The Effects of Relocating the Ku-binding Stem-loop of Telomerase RNA on Telomere Healing Events

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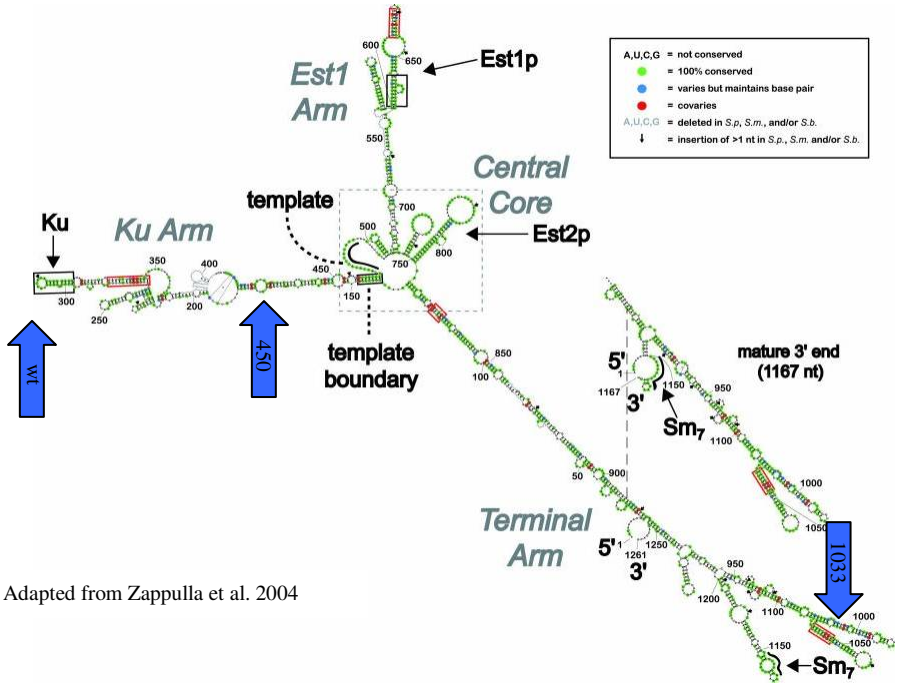

Adapted from Zappulla et al. 2004

Elizabeth Denham

Acknowledgments

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Table of Contents

List of Figures

List of Tables

Abstract

In most eukaryotes, the enzyme telomerase adds telomeric DNA repeats to the 3' ends of chromosomes in order to stabilize them and protect them from degradation. In the budding yeast *Saccharomyces cerevisiae*, telomerase is a ribonucleoprotein complex consisting of multiple protein subunits and an approximately 1.3 kb RNA component termed TLC1. Among the various proteins involved in telomerase, Ku is a heterodimer that binds both to double-stranded DNA and to a 48 nucleotide stem loop on the TLC1 RNA. Beyond its function of extending telomeres at the ends of chromosomes, telomerase can also be instrumental in repairing double-stranded DNA breaks (DSBs) by adding telomeric repeats at the site of the break. This stabilizes the damaged chromosome, but also silences genes proximal to the break. Ku is an important factor in the recruitment of telomerase to these double stranded breaks, so this investigation explored whether TLC1 structural variants with relocated Ku-binding sites are still capable of healing chromosomes via the addition of telomeres. It was determined that the TLC1 RNA is flexible and can retain its function with relocated and additional Ku-binding stem loops.

Introduction

Chromosomes are consistently subject to degradation by nucleases in the cell, an event which can lead to critical damage of essential genes. In order to protect the chromosomes from these harmful enzymes, telomeres are added to the ends of eukaryotic chromosomes. These DNA and protein structures prevent the loss of important genetic information by providing a buffer region between the end of the chromosome and internal coding regions. In most eukaryotes, telomeres take the form of short DNA tandem repeats with associated proteins. In humans, this repeat is T_2AG_3, while in the budding yeast *Saccharomyces cerevisiae* this repeat is $TG_{1\text{-}3}$ (Blackburn, 2001). Subtelomeric regions also consist of repeated sequences. Up to four tandem copies of the highly conserved Y' element exist at about half of all *S. cerevisiae* chromosomes, and the small core X element is centromere-proximal to the Y' and telomeric region of every telomere (Pryde et al., 1997). Yeast and human telomeres end in a 3' single-stranded overhang that is lengthened and shortened during the cell cycle, increasing during S phase (Wellinger et al., 1993).

Most eukaryotes, including humans and yeast, have their telomeres maintained by the enzyme telomerase (Blackburn, 2001). In *S. cerevisiae*, this enzyme is a ribonucleoprotein complex consisting of various proteins, Est1p, Est2p, Est3p, and Sm proteins, and an approximately 1.3 kb RNA component called TLC1 (Singer and Gottschling, 1994). Est2p (TERT in humans) is a reverse transcriptase which utilizes TLC1 (TERC in humans) as a template to add the DNA repeats to the telomere (Lingner et al., 1997). Est1p, Est3p, and the Sm proteins serve as non-catalytic accessory proteins in the complex (Lundblad and Szostak, 1989; Hughes et al., 2000; Seto et al., 1999). Telomerase is also associated with accessory factors on

the chromosome end, such as the protein Ku, a heterodimer that binds both double stranded DNA and a 48-nucleotide stem-loop on TLC1 (Stellwagen et al., 2003). Ku recruits telomerase to the ends of chromosomes by interacting with this stem-loop. When this stem-loop is removed from TLC1, the cells have short but stable telomeres (Peterson et al., 2001). In this case, the Ku-based recruitment pathway no longer functions but supplementary ones, such as that involving Cdc13p, another accessory protein that is essential for telomerase activity *in vivo*, are still active (Nugent et al., 1996).

Chromosomes are not only susceptible to damage at their ends; they also suffer double stranded breaks periodically, as a result of programmed enzymatic breaks, radiation, or spontaneous breaks during replication (Haber, 2000a). In order for the cell to be able to divide properly, these breaks need to be repaired. This primarily occurs by one of two general methods: homologous recombination, which includes single-strand annealing, gene conversion, and break-induced replication, and non-homologous end-joining (NHEJ) (Haber, 2000a). Though these healing mechanisms work well to repair small breaks with overhangs, they have trouble dealing with blunt-ended breaks (Haber, 2000b). Also, they require numerous proteins and enzymes to operate, making the cell vulnerable to mutations in any of a number of genes (Haber, 2000b). Therefore, a backup healing mechanism is in place which involves fewer components and can handle larger, blunt-ended breaks: telomere addition.

Along with its role of adding telomeres to the ends of chromosomes, so called native telomeres, telomerase is also involved in the healing of double stranded breaks through the addition of telomeres at these sites (Stellwagen et al., 2003). Ku is the primary telomerase recruitment factor in this situation. Healing chromosomes in this way causes genes proximal to the break to be silenced, due to the telomere's heterochromatic structure (Renauld et al., 1993).

This aspect of telomere healing can be exploited in the gross chromosomal rearrangement assay as a way to measure the frequency of such healing events (Chen and Kolodner, 1999). This assay utilizes selective media and marker genes to highlight those cells that have repaired double stranded DNA breaks induced by a random mutagenizing agent and lost or silenced the reporter genes. Southern blots can then be used to determine which method of healing the cell underwent. Possible gross chromosomal rearrangements consist of three main classes of genome rearrangements: the deletion of a chromosome arm along with the addition of a new telomere, non-reciprocal translocations with little or no homology at the breakpoint, and interstitial deletions (Myung et al., 2001).

This investigation aimed to determine how relocating the Ku-binding stem-loop within the TLC1 RNA secondary structure affects telomerase's ability to function in the above mentioned capacities. A similar examination involving the Est1p-binding site demonstrated a flexibility of the TLC1 RNA and a conservation of telomerase function (Zappulla and Cech, 2004). The Ku-binding stem-loop was moved from its wildtype position to one of two new sites elsewhere on the RNA structure, at nucleotide position 450 or nucleotide position 1033, or a second copy was added at one of these two sites while the original stem-loop at the wildtype position remained. Determining the flexibility of the telomerase RNA with respect to its Ku-related functions is important in order to better understand the full capabilities of telomerase.

Materials and Methods

Strain Construction

Strains YED001-005 were constructed using the two-step gene replacement method (adapted from Adams et al., 1997). Plasmids pJA002, pJA004, and pJA005 were digested with BsrGI and pJA003 was digested with MfeI prior to a high efficiency LiAc transformation (Gietz and Woods, 2002) into parental strain BY4727.

A TLC1 chemiluminescent probe was made via PCR using primers TLC1 352 fwd and TLC1 649 rvs to amplify TLC1 off pSD120. This TLC1 probe was used to identify, via Southern analysis after a BamHI and SphI digestion, which colonies had been successfully transformed with a plasmid (data not shown). Synthesis of the chemiluminescent probe and Southern detection were done using components of the Genius kit (Roche Chemicals). The cells were then plated to YC+FOA to select for those that had lost the plasmid.

Yeast colony PCR was performed to determine which transformants had integrated the *tlc1* allele into the chromosome (Akada et al., 2000). Primer sets TLC1 352 fwd and TLC1 649 rvs and TLC1 832 fwd and TLC1 1147 rvs were used to amplify the regions surrounding nucleotides 450 and 1033, respectively. Those colonies that had the proper additional stem loop inserted were then tested to determine if they had the wildtype location of the Ku stem loop (nt 280) via PCR using primers TLC1 121 fwd and TLC1 425 rvs.

After it was determined that the proper *tlc1* allele was integrated into the chromosome, the strains had *URA3* inserted at the *HXT13* locus on chromosome V. The *URA3* sequence was amplified from pRS306 via PCR using primers 5'HXT13ko and 3'HXT13ko in order to generate a sequence with tails having homology to *HXT13*. The cells were transformed with the PCR

product by high efficiency LiAc transformation (Gietz and Woods, 2002). A diagnostic PCR (primers 5_HXT13 and 3_HXT13) and a 1.0% agarose gel were performed to verify that *URA3* was integrated at the *HXT13* locus (data not shown).

Southern Analyses

Genomic DNA was isolated from 5 mL YEPD cultures of each strain according to Hoffman and Winston (1987). The DNA was digested with XhoI and analyzed on a 0.8% agarose gel. A Southern blot analysis was performed as described (http://www.fhcrc.org/science/labs/gottschling/). DNA was visualized with chemiluminescent Y' and CA probes to analyze native telomere lengths and with NPR2 and CIN8 probes to analyze GCR healing.

Gross Chromosomal Rearrangement (GCR) Assay

To induce mutations, a 0.22% methyl methanesulfonate (MMS) solution was added to a 5 mL, 1 x 10^7 cells/mL YEPD culture of YED001-005/UCC5114 to reach a final concentration of 0.022% MMS. The cultures were incubated at 30°C for one hour on a rotating wheel before being washed twice with YEPD. The cultures were incubated overnight at 30°C on a rotating wheel. They were each concentrated to 1 mL and plated to 4 YC+CAN+FOA plates. A 10^{-6} dilution was also plated to YEPD to determine cell number (modified from Myung et al., 2001).

Table 1: Strains Used		
Strain	Genotype	Source
BY4727	MATα, his3Δ200, leu2Δ0, lys2Δ0, met15Δ0, trp1Δ63, ura3Δ0	Boeke
YED001	BY4727 tlc1Δ48+1033, hxt13::URA3	This study
YED002	BY4727 TLC1+1033, hxt13::URA3	This study
YED003	BY4727 tlc1Δ48+450, hxt13::URA3	This study
YED004	BY4727 TLC1+450, hxt13::URA3	This study
YED005	BY4727 tlc1Δ48, hxt13::URA3	This study
UCC5114	MATα, his3Δ200, leu2Δ0, lys2Δ0, met15Δ0, trp1Δ63, ura3Δ0, TLC1, hxt13::URA3	Haimberger
YED006	BY4727 tlc1Δ48+1033	This study
YED007	BY4727 TLC1+1033	This study
YED008	BY4727 tlc1Δ48+450	This study
YED009	BY4727 TLC1+450	This study
YED010	BY4727 tlc1Δ48	This study

Table 2: Plasmids Used		
Plasmid	Characteristics	Source
pJA002	tlc1Δ48+1033	J. Arthur
pJA003	TLC1+1033	J. Arthur
pJA004	tlc1Δ48+450	J. Arthur
pJA005	TLC1+450	J. Arthur
pSD120	TLC1	Diede and Gottschling
pRS306	URA3, no ori	Sikorski et al., 1989

Table 3: Primers Used	
Primer	Sequence 5’ to 3’
TLC1 352 fwd	CACTGCTATTGCATTTAGTTGC
TLC1 649 rvs	ATAAAGTGACAGCGCTTAGCA
TLC1 832 fwd	ATTTCCAAATGTGCCCCGTA
TLC1 1147 rvs	AAATGCATCGAAGGCATTAGG
TLC1 121 fwd	ATCATGCAGGCCTCAGAAAT
TLC1 425 rvs	AGGATCGGTACGAAGAAGGAA
5’HXT13ko	ATGTCTAGTGCGCAATCCTCTATTGATAGCGATGGAGATGAGATTGTACTGAGAGTGCAC
3’HXT13ko	TCAATCAGAATTCTTTGAGAACTTCAAAAATTTCTTCCAATCGTGCGGTATTTCACACCG
5_HXT13	ACAGAGTTGTTTGAGGTAAAATTTC
3_HXT13	CGATAATCTTCATAAATTCGTGACA

Results

After generating strains YED001-005, they were analyzed to determine how their TLC1 structural variants would affect native telomere lengths (Figure 1). The Y' probe utilized highlights a subtelomeric region that is present on about half of all yeast chromosomes. Cells with wildtype *TLC1* have telomeres that are approximately 1300 bp in length, while cells with *tlc1Δ48* have telomeres that are approximately 1050 bp in length. The lengths of telomeres can be assessed from the position of the leading edge of the telomeric band. Cells with two copies of the Ku-binding stem loop, either *TLC1+1033* or *TLC1+450*, show telomeres that are about wildtype in length, with *TLC1+450* cells having some that appear slightly longer (about 1350 bp). Cells that have had the Ku-binding stem loop moved from its wildtype location to a novel location have telomeres that are shorter than wildtype, but longer than *tlc1Δ48* cells. Those cells that are *tlc1Δ48+1033* have shorter telomeres than those that are *tlc1Δ48+450*. A second Southern analysis utilizing a CA probe was also performed, yielding similar results (data not shown).

As telomerase also functions to add telomeres at the sites of double stranded DNA breaks, the strains were tested via GCR assay to see how the structural variants affected the cell's ability to recover from such damage. Table 4 holds the calculated frequencies of healing events per cell, which are also displayed graphically in Figures 2 and 3. These frequencies were determined by dividing the number of healing events observed (the number of colonies present on YC+CAN+FOA media plates) by the cell concentration of each culture (determined from colonies on a YEPD plate). Frequencies recorded as being less than a certain value (i.e. <0.09524 events/cell for *tlc1Δ48+1033*) were the result of no colonies forming on

YC+CAN+FOA plates for these cultures. For clarity, these values are represented as zeroes in Figures 2 and 3. The frequencies are grouped by trial number in Figure 2, as frequencies tended to be similarly high or low during individual trials. The frequencies are grouped by genotype in Figure 3 in order to show overall trends. Those frequencies that are substantially higher than others for the same strain may be attributed to so-called "jackpot" events, in which a cell heals itself early on in the recovery period and is able to replicate more than a cell which heals itself late in the recovery period. The middle 50% range of frequencies for each allele is shown in Figure 4, allowing for a second portrayal of trends that helps eliminate the distorting effect of "jackpot" events.

While the GCR assay provides a means to observe how frequently a cell with a TLC1 structural variant is able to heal itself, it does not illuminate what method the cell has used to do this. Healing can be accomplished through one of several ways: the addition of a telomere, non-homologous end-joining, or recombination. To ascertain the method, genomic DNA was prepared from colonies on the YC+CAN+FOA plates, digested with XhoI, and analyzed via Southern analysis using an NPR2 probe (Figure 5). This probe highlights a region of *NPR2*, on chromosome V, that is centromere-proximal to the reporter genes *CAN1* and *URA3* used in the GCR assay. Bands inside the white boxes of Figure 5A and 5B are telomeric, indicating that the chromosomal damage was healed by adding a telomere at the site of the break. The variability of these bands' sizes is a result of the double stranded DNA break and subsequent telomere addition taking place at varying locations in the chromosome, either closer to or farther from the NPR2 probe's target region. Cells could also have recovered through non-homologous end-joining or recombination, which would have yielded well-defined bands that are very distinct from the smeared telomeric bands. Some lanes exhibit both telomeric bands and more well-defined bands

that may indicate one of these methods of healing (i.e. Fig. 5A, *TLC1+1033* leftmost lane), and more analysis would need to be performed to determine a possible cause. These Southern blots were also probed with a CIN8 probe (data not shown), which highlights a region of *CIN8*, the next centromere-proximal gene from *NPR2*, to see if empty lanes may be the result of a loss of *NPR2* during healing, but these lanes remained empty and the analysis therefore inconclusive.

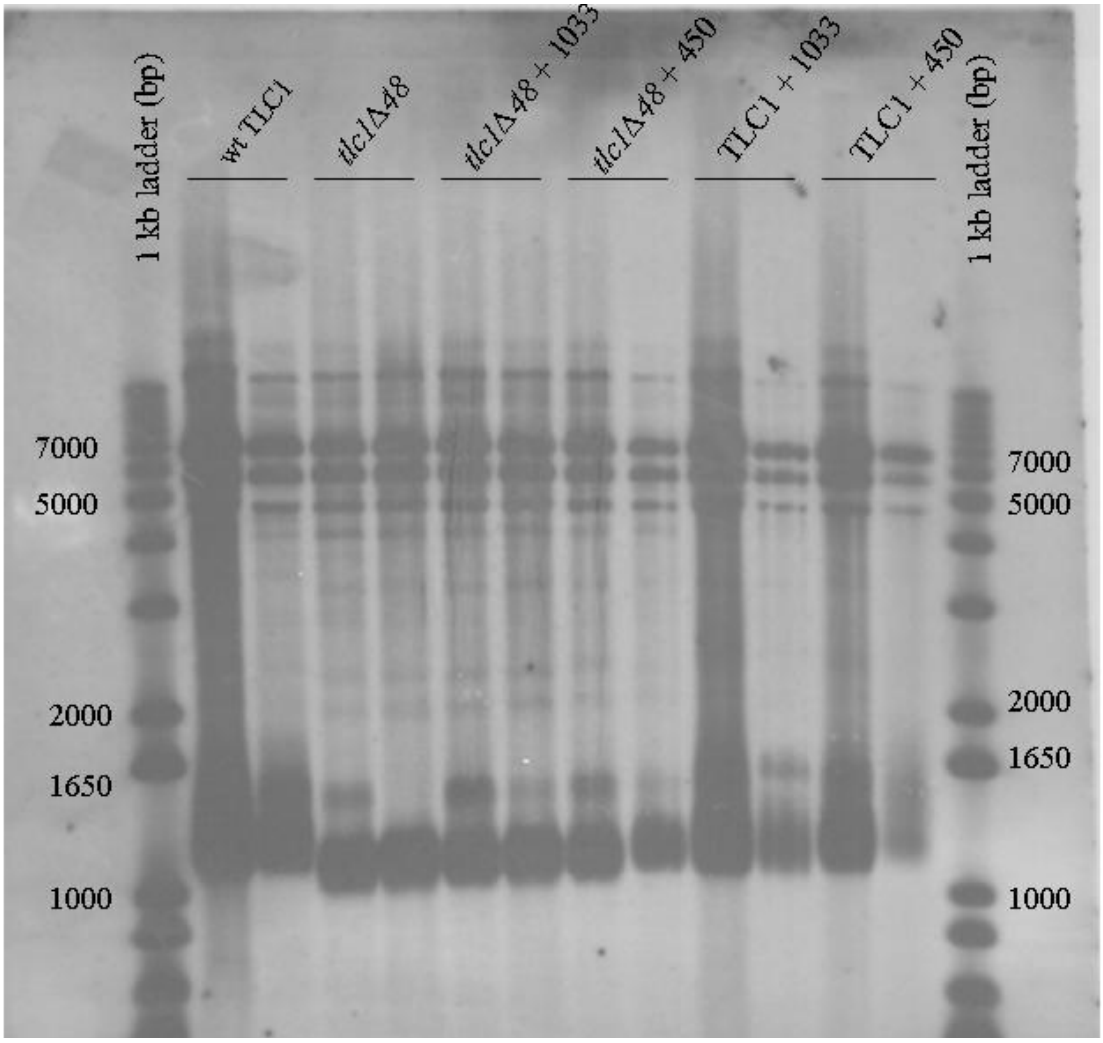

Figure 1. Native telomere lengths of *TLC1* structural variants. Genomic DNA digested with XhoI, resolved by gel agarose electrophoresis, and visualized with a chemiluminescent Y' probe.

Table 4. Frequencies of healing events per cell ($x10^{-8}$).

YED001	**YED002**	**YED003**	**YED004**	**YED005**	**UCC5113**
tlc1Δ48+1033	*TLC1+1033*	*tlc1Δ48+450*	*TLC1+450*	*tlc1Δ48*	*TLC1*
0.7200	0.4337	2.435	14.24	5.083	3.148
3.940	0.08955	2.964	2.919	0.7105	0.2222
4.552	0.1558	3.366	0.7850	0.3529	15.45
2.268	1.200	2.514	20.44	0.9286	1.211
2.870	<0.08219	12.00	<0.09375	0.1538	12.42
4.548	0.04054	4.818	0.4258	6.977	12.53
3.717	<0.03922	9.581	1.325	1.909	6.900
<0.09524	<0.04839	0.08824	0.4845	<0.1250	0.4286
1.286	0.5000	2.604	0.3636	0.1176	3.592
4.143	<0.1224	4.645	3.484	0.9677	2.521
11.54	0.1132	24.63	0.2222	0.2609	3.258
15.38	<0.1154	16.62	0.6818	<0.2500	1.800
6.333	1.385	7.750	3.158	<0.4615	2.250
8.000	1.043	7.862	2.143	0.3810	6.947
	2.800		0.4000		4.098
	0.6316		1.688		2.640
					4.600
					2.222
					2.400

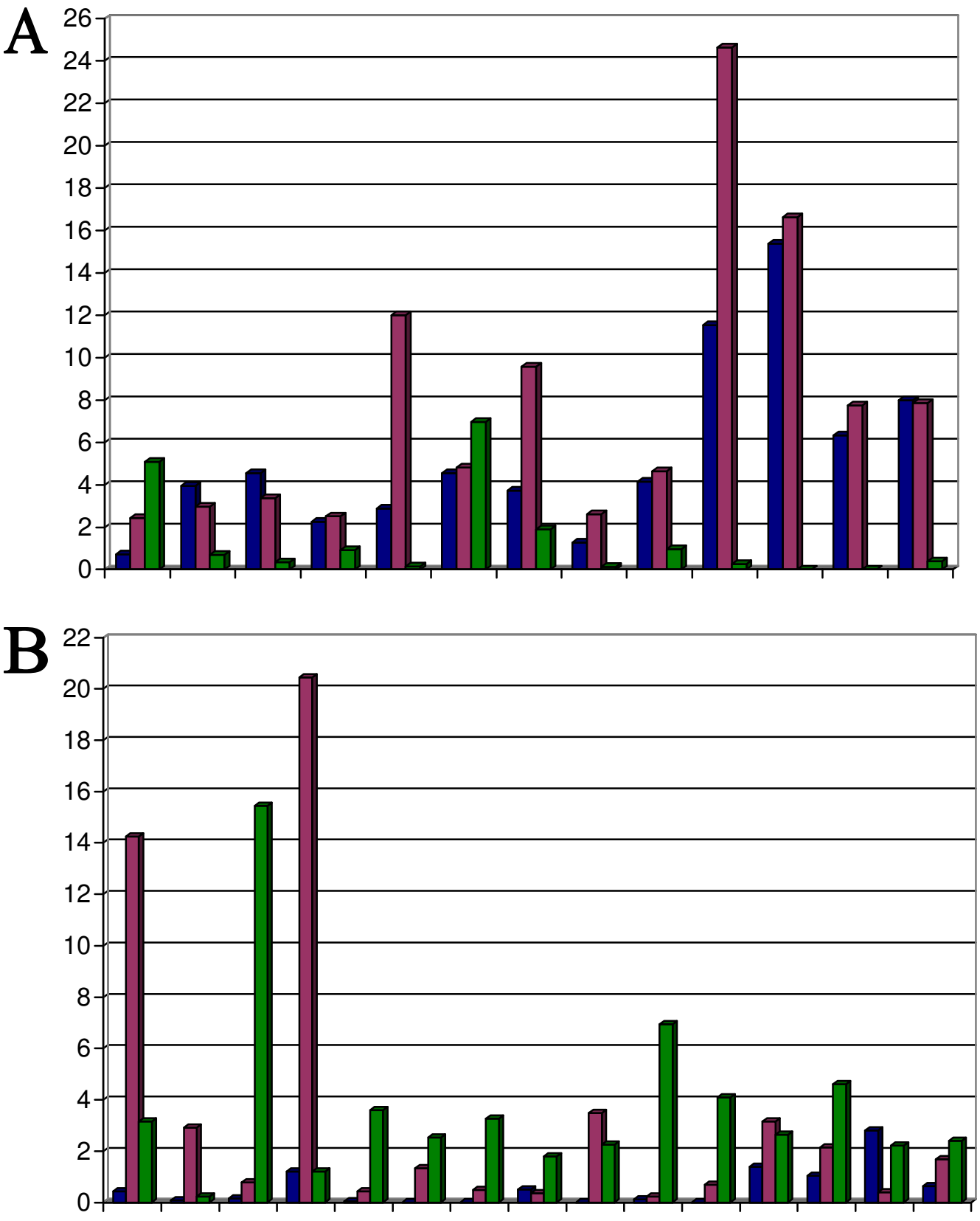

Figure 2. Frequencies of healing events (x10^{-8}). (A) *tlc1Δ48* strains: blue is *tlc1Δ48+1033*, purple is *tlc1Δ48+450*, and green is *tlc1Δ48*. (B) *TLC1* strains: blue is *TLC1+1033*, purple is *TLC1+450*, and green is *TLC1*. Frequencies are grouped by trial number.

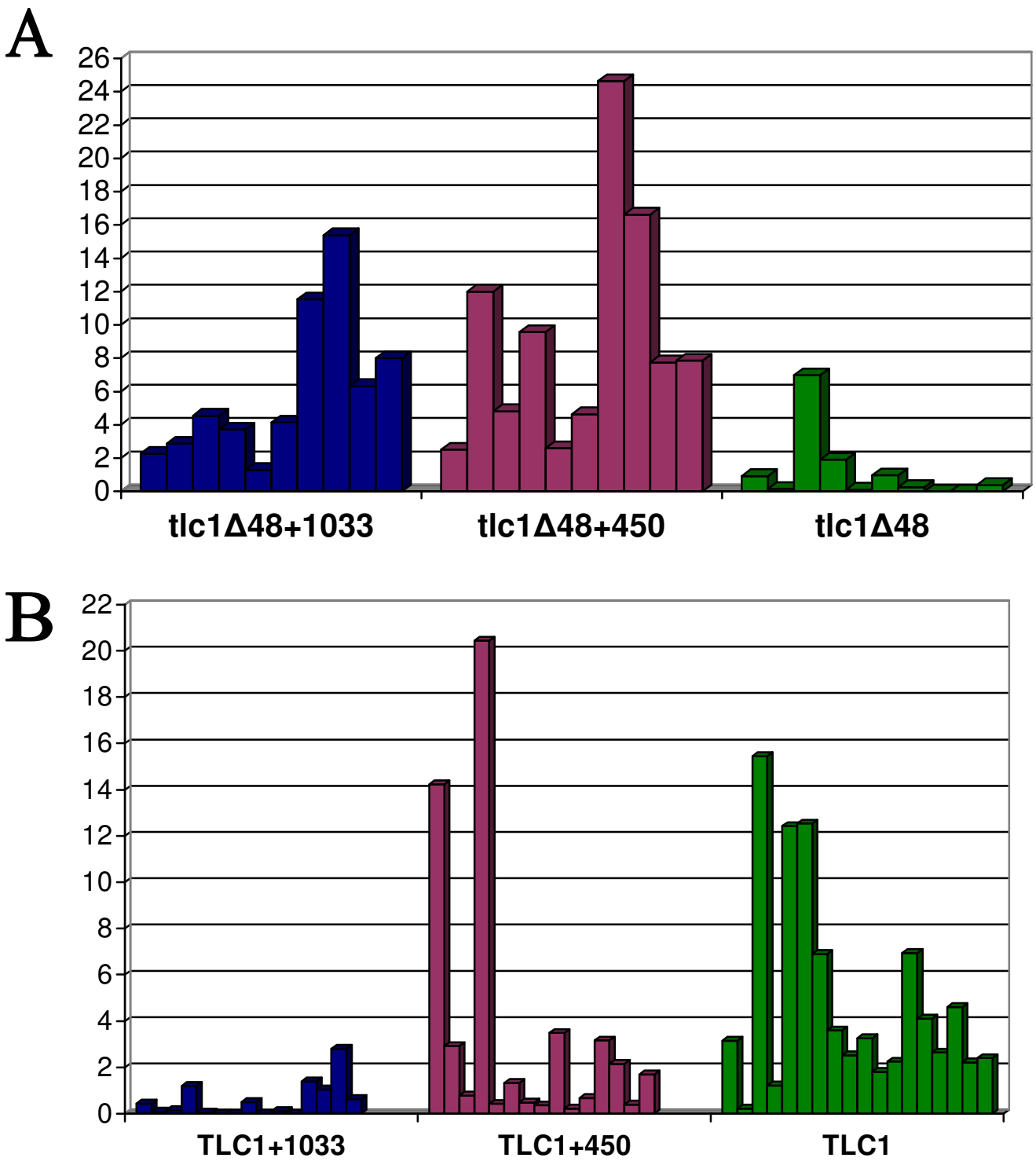

Figure 3. Frequencies of healing events (x10^{-8}) grouped by genotype. (A) *tlc1Δ48* strains: blue is *tlc1Δ48+1033*, purple is *tlc1Δ48+450*, and green is *tlc1Δ48*. (B) *TLC1* strains: blue is *TLC1+1033*, purple is *TLC1+450*, and green is *TLC1*.

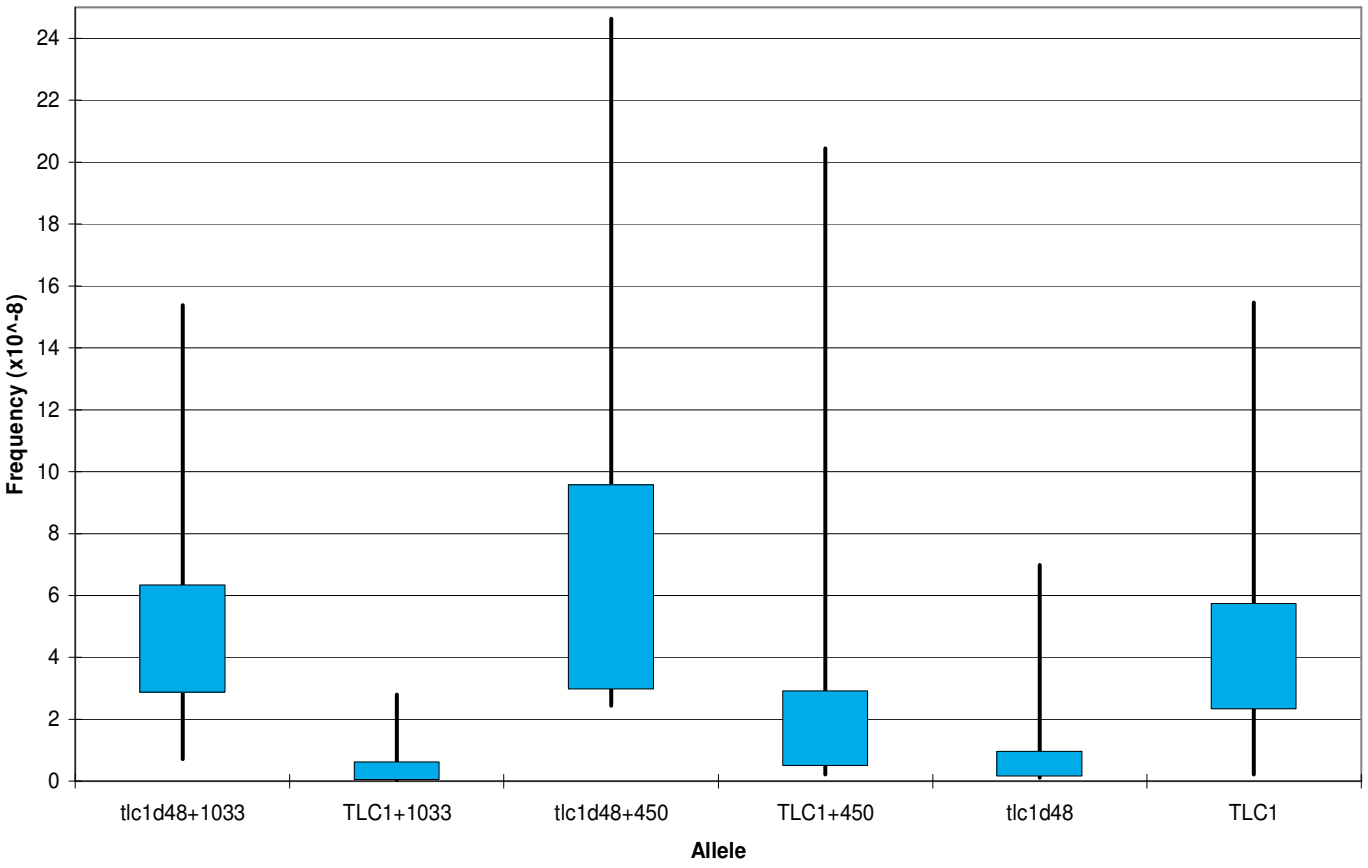

Figure 4. Frequencies of healing events: high, low, and middle 50% ($x10^{-8}$). Bars represent spans from highest frequency to lowest frequency for each allele. Blue boxes represent range of middle 50% of frequencies for each allele.

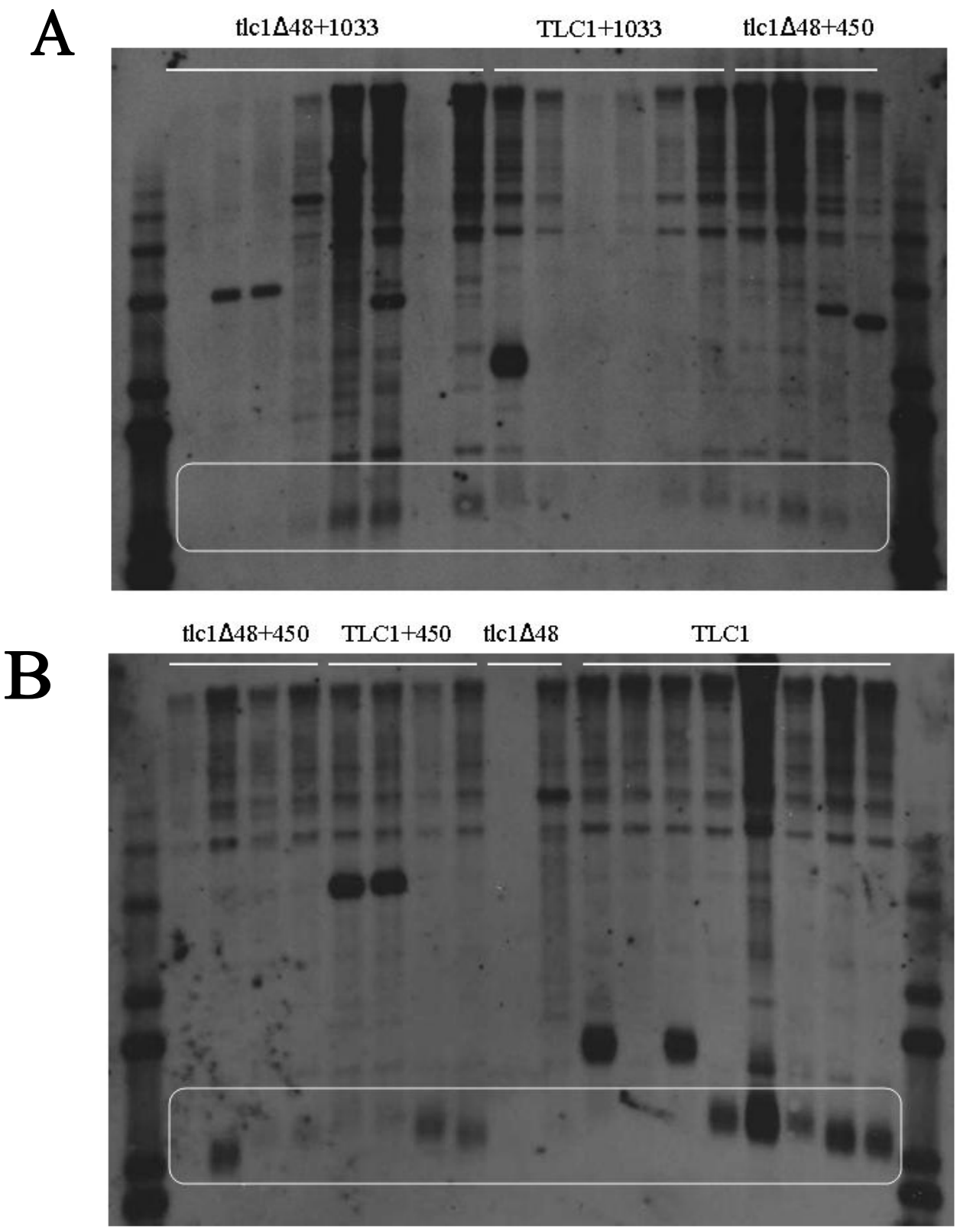

Figure 5. Evidence of telomere healing events. (A) Telomere healing events in *tlc1Δ48+1033*, *TLC1+1033*, and *tlc1Δ48+450* strains. (B) Telomere healing events in *tlc1Δ48+450*, *TLC1+450*, *tlc1Δ48*, and *TLC1* strains. Genomic DNA digested with XhoI, resolved by agarose gel electrophoresis, and visualized with a chemiluminescent NPR2 probe. White boxes surround telomeric bands.

Discussion

Relocating the Ku-binding Stem-loop

Ku recruits telomerase to the ends of chromosomes by interacting with a 48-nucleotide stem-loop on the TLC1 telomerase RNA. Removing this stem-loop disrupts the length of native telomeres, shortening them by about 250 base pairs. Moving the loop from its wildtype location to a new location within the RNA secondary structure causes telomeres to be slightly shorter in length than wildtype. Adding a second stem-loop at these novel locations, while maintaining the one at the wildtype locus, causes telomeres to be equal to or slightly longer than wildtype telomeres. In this capacity, two stem-loops appear to be more readily able to recruit telomerase to the end of the chromosome, and moving the stem-loop to a new place appears to slightly diminish this capacity.

While relocating the Ku-binding stem-loop does not significantly influence the cell's ability to add telomeres to the ends of chromosomes, its ability to repair double-stranded DNA breaks is much more affected. Cells with wildtype *TLC1* heal this damage at a frequency of 2.3 – 5.8 x 10^{-8} events per cell. Unlike its effect on native telomeres, moving the stem-loop to either nucleotide positions 1033 or 450 does not affect the cell's ability to heal DSBs, with healing frequencies of 2.9 – 6.3 x 10^{-8} and 3.0 – 9.6 x 10^{-8} events/cell, respectively. These values are equivalent to or even slightly higher than the wildtype values. Again, the *tlc1Δ48+450* RNA seems to work better than the *tlc1Δ48+1033* RNA, possibly indicating that the 1033 position has some type of inhibitory effect on the function of TLC1. This effect is also seen in the alleles that possess two copies of the stem-loop, where the additional loops seem to decrease telomerase function. While both the *TLC1+1033* (0.05 – 0.6 x 10^{-8} events/cell) and *TLC1+450* (0.5 – 3.0 x

10^{-8} events/cell) RNAs have frequencies lower than wildtype, *TLC1+450* functions to heal cells more often. *TLC1+1033* has a frequency even lower than *tlc1Δ48* (0.2 – 1.0 x 10^{-8} events/cell), providing the strongest evidence that having a Ku-binding stem-loop at nucleotide position 1033 has an inhibitory effect on the TLC1 RNA function of recruiting Ku to double-stranded DNA breaks.

Although moving the stem-loop affects the frequency with which a cell can heal double-stranded breaks, it does not seem to affect the mechanism with which the chromosome is primarily repaired. Telomere healing is evident in each of the six *TLC1* alleles, indicating that while the frequency of repair may change, the underlying function is not completely disrupted.

Future Directions

Additional GCR assays need to be undertaken in order to obtain more frequencies and perform a proper statistical analysis on the data. While the initial trends seen in this investigation provide insight into the Ku-binding stem-loop's effect on telomere healing, more data is needed to make conclusive statements. Furthermore, additional exploration is needed to investigate the non-telomeric bands seen in Figure 5 and determine whether some TLC1 structural variants are less adept at utilizing telomerase to heal double stranded DNA breaks than others.

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