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The Shortest Telomere, Not Average Telomere Length, Is Critical for Cell Viability and Chromosome Stability

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Summary

Loss of telomere function can induce cell cycle arrest and apoptosis. To investigate the processes that trigger cellular responses to telomere dysfunction, we crossed mTR−/− G6 mice that have short telomeres with mice heterozygous for telomerase (mTR+/−) that have long telomeres. The phenotype of the telomerase null offspring was similar to that of the late generation parent, although only half of the chromosomes were short. Strikingly, spectral karyotyping (SKY) analysis revealed that loss of telomere function occurred preferentially on chromosomes with critically short telomeres. Our data indicate that, while average telomere length is measured in most studies, it is not the average but rather the shortest telomeres that constitute telomere dysfunction and limit cellular survival in the absence of telomerase.

Introduction

Loss of telomere function can lead to genetic instability and cancer progression. Normal cells maintain chromosome stability, while cancer cells are characterized by frequent chromosomal changes (Lengauer et al., 1998). Early work of McClintock and Muller showed that loss of a telomere resulted in chromosome end-to-end fusion, chromosomal rearrangements and instability (McClintock, 1942; Muller, 1938). This work defined telomeres as the specialized structures that distinguish chromosomal breaks from natural chromosome ends.

Telomeres consist of a variable number of tandem simple repeated DNA sequences. Telomere length is maintained about an equilibrium length that is characteristic for the given organism. Many processes contribute to establishing this telomere length equilibrium; processes which shorten telomeres, such as incomplete replication and nuclease activity (Greider, 1996), are balanced by de novo telomere repeat addition by telomerase (Greider and Blackburn, 1985). In addition, telomere binding proteins play a major role in establishing the average length at which telomeres are maintained (Lustig et al., 1990; van Steensel and de Lange, 1997). In a population of cells there is a distribution of telomere lengths for each chromosome end. The establishment and maintenance of this telomere length equilibrium requires telomerase.

To understand the role of telomeres in chromosome maintenance in normal and in cancer cells, we generated telomerase null mice mTR−/− (Blasco et al., 1997). In the absence of telomerase, telomere shortening is not balanced by elongation. The first mouse generation lacking telomerase is designated mTR−/− G1, and subsequent generations derived through interbreeding are designated mTR−/− G2 through mTR−/− G6. mTR−/− G1 mice show no phenotype, as only a small amount of (TTAGGG)n telomere repeat is lost from chromosome ends in this first generation. With each successive generation of interbreeding, telomeres become shorter, and in the later generation (mTR−/− G4-G6) mice, chromosome end-to-end fusions are seen in lymphocytes and embryonic fibroblasts (Blasco et al., 1997; Lee et al., 1998). Late generation animals also show apoptosis in germ cells and progressive infertility such that mTR−/− G6 animals are usually not fertile (Hemann et al., 2001b; Lee et al., 1998).

The generation of chromosome end-to-end fusions in late generation mTR−/− mice indicates that progressive telomere shortening results in the loss of telomere function. Different mechanisms that cause telomere dysfunction result in similar cellular consequences (Hemann et al., 2001a). Expression of a dominant negative TRF2, a mammalian telomere binding protein, results in chromosome fusion, cell cycle arrest and p53-dependent apoptosis (Karlseder et al., 1999). Likewise, telomere shortening in mouse results in chromosome fusion, cell cycle arrest, and p53-dependent apoptosis (Blasco et al., 1997; Chin et al., 1999; Lee et al., 1998). Mutations in Taz1, a telomere binding protein in *S. pombe*, result in chromosome fusion and fertility defects (Cooper et al., 1998; Nimmo et al., 1998), phenotypes that are prominent in late generation mTR−/− mice. Finally, alterations in telomere repeats by introduction of mutant telomerase RNA in yeast and mammalian cells also result in chromosome fusion and cell cycle arrest (Guiducci et al., 2001; Kim et al., 2001; Kirk et al., 1997; Marusic et al., 1997; McEachern and Blackburn, 1995). In all of these cases, including telomere shortening, loss of specific proteins at telomeres likely leads to the recognition of chromosome ends as DNA breaks.

While the consequences of telomere dysfunction are well characterized, the primary determinant of the cellular response to global telomere shortening is still not clear. Unlike mutations in telomere binding proteins or alterations in the telomerase template, which likely affect all telomeres in a cell equally, telomere shortening may preferentially affect the function of a subset of telomeres in a cell. One possible mechanism for recognizing telomere dysfunction is that a cell requires maintenance of a certain average telomere length. Upon reaching a sub-threshold number of repeats, a cellular response is elicited. Average telomere length has been shown to correlate with the lifespan of cells in culture (Allsopp et al., 1992), and recent studies have argued that it is the average telomere length in a cell that determines the proliferative potential of a cell line in the absence of telomerase (Lansdorp, 2000; Martens et al., 2000). Similarly, recent models suggest that as telomeres shorten,

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Table 1. Comparison of mTR−/− (iFi) and mTR+/− (iFi) Mouse Phenotypes

Mouse	Testes Weight (% bodyweight)	Testicular Apoptosis (# TUNEL + cells per 100 seminiferous tubules)	Chromosome Fusions/Metaphase
mTR+/−	0.77 ± .08 (n = 12)	31 ± 6 (n = 12)	0 (n = 12)
G3 mTR−/−	0.68 ± .07 (n = 7)	36 ± 7 (n = 3)	0.01 ± .02 (n = 6)
G6 mTR−/−	0.14 ± .03 (n = 12)	85 ± 15 (n = 12)	0.42 ± .37 (n = 12)
mTR−/− (iF1)	0.23 ± .16 (n = 18)	69 ± 16 (n = 5)	0.39 ± .46 (n = 18)
mTR+/− (iF1)	0.74 ± .11 (n = 10)	29 ± 7 (n = 5)	0 (n = 14)

the probability of all telomeres losing function increases. This model suggests that average telomere length contributes to the cellular responses to telomere shortening (Blackburn, 1999, 2000). An alternative hypothesis is that, rather than average telomere length, individual dysfunctional telomeres are recognized as DNA damage and a cellular response is triggered.

To distinguish between the shortest telomeres and decreased average telomere length eliciting cellular responses, we crossed telomerase-deficient mice with short telomeres (mTR−/− G6) and mice heterozygous for telomerase activity (mTR+/−) that had long telomeres. In addition, we used spectral karyotyping (SKY) analysis to examine whether all chromosomes lost function or whether specific chromosomes were involved in end-to-end fusion in late generation knockout mice. We conclude that loss of telomere function occurs preferentially on the shortest telomeres, and that the shortest telomeres, rather than the average telomere length, elicit a cellular response.

Results

Short Telomeres Initiate a Cellular Response in mTR−/− Mice

In the absence of telomerase, progressive shortening of the initial telomere length distribution results in both a decrease in the average telomere length and the generation of extremely short telomeres at a few chromosome ends. To examine whether average telomere length or the shortest telomeres elicit a cellular response, we crossed mTR−/− G6 mice that have short telomeres with mice heterozygous for telomerase activity (mTR+/−) that have long telomeres. Two kinds of F1 offspring were obtained from this intergenerational cross: telomerase-deficient mice (mTR−/− (iF1)) and mice that have telomerase (mTR+/− (iF1)). Both types of offspring inherited 50% long telomeres from the mTR+/− parent and 50% short telomeres from the mTR−/− G6 parent. The average telomere length in these offspring should approximate that of an mTR−/− G3 mouse. mTR−/− G3 mice do not exhibit the infertility and germ cell apoptosis characterized in the later generation (G4–G6) mTR−/− mice (Table 1) (Blasco et al., 1997; Hemann et al., 2001b; Lee et al., 1998). We analyzed testes weight, testicular apoptosis and chromosome fusion in mTR−/− (iF1), mTR+/− (iF1) and compared them to that seen in the parents mTR+/− and mTR−/− G6 and the mTR−/− G3 (Table 1). Both the mTR+/− parent and the mTR−/− G3 mouse showed only background levels of germ cell apoptosis and chromosome fusion. The telomerase null offspring from the intergenerational cross mTR−/− (iF1), however, showed increased germ cell apoptosis and a corresponding reduction in testis weight that was similar to that seen in the mTR−/− G6 mouse. The number of chromosome fusions seen in leukocytes taken from these mice was also very similar in the mTR−/− (iF1) and the parental mTR−/− G6 mouse. In contrast to the telomerase null offspring, the telomerase positive offspring, mTR+/− (iF1), showed wild-type levels of germ cell apoptosis, no decrease in testis weight and no chromosome fusions, indicating that the addition of telomerase rescued these phenotypes. The presence of a significant phenotype in mTR−/− (iF1) mice suggests that the presence of short telomeres is sufficient to elicit a cellular response.

Preferential Elongation of Short Telomeres in mTR+/− (iF1) Mice

To investigate the consequences of telomerase reintroduction in mTR+/− (iF1) mice, we compared telomere length distributions in splenocytes from mTR+/− (iF1) and mTR−/− (iF1) mice with the mTR+/−, mTR−/− G3 and mTR−/− G6 mice. The amount of telomere repeat on each chromosome end was measured on metaphase spreads using quantitative fluorescent in situ hybridization (Q-FISH) (Lansdorp et al., 1996). This method uses directly labeled PNA (peptide nucleic acid) telomere repeat oligonucleotides to quantitate the relative number of repeats on individual chromosome ends. Telomere lengths are represented as arbitrary telomere fluorescence units (TFUs). Results from this experiment for two wild-type, two mTR−/− G6, two mTR−/− G3, three mTR−/− (iF1) and three mTR+/− (iF1) mice are represented as frequency distributions of TFUs (Figures 1A–1E). While there was a significant change in the average telomere length from the mTR+/− (43.99 TFUs) to the mTR−/− G3 (33.7 TFUs), only a small difference in average telomere length was seen between mTR+/− (iF1) mice (34.05 TFUs) and mTR−/− (iF1) littermates (31.18 TFUs). Thus, the telomerase-mediated restoration of telomere function in mTR+/− (iF1) mice occurs without a considerable increase in average telomere length. In contrast, there was a significant difference in the number of very short telomeres between mTR−/− (iF1) and mTR+/− (iF1) mice. mTR−/− (iF1) mice showed an average of 2.4 chromosome ends per metaphase that lacked detectable telomere repeats, while mTR+/− (iF1) mice showed no chromosome ends without detectable telomere repeats (Figures 1C and 1D). Thus, telomere function in mTR+/− (iF1) mice is restored not by global telomere elongation, but by the addition of repeats to the shortest class of telomeres.

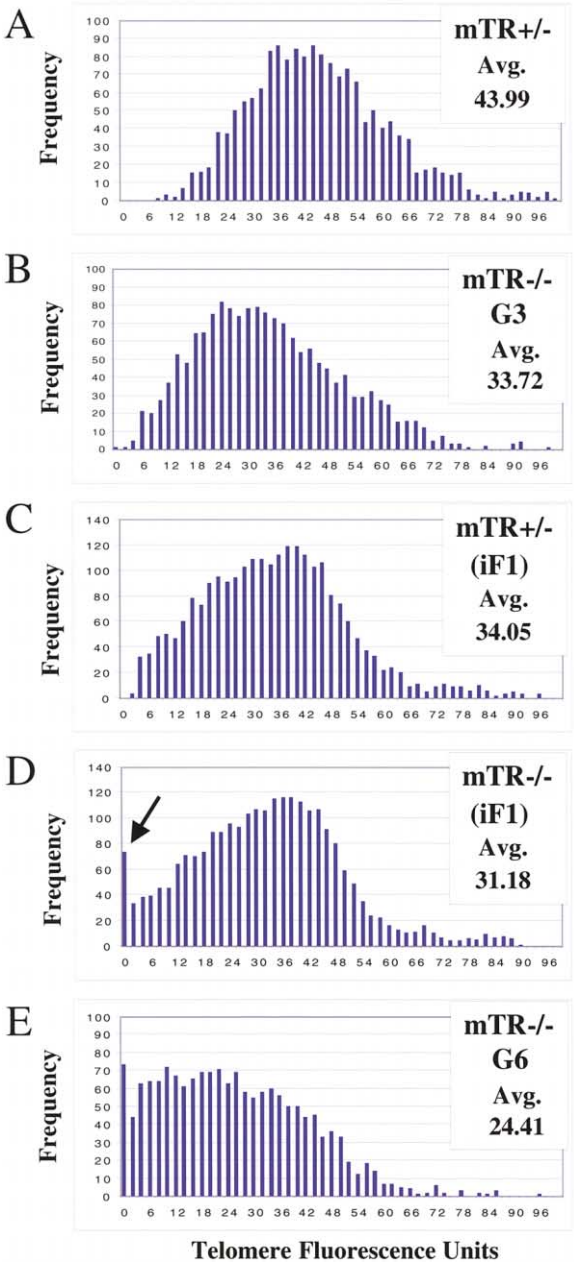

Figure 1. Telomere Length Analysis of mTR+/−, mTR−/− G3, mTR+/− (iF1), mTR−/− (iF1), and mTR−/− G6 Mice

Telomere lengths for each genotype are shown as frequency distributions of telomere signal intensities. Signal intensities from (A) 20 mTR+/−, (B) 20 mTR−/− G3 (C) 30 mTR+/− (iF1), (D) 30 mTR−/− (iF1), and (E) 20 mTR−/− G6 metaphases are shown. Average telomere signal intensities, expressed in telomere fluorescence units, are shown to the upper right of each frequency distribution. The arrow shown in the mTR−/− (iF1) telomere length distribution indicates the class of chromosome ends that lack signal that are specifically absent from the mTR+/− (iF1) telomere length distribution.

Restoration of Telomere Function Occurs Early in Development

To examine the timing of telomerase restoration of telomere function, we examined embryonic fibroblasts (MEFs) from mTR+/− (iF1) and mTR−/− (iF1) mice. mTR−/− (iF1) MEFs showed an average of 1.34 ± 0.98 chromosome ends without detectable telomere repeats per metaphase, while mTR+/− (iF1) MEFs showed only 0.02 ± 0.03 signal-free ends per metaphase (Figure 2B). Examination of cytogenetic abnormalities in these MEFs showed that mTR−/− (iF1) MEFs had an average of 0.27 ± 0.18 chromosome fusions per metaphase, while mTR+/− (iF1) MEFs showed no chromosome fusions (Figure 2A). Thus, the reintroduction of telomerase restored telomere function to mTR+/− (iF1) embryos prior to day 13.5 of development.

To further examine the phenotype of the iF1 MEFS, we examined their radiosensitivity. mTR−/− cells from late generation (G4–G6) mice show enhanced sensitivity to ionizing radiation (Goytisolo et al., 2000; Wong et al., 2000). We subjected early passage MEFs from wild-type, mTR−/− G3, mTR−/− G6, mTR+/− (iF1) and mTR−/− (iF1) mice to increasing doses of X-irradiation. While wild-type, mTR−/− G3 and mTR+/− (iF1) MEFs showed a similar level of growth impairment, mTR−/− (iF1) MEFs and mTR−/− G6 cultures showed a significant reduction in cell survival (Figure 2C). Thus, the short dysfunctional telomeres in the mTR−/− (iF1) MEFs likely mediate the radiosensitivity of these cells.

Nonrandom Distribution of Chromosome Fusions in One mTR−/− Mouse Line

If the shortest telomeres lose function first in mTR−/− mice, then chromosomes with short telomeres should be preferentially involved in end-to-end fusion events. To test this, we analyzed chromosome fusions and chromosomes with short telomeres in splenocytes from two independent lines of mTR−/− mice. Chromosome fusions from a well-characterized line of mTR−/− mice (Blasco et al., 1997; Lee et al., 1998) (referred to here as Line 1) were analyzed using SKY (Liyanage et al., 1996) to identify the specific chromosomes involved in fusions (Figures 3A and 3B). Chromosome end-to-end fusions were found at a variable frequency in individual mice although more fusions were seen in later generation (G4–G6) mice (Figure 4A; and see supplemental Table S1: http://www.cell.com/cgi/content/full/107/1/67/DC1). The overall distribution of chromosome fusions was nonrandom; chromosome 19 was involved in end-to-end fusion in 12/14 mice and chromosome 5 was involved in end-to-end fusion in 10/14. In contrast, chromosomes 3, 9, 10, and Y were never found involved in fusion events in any of the mice from this line (Figure 4A). In our analysis, a given chromosome fusion event in a particular mouse might be counted more than once due to clonal expansion of the cell containing that fusion. To assure that a large number of independent events were represented, we analyzed an average of 26 metaphases per mouse from 14 independent mice. There were no instances in which a particular fusion was seen in all metaphases from a given mouse, indicating that the fusions occurred de novo and were not inherited from the previous generation.

Most of the chromosome fusion events involved the fusion of two chromosomal P-arms (see supplemental Table S1). Mouse chromosomes are telocentric (Kipling et al., 1991); thus fusion of two P-arms can generate a Robertsonian-type translocation with a single functional

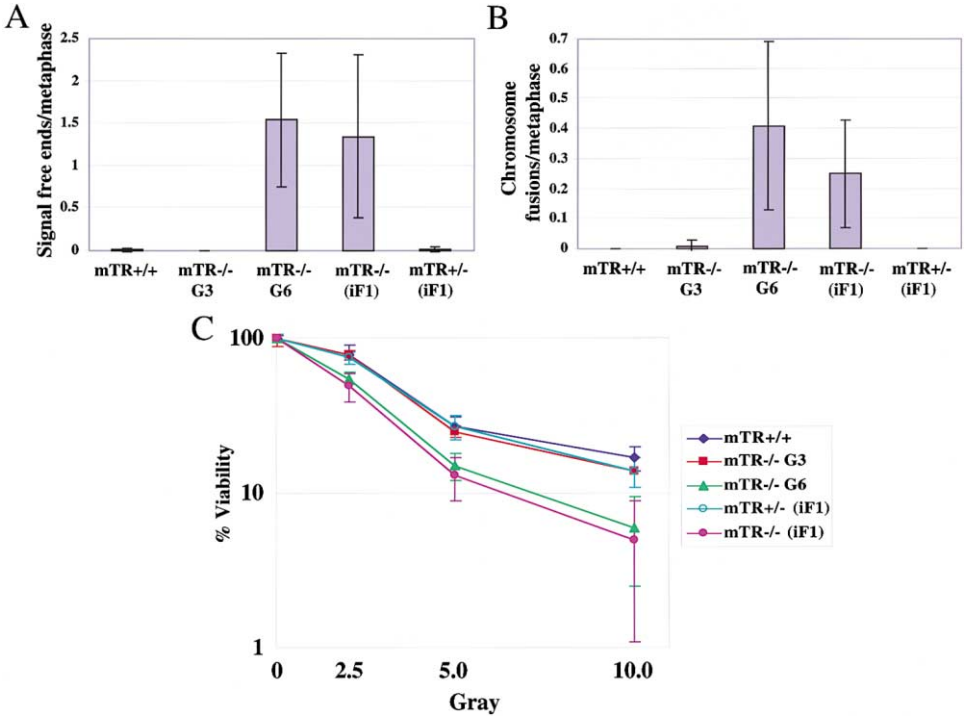

Figure 2. Analysis of mTR−/− (iF1) and mTR+/− (iF1) MEFs

(A) Signal-free chromosome end analysis. 20 metaphases from each of 5 independent mTR+/+, mTR−/− G3, mTR−/− G6, mTR−/− (iF1), and mTR+/− (iF1) were examined for chromosome with signal-free ends. The average number of signal-free ends per metaphase is shown with error bars reflecting the range of the data. The number of signal-free ends in mTR−/− (iF1) MEFs is significantly higher in than in mTR+/− (iF1) MEFs ($p < 0.05$).

(B) Cytogenetic analysis of mTR−/− (iF1) and mTR+/− (iF1) MEFs. 20 metaphases from each of 5 independent mTR+/+, mTR−/− G3, mTR−/−G6, mTR−/− (iF1), and mTR+/− (iF1) embryos were examined for cytogenetic abnormalities. The average number of chromosome fusions per metaphase is shown with error bars reflecting range of the numbers. The number of fusions in mTR−/− (iF1) MEFs is significantly higher in than in mTR+/− (iF1) MEFs ($p < 0.05$).

(C) MEF viability after exposure to ionizing radiation. mTR+/+, mTR−/− G3, mTR−/− (iF1), mTR+/− (iF1), and mTR−/− G6 MEFs were exposed to increasing doses of ionizing radiation. Viability is shown as percent of unirradiated cohorts with error bars reflecting standard deviations. Two independent cultures from each of three independent mice were scored for each data point. Statistical analysis of irradiated cells showed no significant difference in viability between mTR+/+, mTR−/− G3 and mTR+/− (iF1) MEFs ($p > 0.05$). There was a significant difference in viability in these three genotypes when compared to mTR−/− (iF1) or mTR−/− G6 MEFs ($p < 0.005$ at all doses).

centromere (Slijepcevic, 1998). In contrast, chromosome fusions involving either two Q-arms or one P- and one Q-arm are dicentric and are expected to lead to anaphase bridges and subsequent chromosome instability (de Lange, 1994; McClintock, 1942). These P-P fusions may occur more frequently than P-Q or Q-Q fusions, or they may simply be observed more frequently since they can give rise to a stable chromosome. We conclude that, in this line of mice, fusions most frequently involve chromosomes 19 and 5.

Chromosomes Frequently Involved in Fusions Have the Shortest Telomeres

To examine whether the presence of short telomeres correlated with the chromosome fusions, we performed Q-FISH on splenocytes from late generation mTR−/− mice. Wild-type mice analyzed using this technique show a signal on every chromosome end, while late generation mTR−/− G4–G6 mice show an increasing number of chromosomes where no signal is detectable (Blasco et al., 1997) (data not shown). Metaphase spreads were analyzed by Q-FISH to determine which telomeres lacked a signal, followed by SKY to determine the identity of the chromosome (Figures 3C and 3D). The set of chromosomes that lacked telomere signal were similar to the set frequently involved in fusion. For example, chromosome 19 lacked a telomere signal in 9/9 mice and chromosome 5 lacked telomere signal in 7/9 mice examined (Figure 4B). To determine whether the apparent correlation of chromosomes with signal-free ends being more likely to fuse was significant, we performed a statistical test. For each mouse where signal-free ends were identified, we compared the observed fraction of chromosomes with a signal-free end that were involved in fusion versus the calculated expected probability that that chromosome would randomly be involved in a fusion with any other chromosome. Mice with fewer than two fusions were excluded as there were not enough data to reliably do the statistical test. The P values were significant for all mice analyzed (with a range of $< 2.7 \times 10^{-2}$ to $< 1 \times 10^{-6}$) (see supplementary Table S1). Thus, while there is not an exact one-to-one correspondence between telomeres lacking signal and chromosome fusion, we conclude a

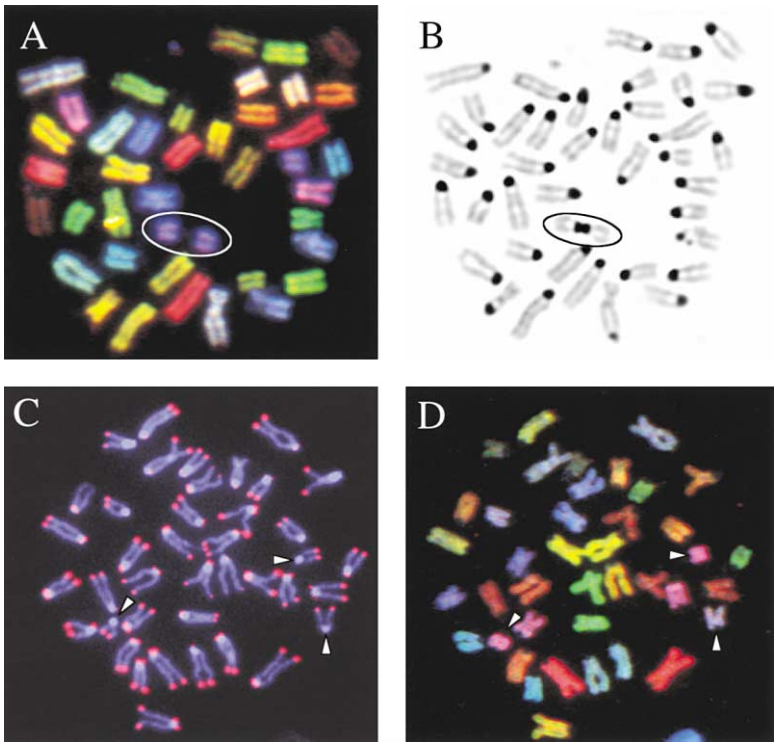

Figure 3. Chromosome Fusions and Signal-Free Ends

(A) Representative metaphase spread stained by SKY, showing a chromosome 19-19 P-P-arm fusion (circled).

(B) The same metaphase shown in (A) was stained with DAPI to identify fused chromosomes.

(C) Representative metaphase spread stained by Q-FISH to identify signal-free ends.

(D) The same metaphase shown in (C) was stained and analyzed by SKY to identify which chromosomes had lost telomere signal.

chromosome with a signal-free end has a greater probability of fusion than a chromosome containing telomeric repeats.

This data indicates that the shortest telomere is the most prone to lose function and undergo fusion with another short telomere suggesting that two short telomeres may need to be present in a cell for a fusion event to take place. Additional evidence for the requirement for two short telomeres comes from an examination of trisomies in the metaphases analyzed. 27% (52/192) of the P-P fusions occurred between two copies of the same chromosome. In these cases, cells were frequently trisomic for the chromosome involved in the fusion event (83% (43/52)). For example, most metaphases containing a 19:19 fusion also had an additional, nonfused copy of chromosome 19 (see supplementary Table S1). This suggests that a copy of chromosome 19 may have fused with its sister after replication and created a Robertsonian fusion with one functional centromere.

Analysis of Chromosome Fusions Caused by Telomere Dysfunction

To understand the nature of the telomere dysfunction that might lead to chromosome fusion we examined fusion junctions in late generation mTR−/− mice. Telomeres on the P-arms of mouse chromosomes are located immediately distal to minor satellite repeats (Kipling et al., 1991). Previous analysis of mouse Robertsonian fusions showed that the fusion junctions consist of minor satellite sequences from one chromosome fused head-to-head with minor satellite sequence from the other fusion partner, with no intervening telomere repeats (Garagna et al., 1995, 2001). These fusions also show a considerable loss of minor satellite repeat at fusion junctions. To determine the structure of P-arm fusions in our mice, we cloned and sequenced a number of fusion junctions. We used a single minor satellite-specific primer directed 5′ to 3′ toward the telomere to amplify DNA from wild-type or late generation mTR−/− splenocytes. No specific products were generated from wild-type DNA, while specific bands were generated from the late generation mTR−/− mouse DNA (data not shown). Sequence analysis of clones obtained from mTR−/− G5 or G6 DNA revealed that the fusion junctions consisted of minor satellite sequence fused directly with minor satellite sequence (Figure 5A). In all cases, fusion junctions contained no telomere repeat sequence. The structure of the fusion junctions was similar to fusions found in yeast cells that lack telomerase activity (Hackett et al., 2001). Interestingly, all eight fusions showed regions of microhomology at the fusion junctions. These regions of microhomology are characteristic of fusion events formed by several different non-homologous end joining (NHEJ) mechanisms (Feldmann et al., 2000; Kramer et al., 1994). These data suggest that, in these cases, dysfunctional telomeres were resected back to regions of microhomology within minor satellite repeats and the chromosomes were fused together by NHEJ.

While PCR analysis of fusion junctions demonstrated that minor satellite to minor satellite fusions could occur in mTR−/− G5–G6 mice, it was unclear whether these fusions represented the majority of fusions in these mice. To examine fusion junctions more quantitatively, we performed FISH using a probe specific for mouse minor satellite repeats on 30 metaphases from two different mice (Figure 5B). Minor satellite signal was quantitated on both normal chromosome ends and on fusion junctions. All fusions showed decreased signal relative

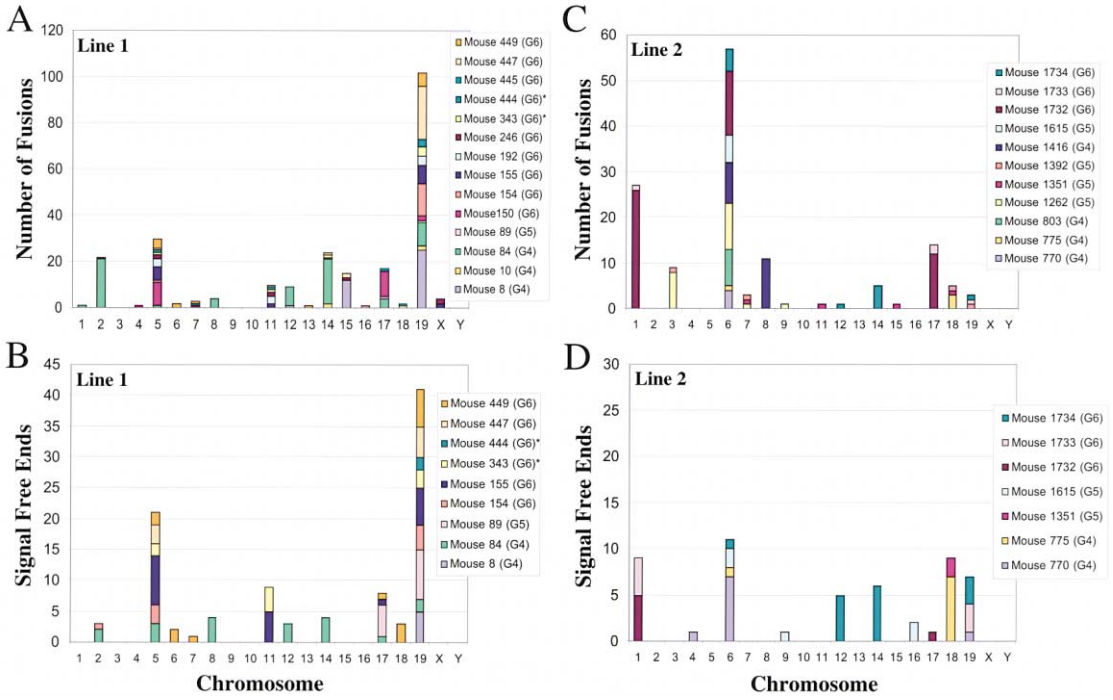

Figure 4. Chromosome Fusions and Signal-Free Ends in Two Independent Mouse Lines

(A) Frequency of fusions for each chromosome in mouse Line 1.

(B) Frequency of signal-free ends on specific chromosomes in mouse Line 1.

(C) Frequency of fusions for each chromosome in mouse Line 2.

(D) Frequency of signal-free end on specific chromosomes in mouse Line 2. Each color denotes data from an individual mouse.

to the average of all normal minor satellite signals. The amount of minor satellite signal loss ranged from 2.5% to 55%. The average decrease in signal intensity at fusion junctions was 21% ($P < .0001$). The loss of minor satellite sequence at fusion junctions further suggests that no telomere sequence is present at fusion junctions in late generation mTR−/− mice.

An Independent mTR−/− Line Has Different Short Telomeres and Different Chromosome Fusions

The association of short telomeres and chromosome fusions suggests that, in this line of telomerase null mice, specific chromosomes have telomeres that are reproducibly shorter than other chromosome ends. Experiments in yeast have shown that telomere length is clonal and that apparent changes in telomere length can be due to founder effects in a clone (Shampay and Blackburn, 1988). Given that each line of mTR−/− (G1–G6) mice is established by only a few initial fertilization events, a founder effect might contribute to the nonrandom distribution of short telomeres. To determine whether the breeding scheme used to generate the Line 1 mice stochastically fixed a particular distribution of telomere lengths, we carried out both SKY and Q-FISH analysis on a second line of telomerase null mice. This line was extensively backcrossed onto the C57BL/6J genetic background before mTR+/− heterozygotes were crossed. In this second line of mice (Line 2), we examined an average of 54 metaphases per mouse from 11 independent mice. We again found a nonrandom distribution of chromosome fusions; however, the chromosomes involved were different than those seen in Line 1 (Figures 4C and 4D). In 8 of 11 mice examined, chromosome 6 was involved in fusions. In contrast to Line 1, no fusions of chromosome 5 were seen and only 3 of 11 mice showed fusion of chromosomes 19. Q-FISH analysis of metaphases from Line 2 mice showed chromosome 6 most frequently lacked a telomere signal. Thus again, in this second line of mice, the chromosomes that fused were most often those with the shortest telomeres; however, the identity of the chromosomes involved differed from those seen in Line 1 mice.

Telomere Lengths in Wild-Type Mice Are Not Chromosome Specific

The identification of different sets of nonrandom chromosome fusions in two independent lines of telomerase null mice suggested that no particular chromosome had a telomere distribution that is consistently shorter than other telomeres in wild-type mice. Rather, the data is consistent with the hypothesis that each chromosome has a distribution of telomere lengths and at each fertilization event a new distribution is established from the randomly chosen initial telomere lengths in the fertilized egg. To test this hypothesis directly, we looked at telomere length distributions on individual chromosomes in wild-type mice using SKY and Q-FISH. Twenty metaphases from five wild-type mice were analyzed and the distribution of telomere lengths on each chromosome arm was compared to the distribution on all other chro-

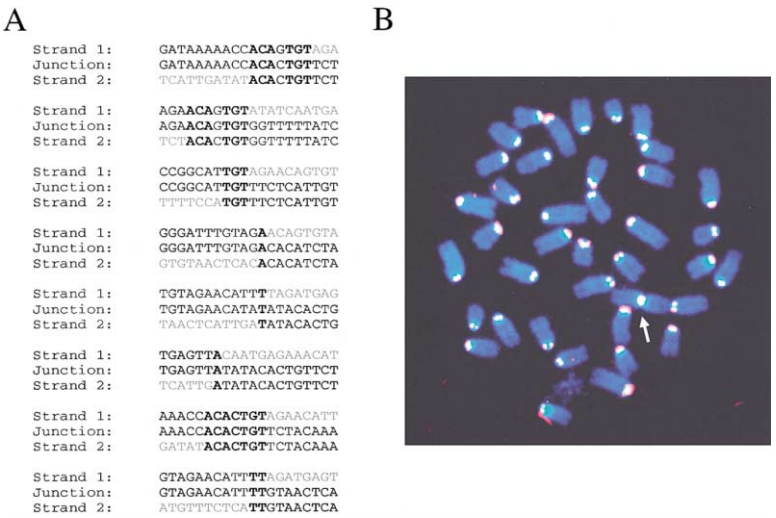

Figure 5. Analysis of Fusion Junctions in Late Generation mTR−/− Splenocytes

(A) Fusion junction sequences from mTR−/− G5 or G6 splenocyte DNA. Eight fusion junctions, with surrounding sequence, are shown. The middle sequence represents the fusion junction, while the sequences above and below represent where the fusion junction occurs in the context of normal minor satellite sequence. Letters in bold indicate regions of microhomology, and letters in gray indicate sequences that were removed in forming the fusion.

(B) Representative FISH image stained for mouse minor satellite DNA. Signals at chromosome fusion junctions (shown with an arrow) were quantitated relative to minor satellite signals present at unfused mouse chromosomes. Chromosomes were counterstained with DAPI.

mosome arms in each mouse (Figure 6). As a control for the inherent variability of the quantitative fluorescence method, we compared sister chromatid signals of 120 telomeres where the signal for each sister was well separated. Since sister chromatids are replication products of each other, they are expected to have very similar telomere signal intensities. This was the case, as the correlation coefficient was 0.80. We next examined whether any telomere length distribution on a given chromosome was significantly different than the telomere length distributions of all of the other chromosomes using a Wilcoxon rank sum test (see Experimental Procedures). This method compares each distribution against all of the other distributions to determine if there is a significant deviation in one from the set of the other distributions. In any given mouse, one or several chromosome ends had length distributions that were significantly different than the other chromosome ends. However, in no case did any particular chromosome consistently have a shorter or a longer distribution in all of the mice examined. Thus, we conclude that there is no particular chromosome end that has a reproducibly shorter telomere length distribution than the other telomeres in wild-type mice.

Discussion

Critically Short Telomeres Cause the Phenotype in Late Generation mTR−/− Mice

Telomeres play an important role in maintaining genomic stability in mammalian cells. Uncompensated telomere shortening leads to telomere dysfunction and subsequent chromosome rearrangement, cell cycle arrest, and apoptosis. Previous studies have followed changes in average telomere length with the onset of specific phenotypes. Our data suggest that it is not average telomere length, but rather individual critically short telomeres that trigger cellular responses to the loss of telomere function.

Recent experiments have proposed that telomere length is not the sole determinant of telomere function because, in some cases, telomere dysfunction occurred when the average telomere length was relatively long (Zhu et al., 1999). The inability to visualize the length of individual short telomeres in a distribution of terminal restriction fragments on a Southern blot makes it difficult to interpret the cause of telomere dysfunction. Our data suggest that critically short telomeres are both necessary and sufficient for producing the phenotypes seen in late generation mTR−/− mice.

Based on these results, a recent model for initiation of telomere dysfunction may need to be refined. This model suggests that all telomeres in a population have a certain probability of becoming "uncapped," or dysfunctional, and as the average telomere length in the population decreases, the probability of all telomere dysfunction increases (Blackburn, 2000). From this model one would predict that as telomeres shorten, a random chromosome, possibly one with a long telomere, will undergo chromosome fusion. Instead, we find that only chromosomes with the shortest telomeres are involved in fusions and that short telomeres are responsible for the phenotype in mTR−/− (iF1) mice. Perhaps these dysfunctional telomeres have fallen below a threshold of telomere repeats necessary for efficient binding of telomere-associated proteins such as TRF2 (Broccoli et al., 1997), or the formation of telomere loop structures may be impaired (Griffith et al., 1999).

Restoration of Telomere Function upon Reintroduction of Telomerase

The reintroduction of telomerase restored telomere repeats to the shortest telomeres in the mTR+/− (iF1) mice. Strikingly, the average telomere lengths in mTR−/− (iF1) F1 and mTR+/− (iF1) mice were very similar. Thus, telomerase is apparently targeted to the shortest, and possibly the dysfunctional telomeres in the population. The mechanism by which short telomeres become preferential substrates for telomerase elongation is unclear. Perhaps telomerase recruitment to telomeres occurs more efficiently when telomere proteins are absent or inefficiently bound. Another possibility is that proteins that recognize telomere dysfunction also recruit telomerase to chromosome ends. Recent experi-

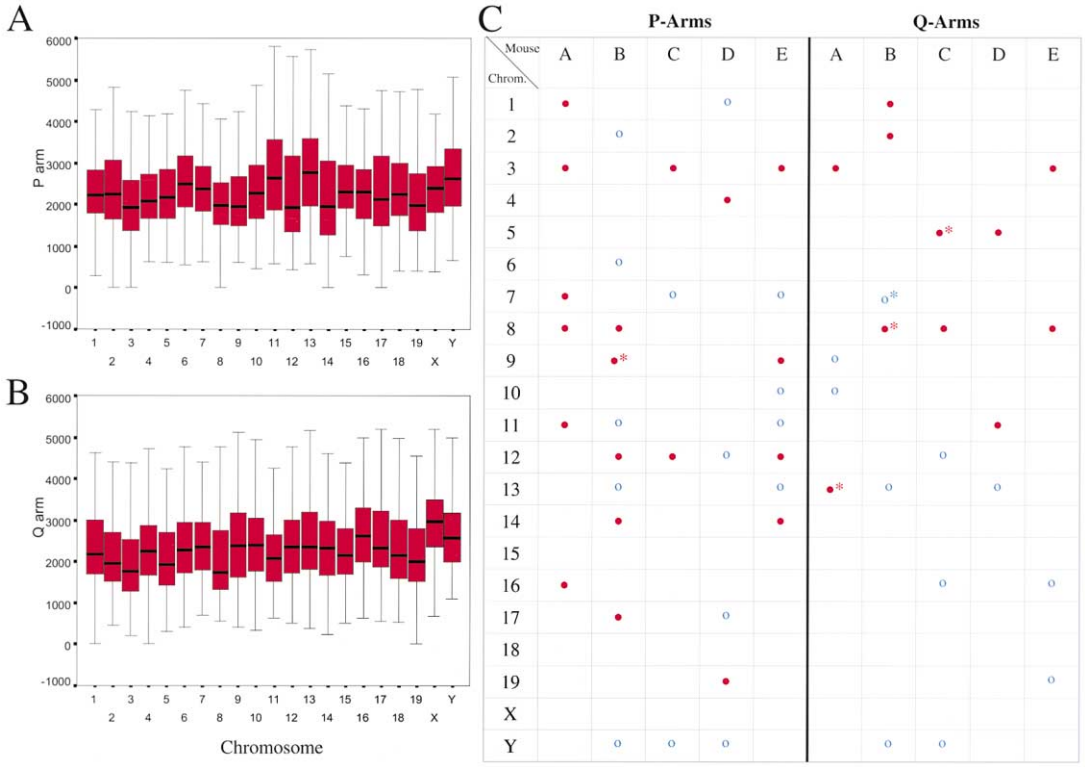

Figure 6. Relative Telomere Signal Intensities for Five Unrelated C57BL/6J Mice

(A) Box plot of P-arm telomere lengths for all five mice. Intensities are provided in telomere fluorescence units. At least 20 metaphases were examined for each mouse. Each box plot shows the mean (black bar), the 50th (red box), and 90th (thin lines) percentages of the distribution.

(B) Box plot of Q-arm telomere lengths for all five mice.

(C) Table summarizing the results of the Wilcoxan rank sum statistical test for all five mice, labeled A, B, C, D, and E. Blue circles represent significantly longer telomere distributions, and red dots represent significantly shorter telomere distributions (see Experimental Procedures). The asterisks represent differences in telomere lengths that were only weakly significant ($0.6 > P > 0.4$).

ments have shown, for example, that the yeast ku protein, which is involved in DNA end-joining, might also recruit telomerase RNA to chromosome ends (Peterson et al., 2001).

The preferential elongation of short telomeres may explain previous results in which limiting amounts of telomerase maintained telomere function, despite decreases in overall telomere length (Chiu and Harley, 1997; Ouellette et al., 2000; Zhu et al., 1999). This process may represent an uncoupling of telomere length maintenance from the maintenance of telomere function. Although a wild-type telomere length distribution is not restored in mTR+/− (iF1) mice, telomere function is restored. Thus, the essential role of telomerase may not be the maintenance of an average telomere length, but rather the maintenance of telomere function at critically short telomeres.

Fusions Involve Chromosomes with the Shortest Telomeres

Because cancer cells often have specific characteristic chromosomal rearrangements, it is important to know whether initial telomere length leads to a bias in which chromosomes undergo rearrangement. A previous study in human cells suggested that the telomere on 17p is characteristically shorter than other telomeres in normal human cells (Martens et al., 1998). These authors suggested chromosome 17 might more be more frequently involved in rearrangements and that the location of p53 on 17p may contribute to the frequent loss of p53 in human tumors.

To establish whether telomere length may play a role in specific chromosomal rearrangements in mice, we examined whether there is a preference for fusion of certain chromosomes relative to others in telomerase null mice. While individual lines of mice had characteristic patterns of chromosome fusion, these patterns were not consistent between independent mTR−/− lines. We propose that, in the absence of telomerase, telomere length is fixed at fertilization; the shortest telomere remains the shortest while the entire distribution becomes progressively shorter with each generation. Thus in a particular line of mTR−/− mice, the specific chromosomes which happened to be the shortest when the line was established will preferentially undergo fusion. This stochastic determination of the shortest telomere in different mouse lines may explain the previous discrepancy between two studies that found different chromosomes most frequently involved in chromosome fusion in mTR−/− mice (Hande et al., 1999; Lee et al., 1998).

Stochastic determination of telomere length is well documented in yeast. When a culture of yeast cells is

grown and plated, independent single colonies have distinct distributions of telomere lengths on any given chromosome end (Shampay and Blackburn, 1988). This is because the initial telomere length in the single cell that established the colony determined the starting point for the new length equilibrium. Since wild-type yeast have telomerase activity, and telomerase is necessary to maintain the length equilibrium, a new equilibrium is established using the founder telomere length as the starting point. Such clonal establishment of individual telomere length likely also occurs in mice. Thus, the establishment of a line of mice fixes telomere length on specific chromosomes at fertilization. In the case of the telomerase null mice, however, a new length distribution cannot be established and the identity of the chromosome that happened to have the shortest telomere is maintained through subsequent generations of breeding.

Possible Mechanisms for Generating Chromosome Fusions

The correlation between the chromosomes with the shortest telomeres and those involved in fusion, together with the lack of telomere signal at the fusion junction, implies that both chromosomes involved in fusion may have very short telomeres. In addition, the loss of minor satellite sequence at the junction also suggests that two dysfunctional telomeres may have been processed to generate two ends that resembled a double-strand break. Finally, the fact that the fusion junction sequence contains microhomology characteristic of NHEJ events is consistent with a model in which telomere dysfunction results in transient chromosome end degradation followed by NHEJ-mediated fusion of broken chromosome ends. A similar requirement for two broken ends to generate chromosome fusions was documented by McClintock in maize (McClintock, 1941, 1942). One mechanism for generating two dysfunction telomeres for chromosome end-to-end joining is the fusion of a chromosome to its own replication product after S phase. Thus, the mechanism for generating fusion chromosomes and trisomies may be analogous to the mechanism that McClintock proposed for the "chromatid" type breakage-fusion-bridge-cycle (McClintock, 1939).

Experimental Procedures

Telomere Length Analysis

Q-FISH was performed using a Cy3-labeled $(CCCTAA)_3$ PNA oligonucleotide (PE Biosystems) as described (Lansdorp et al., 1996). Metaphase spreads were counterstained with DAPI. Images were acquired using a Zeiss Axioskop microscope and IP-Lab Spectrum acquisition software. FluoreSpheres® fluorescent beads (Molecular Probes) were used to monitor signal intensity loss during microscope use.

Cell Culture Radiation Study

MEFs were prepared from day 13.5 embryos as described (Blasco et al., 1997). Irradiation experiments were performed as described (Wong et al., 2000). Early passage MEFs (PD 4) were irradiated using a $Cesium^{137}$ source at a dose of 0.77 grays/min to a final dose of 0, 2.5, 5, or 10 grays. After irradiation, 1×10^5 cells were plated onto 10 cm culture plates and grown for 5 days. Cells were then trypsinized, stained for viability with trypan blue, and counted.

Metaphase Preparation

Metaphase preparations were obtained from either cells arrested in vivo, or in vitro. Mice were injected intraperitoneally with colcemid (Gibco BRL) at a dose of 1 mg/kg and spleen and bone marrow lymphocytes were harvested. The cells were washed in PBS, swelled with 0.075 M KCl at 37°C for 15 min and fixed in methanol:acetic acid (3:1). Cell suspensions were then dropped on chilled slides and dried overnight. Alternatively, Cells were harvested from spleens and cultured in 100 ml RPMI 1640 w/L-glutamine (Gibco BRL) supplemented with 1× penicillin-streptomycin-glutamine, 10% heat inactivated fetal bovine serum, 10 mM HEPES buffer, 1 mM sodium pyruvate, (Gibco BRL), 1 mg LPS (Sigma), 1000U IL-2 (Roche), and 500 μg ConA (Sigma). After 48 hr the cells were arrested with 0.1 μg /ml colcemid (Gibco BRL) for 30 min, and metaphases were prepared as described above.

Q-FISH/SKY

Spectral karyotyping was done as described (Liyanage et al., 1996). A chromosome fusion was identified if there was no gap between centromeres in the DAPI image and there was precise alignment of the two centromeres. Chromosome ends were scored as signal free when no telomere repeats were detected, even after overexposure. After telomere FISH analysis, coverslips were removed from slides by washing in 2× SSC. The slides were then prepared for hybridization with the SKY probe, starting with the slide denaturation step of the SKY protocol.

Sequencing Fusion Junctions

Minor satellite-specific primers were designed from published minor satellite sequence (Pietras et al., 1983). Primer sequences were as follows: MSF1, 5′ TCGTTGGAAACGGGATTTGTAG 3′; MSF2, 5′ CATTCGTTGGAAACGGGATTTG 3′; and MSR1, 5′ TCCCGTTTCCAA CGAATGTG. PCR analysis of fusion junctions was performed using either MSF1 or MSF2 as the sole primer in the reaction. Input DNA was derived from splenocytes from both mTR+/+ and mTR−/− G5–G6 mice. Reactions were run for 30 cycles of 15 s at 95°, 1 min at 60°, and 2 min at 72°, using standard PCR reaction conditions. PCR products were isolated from agarose gels using a Qiaquick™ Gel Extraction Kit (Qiagen). Purified PCR products were TA cloned using a TA cloning kit (Invitrogen) and the DNA insert was sequenced.

FISH Analysis of Fusion Junctions

A 500 bp minor satellite probe was generated by PCR cloning of genomic DNA using MSF2 and MSR1 primers described above. The minor satellite probe was purified and labeled with biotin-16-dUTP by nick translation (Langer et al., 1981). The reaction was then ethanol precipitated and resuspended in a 70% formamide, 2× SSC, 10% dextran sulfate, and 25 mM NaH_2PO_4 solution. Prior to hybridization, the minor satellite probe was denatured at 75° for 5 min.

Metaphase chromosome spreads were prepared from mTR−/− G6 splenocytes as described above, denatured in 70% formamide/2× SSC at 70° for 2 min, and then dehydrated through a graded cold ethanol series. Hybridization was performed as described (Garagna et al., 1995).

Images were acquired using a Zeiss Axioskop microscope and IP-Lab Spectrum acquisition software. Signal intensities were calculated using designated software provided by the Lansdorp laboratory (Lansdorp et al., 1996). 30 metaphases from 2 mTR−/− G6 mice were scored. The ratio of the minor satellite signal intensity at fusion junctions relative to the average minor satellite signal intensity at all unfused centromeres was calculated for each metaphase.

Statistical Methods for Comparing Telomere Length Distributions

At least 20 metaphases from 5 independent 10-week-old male C57BL/6J mice were prepared from splenocytes as described above. Telomere lengths were first quantified by Q-FISH, and each metaphase was subsequently analyzed by SKY. The distribution of telomere lengths for each chromosome was determined. The chromosome specific telomeric length distributions were analyzed via Wilcoxon's Rank Sum test using the statistical program SPSS version 10.0. This analysis established whether any telomere distri-

bution was significantly shorter or longer that all of the other telomere distributions. To ensure the two samples (*n*1 and *n*2) in Wilcoxon's test were independent of each other, the telomere lengths from each chromosome end were compared with a group of all of the other chromosome ends except the specific telomere distribution being analyzed.

mTR−/− Mouse Lines

Two independent lines of mTR−/− mice were analyzed in this study. Line 1: mTR−/− mice bred essentially generated as described (Blasco et al., 1997; Lee et al., 1998). The genetic background was 60% C57BL/6, 37.5% 129/Sv, and 2.5% SJL (Blasco et al., 1997; Lee et al., 1998). Line 2: Heterozygous mTR+/− mice initially derived from Line 1 were backcrossed to C57BL/6J mice (Jackson Laboratory) 6 times.

Intergenerational Mouse Crosses

mTR+/− males were crossed with mTR−/− G6 females. This breeding strategy was chosen to prevent the potential introduction of telomerase activity from mature oocytes into telomerase-deficient offspring. Telomerase-deficient offspring from this cross were designated mTR−/− (iF1) mice, while heterozygous offspring were designated mTR+/− (iF1) mice.

Age-matched adult mice (3–5 months) were used for the analysis of mTR+/−, mTR−/− G3, mTR−/− G6, mTR−/− (iF1), and mTR+/− (iF1) phenotypes. Testicular apoptosis was examined by TUNEL analysis of testis sections as described (Hemann et al., 2001b).

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