} lane of every clone) and \textit{EcoRI}+\textit{RsaI} (3\textsuperscript{rd} lane of every clone) digested DNA from the A1 clone (in Figure 2A) with (A1 \textit{msh2-\Delta}) or without (A1 \textit{MSH2}) successful deletion of \textit{MSH2}. Each clone is shown at each of 3 serial streaks. The DNA was separated on a 3\% agarose gel. The A1 clone from Figure 2A is also shown. (C) Diagram summarizing the experimental outline and results from generating \textit{stn1-M1 ter1-\Delta MSH2} and \textit{stn1-M1 ter1-\Delta msh2-\Delta} mutants from ApaL precursors. Only a minority of newly generated mutants retain both wild type telomeric repeats (white boxes) and ApaL repeats (grey boxes) in their lengthened telomeres. The \textit{stn1-M1 ter1-\Delta MSH2} clones such as the A1 clone are shown as hypothetically being derived from a newly established mutant cell with long telomeres with a strongly repeating pattern of wild type and ApaL repeats ("strong repeating pattern"). By the time enough cells are available for DNA analysis, recombination is likely to have partly degraded the initial pattern ("intermediate repeating pattern"). Subsequent passaging of these cells leads to further pattern degradation ("weak repeating pattern"). In contrast, an equivalent generation of \textit{stn1-M1 ter1-\Delta msh2-\Delta} mutants (left side of figure) leads to no sign of repeating patterns in cells retaining both ApaL and WT repeats, even immediately after their isolation.
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Analysis of 76 newly generated clones of \textit{stn1-M1 ter1-\Delta} mutants from long Bcl precursors showed somewhat different results (Figure 4, column 2). While 53 clones (70\%) showed no amplification or spreading of Bcl repeats, the other 23 clones (30\% of the total) did show some degree of amplification and spreading of Bcl repeats. Most notably, telomeric signals in three of these clones, including clones 6 and 13 in Figure 3F, can be completely or nearly completely cleaved away by \textit{Bcl} digestion, suggesting that telomeres in these clones were composed mostly or entirely of Bcl repeats (Figure 4). Consistent with this interpretation, the Bcl repeat-specific signals of these three clones were intense in \textit{EcoRI} digests but were eliminated by \textit{Bcl} digestion (Figure 3G). Furthermore, the long smeared subtelomeric signal of these clones in \textit{EcoRI} digests was largely or entirely cleaved by \textit{Bcl} digestion.
into fragments that were generally <2 kb (Figure 3H). Interestingly, these three clones showed no amplification of the subtelomeric HIS3 gene (Figure 3I and data not shown), suggesting that the Bcl repeats were amplified by inter-telomeric recombination rather than by subtelomeric duplication.

As indicated in Figure 4, 8 of the 76 clones derived from the long Bcl precursor (including clone 1 in Figure 3F-3I) were classified as having intermediate amplification and spreading of Bcl repeats based on: 1) total telomeric signal that was partially cleaved by BclI (Figure 3F); 2) Bcl-specific telomeric signal that was at least 5 times that of the precursor (Figure 3G and data not shown); and 3) subtelomeric signal that was substantially cleaved into one or more short fragments by BclI digestion (Figure 3H). These results suggest that both Bcl and WT repeats were amplified into fragments that were generally <2 kb (Figure 3H). Interestingly, these three clones showed no amplification of the subtelomeric HIS3 gene (Figure 3I and data not shown), suggesting that the Bcl repeats were amplified by inter-telomeric recombination rather than by subtelomeric duplication.

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Figure 4. Frequency of spreading of sequence from a single tagged telomere to other telomeres in K. lactis stn1-M1 mutants with different genetic backgrounds. Columns 1–8 show histograms of the frequencies of four different types of outcomes (none, slight, intermediate and complete/near complete) with regard to the extent and spreading of ApaL repeats in newly generated stn1-M1 mutants. Numbers above each column indicate the total number of clones examined. Numbers within histograms indicate the number of clones of each type when those numbers were above zero. Definitions of the outcome types are as follows. None: defined by each of the following being true: 1) total EcoRI-digested telomeric signal is reduced no more than 1.5 fold by additional Bcl digestion; 2) Bcl specific telomeric signal is less than 2.5 fold more than that of “precursors”; 3) EcoRI-digested subtelomeric signal larger than 2 kb is reduced no more than 1.5 fold by EcoRI digestion. Slight: defined by each of the following being true: 1) total EcoRI-digested telomeric signal is reduced no more than 3 fold by additional Bcl digestion; 2) Bcl specific telomeric signal is less than 5 fold more than that of “precursors”; 3) EcoRI-digested subtelomeric signal larger than 2 kb is reduced no more than 1.5 fold by EcoRI digestion. Intermediate: defined by each of the following being true: 1) total EcoRI-digested telomeric signal is reduced no more than 3 fold by additional Bcl digestion; 2) Bcl specific telomeric signal is 5 fold or more higher than that of “precursor”; 3) EcoRI-digested subtelomeric signal larger than 2 kb is reduced more than 1.5 fold by EcoRI digestion. Complete/Near Complete: defined by each of the following being true: 1) total EcoRI-digested telomeric signal is reduced at least 3 fold by additional Bcl digestion; 2) Bcl specific telomeric signal is 8 fold or more than that of “precursors”; 3) EcoRI-digested subtelomeric signal larger than 2 kb is reduced more than 1.5 fold by Bcl digestion.

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and copied onto multiple telomeres in these mutants. This group of clones had no obvious telomeric fragments of 50–500 bp in EcoRI+BclI digests (Figure 3F and data not shown), suggesting that the amplified Bcl and WT repeats were not generally interspersed closely together. Indeed, the signal from WT repeats that remained after BclI digestion was generally very long, suggesting that WT repeats in these clones were often in long continuous arrays (Figure 3F and data not shown). Twelve other clones (including clone 4 in Figure 3F–3I) were classified as having slight amplification and spreading of Bcl repeats based on lesser degrees of both amplification of Bcl repeats and subtelomeric signal that was cleaved by BclI (Figure 4, column 2).

One clone of the std1-M1 ter1-Δ mutant, clone 15 in Figure 3F–3I, showed a unique outcome. It displayed a ~9–10 kb band that hybridized intensely with both telomeric and subtelomeric probes but was resistant to BclI digestion (Figure 3F and 3H). These results are consistent with the possibility that this clone contained tandem arrays composed of both WT telomeric repeats and subtelomeric sequences, but not Bcl repeats. HIS3 was detected in the clone but apparently was not within the amplified fragment (Figure 3I). Conceivably, the putative tandem arrays originated from a native telomere rather than the Bcl telomere. This clone is reminiscent of type I post-senescent survivors in Saccharomyces cerevisiae, which are characterized by amplified subtelomeric Y′ elements and short tracts of telomeric repeats [31,54]. Although type I-like survivors with alternating telomere and non-telomeric sequences can occur in K. lactis cells that are transformed with a circle containing telomeric repeats and the URA3 gene [35,48], to our knowledge, this is the first report of RTE amplifying subtelomeric sequences in K. lactis.

23 of 76 std1-M1 ter1-Δ mutants (30%) derived from long Bcl precursors had lost HIS3 signal (Figure 3I and data not shown). All 23 of these clones showed no amplification and spreading of Bcl repeats. This loss rate of HIS3 was significantly less (p<0.001, in Fisher exact test) than that seen in mutants derived from normal Bcl precursors where 41 of 55 clones (75%) showed no HIS3 signal. This result suggests that a long telomere stabilizes the adjacent subtelomeric sequences from being lost during the establishment of an std1-M1 ter1-Δ mutant.

The absence of mismatch repair facilitates the spreading of sequence from a single tagged telomere to other telomeres

To test whether the low frequency of spreading Bcl repeats to other telomeres in std1-M1 ter1-Δ mutants was affected by mismatch repair, we constructed normal length Bcl precursor and long Bcl precursor strains containing pSTN1-TER1 and a msh2-2 mutation (Figure 5A). After screening for loss of pSTN1-TER1, we identified 55 std1-M1 ter1-Δ msh2-2 mutants from three
normal length Bcl precursors and 38 mutants from two long Bcl precursors. The results showed that the msh2-D background permitted a substantially higher frequency of amplification and spreading of Bcl repeats than a MSH2 background did. Eight of 55 stn1-M1 ter1-D msh2-D mutants (15%) derived from normal length Bcl precursors (including clones 1–2 in Figure 5B–5E) showed complete or near complete spreading of Bcl repeats and the remaining mutants (45 of 55; 81%), including clones 5 and
11–13 in Figure 5B–5E, showed no amplification (Figure 4, column 3).

Remarkably, 30 of 38 mutants (78%) derived from long Bcl precursors (including clones 5–7, 9, and 10 in Figure 5F–5I) showed complete or near complete spreading of Bcl repeats (Figure 4, column 4). At least 25 of these clones appeared to contain a small percentage of WT repeats interspersed among the Bcl repeats, as indicated by telomeric signal of <500 bp present in EcoRI+BclI digestion (e.g., clones 5–7, 9, and 10 in Figure 5F). However, as these telomeric signals were not intense, it is likely that the WT repeats were not interspersed throughout the telomeres. Only two clones (6%), including clone 8 and 11 in Figure 5F–5I, showed intermediate amplification and spreading of Bcl repeats (Figure 4, column 4). As the disruption of mismatch repair presumably permits Bcl repeats to recombine with WT repeats in an unperturbed fashion, we conclude that the long telomeres formed during the establishment of an stn1-M1 ter1-A mutant are regularly derived primarily from a single telomere source.

45 of 55 (82%) stn1-M1 ter1-A msh2-A mutants derived from normal Bcl precursors (Figure 5E and data not shown) and 21 of 38 (55%) mutants derived from long Bcl precursors (Figure 5I and data not shown) had lost the HIS3 genes. This difference was significant (p = 0.0005 in Fisher exact test). Strikingly, many clones that had complete or nearly complete spreading of Bcl repeats (4 of 8 mutants from the normal length Bcl precursor and 14 of 30 from the long Bcl precursor) had lost the HIS3 genes (clone 5 and 7 in Figure 5I and data not shown). On the other hand, 4 of 18 clones that had complete or near complete spreading of Bcl repeats contained estimated 5–10 copies of HIS3 genes (data not shown). These results showed that the spreading of Bcl repeats to all other telomeres could occur either with or without concurrent spreading of the subtelomeric HIS3 to other telomeres.

The presence of telomerase inhibits the spreading of sequence from a single tagged-telomere to other telomeres

To test the effect of telomerase on amplification and spreading of Bcl repeats, we constructed normal length Bcl precursors and long Bcl precursors in both stn1-M1 TER1 MSH2 and stn1-M1 TER1 msh2-A backgrounds that were complemented by plasmid pSTN1. These precursors were similar to those used above except that the subtelomeric marker gene was URA3 and the complementing plasmid carried STN1 and HIS3 (Figure 6A). We generated stn1-M1 mutants from all four precursor types by screening for loss of pSTN1 after streaking onto YPD plates and

Figure 6. Spreading of a telomeric sequence from a single telomere source during RTE in stn1-M1 TER1 msh2-A cells. (A) Experimental outline showing generation of stn1-M1 TER1 msh2-A mutants from STN1 TER1 msh2-A precursors containing a single telomere composed of Bcl repeats that is either normal length or 2x longer than normal length. The sequences of a WT repeat and a Bcl repeat are indicated. (B) Southern blot, hybridized with a telomere probe, of EcoRI (indicated by “−”) and EcoRI+BclI (indicated by “+”) digested DNA from six newly generated stn1-M1 TER1 msh2-A clones from long Bcl precursors in a TER1 msh2-A background as well as one normal length Bcl STN1 TER1 msh2-A precursor control. (C–E) Same filter as in panel B after stripping and rehybridization with a Bcl telomere probe, subtelomeric probe and URA3 probe, respectively. doi:10.1371/journal.pgen.1003017.g006
identifying clones with the rough colony phenotype characteristic of $stn1-M1$ mutants. Results from this showed that telomerase significantly inhibited the amplification and spread of Bcl repeats (Figure 6; Figure S3; and Figure 4, columns 5–6). None of 37 $stn1-M1$ TER1 MSH2 clones derived from normal-length precursors showed amplification and spreading of Bcl repeats (Figure S3A–S3D and Figure 4, column 5), and only 1 of 38 $stn1-M1$ TER1 MSH2 clones from the long precursors showed detectable amplification and spreading (clone 9 in Figure S3E–S3H and Figure 4, column 6). The one mutant that did show amplification (an intermediate level) was estimated to contain three copies of URA3 (data not shown). This may suggest that amplification and spreading of Bcl repeats in this clone occurred primarily through subtelomeric BIR events that produced extra copies of the tagged telomere rather than by telomere-telomere recombination. Similar outcomes were seen with $stn1-M1$ TER1 msb2-A mutants. There, the frequency of amplification and spreading of Bcl repeats was markedly lower than that seen in $stn1-M1$ ter1-A msb2-A mutants (Figure 4, columns 7–8). None of 28 $stn1-M1$ TER1 msb2-A mutants obtained from the long precursors showed complete amplification and spreading of Bcl repeats, and only 7 of 28 mutants showed intermediate or slight amplification. Clone 9 in Figure 6B–6E is the sole example of intermediate amplification. Among $stn1-M1$ TER1 msb2-A mutants derived from a normal length Bcl precursor, only 3 of 25 mutants, including clones 6 and 7 in Figure S3I–S3L, showed slight Bcl amplification (Figure 4, column 7).

The subtelomeric URA3 marker was comparatively stable in the $stn1-M1$ TER1 mutants. Only 7 of 67 $stn1-M1$ TER1 msb2-A mutants and no $stn1-M1$ TER1 MSB2 mutants (0 of 39) had lost URA3 (Figure S3D, S3H, S3L, Figure 6E; and data not shown). These results suggest that an active telomerase in $stn1-M1$ mutants helps to stabilize the adjacent subtelomeric sequences against loss or amplification.

**Discussion**

Accumulated previous evidence suggests that a roll-and-spread mechanism is involved in generating the moderately elongated telomeres formed in *K. lactis* ter1-A post-senescence survivors (reviewed in [27]). Our studies here suggest that this mechanism, involving amplification of sequence from a single t-circle is also involved in establishing the more ALT-like highly elongated telomeres in the *K. lactis* stn1-M1 mutant. Some support for the importance of t-circles in *stn1-M1* cells already existed. Previous data had shown that t-circles are abundant in *stn1-M1* cells [38]. Additionally, a recent study showed that introducing t-circles into *stn1-M1* cells helps to stabilize the adjacent subtelomeric sequences against loss or amplification.

Establishment of Long Telomeres

When all twelve telomeres are the same length and each has the same frequency as recombination between two wild type telomeres in *K. lactis* ter1-A mutants, the percentage of *stn1-M1* mutants that displayed complete or near complete amplification and spreading of Bcl repeats was 14% in mutants derived from the normal length Bcl precursors and 78% in mutants derived from the long Bcl precursors (Figure 4). These results are very similar to those seen in an earlier study with *K. lactis* ter1-A mutants, where total spreading of Bcl repeats to all telomeres from a single normal length Bcl telomere and a single long Bcl telomere was measured at 10% and 94%, respectively [37]. As was proposed with ter1-A mutants, we suggest that a roll-and-spread mechanism, where rolling circle amplification of a single t-circle followed by spreading of that sequence to all telomeres by BIR events, can account for these observations. When all twelve telomeres are the same length and each has the same chance to be amplified and spread, the predicted frequency of clones where Bcl repeats have been amplified and spread would be roughly one twelfth. The much greater frequency of spreading of Bcl repeats that is observed from mutants derived from the long Bcl precursors indicates that a longer telomere is much better able than a shorter telomere at promoting the spread of its sequence to other telomeres. The roll-and-spread model predicts that this could occur because the long Bcl telomere is used directly as a template for lengthening other telomeres and/or is better at forming t-circles. Although t-circles are common in established *stn1-M1* cells, the fact that all amplified telomeric sequences can be derived from a single telomere may suggest that t-circle formation is limiting during the initial establishment of the mutant state.
Additionally, the copying of sequence from a lengthened telomere onto other telomeric ends might be facilitated by the physical clustering of uncapped telomeres. In favor of this, it has been reported that multiple double-strand DNA breaks (DSBs) are often recruited to a single Rad52-containing focus for DNA repair in *S. cerevisiae* [53].

The amplification and spreading of Bcl repeats in newly generated *stn1-M1* mutants was strongly inhibited by the mismatch repair system. This is not surprising as mismatch repair can inhibit recombination involving sequences with imperfect homology [50]. For example, in *S. cerevisiae*, 1% and 6% sequence divergence can reduce mitotic recombination ~20 and ~140 fold, respectively [56]. Therefore, the 4% sequence divergence between Bcl repeats and WT repeats in our study may significantly reduce the recombination between the telomeres containing them. Mismatch repair has been suggested to inhibit recombination by rejecting or processing the heteroduplex formed by strand invasion occurring with homeologous sequences [50]. Loss of *MSH2* was previously shown to facilitate the recombination that generates post-senescence survivors in telomerase deletion mutants of *S. cerevisiae* and *K. lactis* [57]. This was attributed to increased recombination between the homeologous telomeric repeats in *S. cerevisiae* and between the homeologous subtelomeric sequences in *K. lactis*. Deficiency in mismatch repair can also facilitate ALT-like telomerase-independent telomere elongation in human colon cancer cell lines and in gastric carcinomas [20,58]. Conceivably, this effect stems from the degenerate telomeric repeats that are common in the basal regions of human telomeres [59–60].

In *stn1-M1* mutants formed in a *ter1-A* MSH2 background, there was not only a substantial reduction in the frequency of clones exhibiting total spreading of Bcl repeats but also, when spreading was observed, it was far more likely to be partial, accompanied by substantial amplification of WT repeats as well. We suggest that in these clones, formation of a t-circle from the Bcl telomere will occur efficiently (as no mismatches would be present during intratelomeric recombination) but copying sequence from a t-circle composed purely of Bcl repeats onto any of the 11 wild type telomeres would be impeded. When copying of a Bcl repeat t-circle did occur, the impeded ability of the Bcl repeats to recombine with the resident wild type telomeres could allow time for wild type t-circles to form and have their sequences amplified.

Interestingly, mismatch repair was apparently not an appreciable barrier to recombination between Bcl and WT repeats in *STN1* during the formation of post-senescence survivors in *ter1-A* mutants [37]. One possibility to account for this is that the extremely short telomeres in senescing cells of these mutants recombine in a different way than the somewhat longer telomeres in newly forming *stn1-MI* mutants. This is supported by the fact that the type II RTE in *S. cerevisiae* depends on a pathway involving Rad50 when occurring at very short telomeres in a senescing telomerase deletion mutant but depends on the more standard Rad51-dependent pathway when occurring at longer telomeres uncapped by defects in telomere capping proteins [30,40]. Also, Rad51-dependent recombination is inhibited by mismatch repair 15-fold more than Rad50-dependent recombination [61]. The formation of post-senescence survivors in *K. lactis* *STN1* *ter1-A* mutants has recently been shown to require both the Rad50- and the Rad51- pathways (Basenko and McEachern, unpublished data).

Telomerase is active in *stn1-M1* cells, but its presence does not grossly affect the phenotype of the mutant [42]. This indicates that the recombination by itself is capable of generating and maintaining the very long and unstable telomeres of *stn1-M1* cells and that the recombinational processes of type IIR RTE in *stn1-M1* cells are not suppressed by the presence of telomerase. Our results here demonstrate that the presence of telomerase at the establishment of the *stn1-M1* state can largely inhibit the amplification and spreading of Bcl repeats. While we cannot rule out the possibility that telomerase fundamentally alters the mechanism by which telomeres are maintained in *stn1-M1* cells, we believe this is unlikely. Rather, we suggest that sequence addition by telomerase masks the effects of recombination. In particular, we suggest that the *stn1-M1* mutation, like certain telomeric repeat mutations shown to produce type IIR RTE, is disrupted not only in telomeres’ ability to block recombination but also in their ability to negatively regulate sequence addition by telomerase [37,41]. Consistent with this possibility is our observation that telomerase inhibits the spreading of Bcl repeats not only from a normal length telomere, but also from long Bcl telomere in *stn1-M1* cells. The latter is resistant to telomerase addition in wild type *K. lactis* cells [37]. With multiple telomerase-synthesized WT telomeric repeats added onto the ends of both long and normal length Bcl telomeres, t-circles formed from terminal deletions of these telomeres would likely often be composed only of WT repeats. This would of course interfere with the ability of the Bcl repeats to amplify and spread to other telomeres. The relative contribution of recombination and telomerase to new telomeric repeat synthesis in *stn1-M1* TER1 cells is not fully known. In experiments we performed where TER1-20C(ApaL) was present during the establishment of newly generated *stn1-M1* mutants, we found that ApaL repeats were present but only as a minority of the telomeric repeats in each of multiple clones examined (data not shown). This argues that recombination is the predominant mechanism for telomeric repeat amplification in *stn1-M1* cells. It is reasonable to believe, however, that the contribution of telomerase might be greatest at the earliest stages of formation of the *stn1-M1* state, before recombination has abundant long telomeric sequences available for it to copy.

One unexpected result from our work was that precursor cells with ApaL repeats present at the termini of all telomeric ends produced *stn1-M1* mutants where the ApaL repeats were generally completely absent. This occurred in both *msb2-A* and *MSH2* backgrounds and therefore does not appear to require mismatch repair. As the ApaL telomeric base change does not appear to alter telomere function [49], this effect is also not likely to be due to selection against amplification of ApaL repeats. The simplest explanation for this outcome would be that a significant fraction of telomeric termini, at least a third of the ~350–600 bp telomeres by our estimates, is routinely deleted prior to the initiation of recombination events that elongate telomeres when the *stn1-M1* mutant state is established. Telomere shortening also precedes RTE in yeast telomerase deletion mutants. In that case, however, the shortening occurs very gradually over many tens of cell divisions from replicative sequence loss [54,62–63]. Such gradual telomere shortening cannot explain the terminal sequences loss we see in *stn1-M1* cells. Most newly generated spores of the *stn1-M1* mutant die within a few cell divisions [42]. This indicates that they experience a severe growth problem immediately after their generation and suggests that the terminal telomeric loss occurs very rapidly. Indeed, the rapid telomere deletion might help account for the poor viability of *stn1-M1* spores.

A number of mechanisms have been proposed for generating telomeric deletions (for a review see [64]). One well studied mechanism is telomere rapid deletion (TRD) which is thought to be an intratelomeric recombination event that occurs after a telomeric 3’ overhang strand invades into the double-stranded region of the same telomere (for a review see [65]). TRD can lead to telomere deletion in wild type yeast cells that contain artificially
elongated telomeres [66–68]. Processes similar to TRD, requiring the recombination protein XRCC3, can lead to shortening of both normal and dysfunctional mammalian telomeres [18,69]. TRD has been proposed to be a mechanism that can generate t-circles [34,68].

An obvious question that arises from our data is why telomeric truncations would predominate at the earliest stage of the formation of a mutant that ultimately generates and maintains highly elongated telomeres. At least two possibilities exist. One is that the physiological conditions at the earliest stage of stn1-M1 mutants, when telomere deletions occur, are different from those at later stages, when telomere elongation predominates. Conceivably, later stages might be influenced by the presence of chronic DNA damage and react differently to dysfunctional telomeres compared to cells at the earliest stage. This idea is supported by the finding that *S. cerevisiae* telomerase deletion mutants showed expression changes in hundreds of genes during the senescence caused by shorting telomeres [70]. Another possibility is that telomeric deletions are always more frequent than recombination events that lengthen telomeres in stn1-M1 cells. In this scenario, net lengthening might only predominate over shortening once long telomeres or t-circles are present and abundant enough to serve as efficient templates for elongation events that can generate long extensions. Some observations support this possibility as well.

In wild type *K. lactis* cells, deletions from TRD are approximately an order of magnitude more frequent than telomere elongation by recombination [67]. Also, telomeric deletions are very frequent in *stn1-M1* cells and in other mutants undergoing type IIR RTE [41,48].

Taken together, our results suggest that the establishment of long and heterogeneous telomeres in *stn1-M1* via type IIR RTE may involve the following events as summarized in Figure 7. First, upon initiating the *stn1-M1* state, the uncapped telomeres rapidly undergo net deletion to generally lose at least one third of the repeats from telomeric termini. Next, an occasional telomeric recombination event results in the production of a small t-circle that is used as a template to lengthen one or more telomeres. These t-circles are likely derived from the more basal repeats of a telomere as indicated by the absence of amplification of ApaL repeats in most clones in our experiments. Finally, the initial elongated telomere(s) serve as the templates for lengthening most or all remaining telomeres, generally before other t-circles can form and compete for being copied. Another study of ours [48] that examined the stability of tandem arrays at telomeres in *stn1-M1* cells, suggests that once the long telomere state is established, the maintenance of it likely includes secondary formation and copying of t-circles. However, the spreading of sequence from a single source to all telomeres in these secondary amplifications was

**Figure 7. Modified roll-and-spread model of recombinational telomere elongation during the establishment of *stn1-M1 ter1-Δ* cells.**
1. Upon loss of the complemented wild type *STN1* and *TER1* alleles, the terminal telomeric repeats are subject to rapid deletion; (2) A rare formation of a t-circle, likely as a deletion product of a single telomere. As 11 of 12 telomeres are composed of wild type repeats, most t-circles formed would have wild type repeats, as indicated by the width of the arrows; (3) At least one telomere is elongated through rolling circle synthesis using the t-circle as the template; (4) the sequence of the elongated telomere is spread to most or all other telomeres.

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were run on 0.8% or 3% agarose gels and then transferred onto Hybond N+ and visualized by autoradiography. Hybridization with various probes were done using standard methods [37].

Establishment of Long Telomeres

The yeast genomic DNA sample from clone A1 in Figure 2A was partially digested with ApalI. This was terminated by an equal volume of 12.5 mM EDTA added to the digestion reaction. The ApalI partially digested DNA was ligated with the ApalI-digested pACYC177 plasmid, and transformed into DH5α cells. The clones with telomeric fragments were confirmed by Southern blot hybridization to telomere probe. Positive clones were then sequenced.

Southern hybridizations

Yeast genomic DNA samples digested with restriction enzymes were run on 0.8% or 3% agarose gels and then transferred onto Hybond N+ membrane in 0.4 M NaOH. All hybridization were carried out in Na2HPO4 and SDS as described [79]. The γ32P-labeled telomeric probe is Klac 1–25 [63]. The temperature of Chromea dye was used to estimate the telomeric content of the plasmid DNA [64]. The probe was then hybridized to the Southern blots at 50°C for 16 h. After hybridization, the blots were washed with 2× SSC, 0.1% SDS at 60°C for 15 min, and then exposed to Kodak X-OMATAR film with an intensifying screen. The signals were quantified with a densitometer [65].

Materials and Methods

Strains and plasmid

All K. lactis strains used were derivatives of wild type (WT) 7B520 [74]. K. lactis sln1-M1, sln1-M1 ter1-Δ strains were described previously [42]. The precursors of K. lactis sln1-M1 msh2-Δ and sln1-M1 ter1-Δ msh2-Δ were described as follows. The MSH2 gene was first amplified as a 4.4 kb fragment from genomic DNA of pSTN1 by PCR (forward primer: 5’ ACGAGCTCTGGGAACCCATTTGTTGACTA3’; reverse primer: 5’ ACCGAGATGTCAGACCAGCAATTTCGTTG3′) using the genomic DNA of the 7B520 strain as the template. Second, the PCR fragment, which contains flanking SacI and XhoI sites, was inserted into the polylinker SacI and XhoI sites of pKL313/HIS3 [51] to generate the pSTN1. Plasmids pSTN1-TER1 and pSTN1-TER1(-Apal) were constructed as follows. First, the STN1 gene was cloned as a 3.4 kb fragment from genomic DNA of 7B520 cells by PCR (forward primer: 5’ ACGAGCTCTGGGAACCCATTTGTTGACTA3’; reverse primer: 5’ ACCGAGATGTCAGACCAGCAATTTCGTTG3′) into the polylinker SacI and XhoI sites of pCXJ18 [76] to result in plasmid pCXJ18-STN1. The 2.6 kb TER1 or TER1(-Apal) gene fragments flanked by XhoI and KpnI sites were obtained from pJR31 [37], and pJR31 derivative that contained a mutation in the template of TER1 changing T20 to C to create an ApalI restriction site by oligonucleotide mediated site-directed mutagenesis as described elsewhere [77]. Subsequently, the 2.6 kb TER1 or TER1(-Apal) fragment was cloned into the pCXJ18-STN1 to generate pSTN1-TER1 or pSTN1-TER1(-Apal). The URA3-tagged single Bcl telomeres containing ~11 and ~40 Bcl-telomeric repeats were described before [37]. The HIS3-tagged single Bcl telomeres were based on the URA3-tagged single Bcl telomeres, which were cleaved by PstI and XhoI to excise the URA3 gene and replace it with a 1.2 kb HIS3 fragment amplified from pKL313 [51] by PCR (forward primer: 5’ ACGAGCTCTGGGAACCCATTTGTTGACTA3’; reverse primer: 5’ ACGAGCTCTGGGAACCCATTTGTTGACTA3’). Alternatively, the URA3 and HIS3 genes were cloned into the polylinker SacI and XhoI sites of pCXJ18 [76] to result in plasmid pCXJ18-STN1 to generate pSTN1-TER1 or pSTN1-TER1(-Apal).

K. lactis transformation was done by electroporation as described for S. cerevisiae [78]. Passaging of complemented cells was carried out by serial streaking of single colonies on rich medium (YPD plates) at 30°C. Strains were streaked every 3 days down to single cells that grew into colonies. Each streak was estimated to be 20–25 cell divisions.

Telomere cloning and sequencing

exo-III1, exo-III2 and exo-III5 were generated by PCR amplification of genomic DNA as described [37]. Telomeric repeats were amplified by PCR using telomere specific primers as described previously [37]. The PCR products were cloned into the pUC18 vector and sequenced as described [37].

Southern hybridizations

The yeast genomic DNA samples digested with restriction enzymes were run on 0.8% or 3% agarose gels and then transferred onto Hybond N+ membrane in 0.4 M NaOH. All hybridization were carried out in Na2HPO4 and SDS as described [79]. The γ32P-labeled telomeric probe is Klac 1–25 [63]. The temperature of
hybridization and washing for this probe was between 45–50°C. The γ-32P-labeled Bcl telomeric probe was KTelBcl (GATCAGGTATGGTG) [51]. The temperature of hybridization and washing was 40°C and 34–36°C respectively. The subtelomeric probe was generated from pKL11-B [Insert of ~1 kb telomeric EcoRI-Smal fragment into pBluescript SK−], which was digested with XhoI and ligated back together to excise all the telomeric sequence and was then digested by EcoRI and XhoI to generate a ~600 bp subtelomeric fragment for probe. The URA3 probe was described before [80]. The RAD50 gene probe was the purified PCR product from K. lactis genomic DNA (Forward primer: 5’GATAGGTACCGCGACCAA3’; Reverse primer: 5’GCGTATTAGAGGACGCAATTC3’). Subtelomeric, URA3, and RAD50 probes were prepared using a random priming kit (NEB). Temperature of hybridization and washing for these probes was 65°C. The membranes were autoradiographed and visualized using a Molecular Dynamics Storm PhosphorImager.

Supporting Information

Figure S1 The structure of two telomeres cloned from ApaL precursors. (A) Sequences of two cloned telomeres from ApaL precursors. The sequences of WT repeats (denoted as WT) or ApaL repeats (denoted as ApaL) and half WT repeats (denoted as 1/2WT) are shown. The point mutation in the ApaL repeat is denoted by the asterisk. The number of repeats is shown in the subscript. (B) A potential mechanism that could generate the half WT repeat. The top panel shows the K. lactis telomerase RNA template region (bottom strand) with the imperfect 9 nt terminal repeats (arrows) that function during the translocation step of telomeric repeat synthesis [81]. The top strand indicates the 25 nt telomeric repeat expected to be synthesized. While the normal translocation of telomerase (middle panel) leads to the synthesis of a whole repeat, an abnormal translocation of telomerase (lower panel) due to a particular misalignment between the template and telomeric DNA could generate the observed half WT repeat. The sequence of the repeat that could be synthesized in each case is shown underlined. Drawings depict synthesis stopping before the last nt of the template, consistent with in vitro data [92].

Figure S2 Sequences of telomeric fragments cloned from the A1 clone. Shown are diagrams of 38 telomeric fragments cloned from ApaLI partially digested DNA from the A1 stn1-M1 ter1-Δ clone derived from an ApaL precursor strain. The sequences and names of different boxes illustrated in the patterns are shown. The number of clones recovered with the same DNA sequence is indicated at left.

(TIF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: MJM JX. Performed the experiments: JX. Analyzed the data: MJM JX. Contributed reagents/materials/analysis tools: JX. Wrote the paper: MJM JX.

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