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The Rate of Increase of Short Telomeres Predicts Longevity in Mammals

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SUMMARY

Aberrantly short telomeres result in decreased longevity in both humans and mice with defective telomere maintenance. Normal populations of humans and mice present high interindividual variation in telomere length, but it is unknown whether this is associated with their lifespan potential. To address this issue, we performed a longitudinal telomere length study along the lifespan of wild-type and transgenic telomerase reverse transcriptase mice. We found that mouse telomeres shorten ∼100 times faster than human telomeres. Importantly, the rate of increase in the percentage of short telomeres, rather than the rate of telomere shortening per month, was a significant predictor of lifespan in both mouse cohorts, and those individuals who showed a higher rate of increase in the percentage of short telomeres were also the ones with a shorter lifespan. These findings demonstrate that short telomeres have a direct impact on longevity in mammals, and they highlight the importance of performing longitudinal telomere studies to predict longevity.

INTRODUCTION

Telomeres are repeated DNA nucleoprotein structures at the ends of eukaryotic chromosomes (Blackburn, 1991; de Lange, 2005) that protect them from degradation and DNA repair activities, and are essential for chromosomal stability (Chan and Blackburn, 2002). Telomere repeats can be added de novo by telomerase, a reverse transcriptase that elongates telomeres in cells in which it is expressed (Blackburn, 2005; Flores et al., 2006; Greider, 1998; Greider and Blackburn, 1985; Marion et al., 2009). In telomerase-negative cells, telomeres shorten with each round of cell division as a result of end replication and DNA-degrading activities. Short telomeres are passed onto daughter cells, and thus telomere shortening is exacerbated by cell division and increasing age in both humans and mice (Flores et al., 2008; Harley et al., 1990). Critically short telomeres can trigger a persistent DNA damage response that leads to cellular senescence and/or apoptosis (Collado et al., 2007; Deng et al., 2008), eventually compromising the regenerative capacity and function of tissues (Blasco, 2007). Short telomeres are proposed to be sufficient to cause the degenerative pathologies associated with aging even in the presence of telomerase activity (Armanios et al., 2009; Hao et al., 2005).

In further support of this notion, both telomerase-deficient mice and human diseases due to mutations in telomerase components result in accelerated-aging phenotypes and decreased longevity due to premature depletion of stem cells and subsequent organ/tissue failure (Armanios et al., 2007; Blasco et al., 1997; García-Cao et al., 2006; Herrera et al., 1999; Mitchell et al., 1999; Tsakiri et al., 2007; Vulliamy et al., 2001; Yamaguchi et al., 2005). Telomere shortening associated with normal aging can be influenced by known risk factors for disease and premature death, such as psychological stress, smoking, cognitive impairment, and obesity (Canela et al., 2007; Cawthon et al., 2003; Cherkas et al., 2006; Epel et al., 2004; Valdes et al., 2005). However, whether the rate of telomere shortening in individual mammals has an influence on their longevity remains unexplored. In this regard, although serial telomere length measurements were previously obtained in baboons, a correlation with longevity was not sought (Baerlocher et al., 2003). Of interest, it was recently reported that telomere length measured at 25 days of age is a predictor of lifespan in birds (Heidinger et al., 2012).

Evidence from telomerase loss-of-expression and gain-of-expression transgenic mouse models suggests that telomere length may be rate limiting for mouse longevity (García-Cao et al., 2006; Tomás-Loba et al., 2008). Nonetheless, a role for telomere length in normal mouse aging is brought into question by the fact that although mice have very long telomeres compared with humans (∼50 kb in young C57BL/6 mice, compared with ∼15 kb in young humans) (Gomes et al., 2011; Wright and Shay, 2000), mice have a much shorter maximal lifespan (∼5 years in mice of mixed background homozygous [−/−] for the *GHR/BP* gene) (Brown-Borg and Bartke, 2012) than humans (122 years). Although numerous studies have measured the average rate of telomere shortening per year in human population studies, which ranges from 31 base pairs (bp)/year to 72 bp/year in peripheral blood cells (Canela et al., 2007; Hastie et al., 1990; Slagboom et al., 1994; Vaziri et al., 1993), this rate has not been obtained in mice. Moreover, only one study has linked the increase in the percentage of short telomeres with age in humans, which was calculated as a 0.6% increase per year (Canela et al., 2007).

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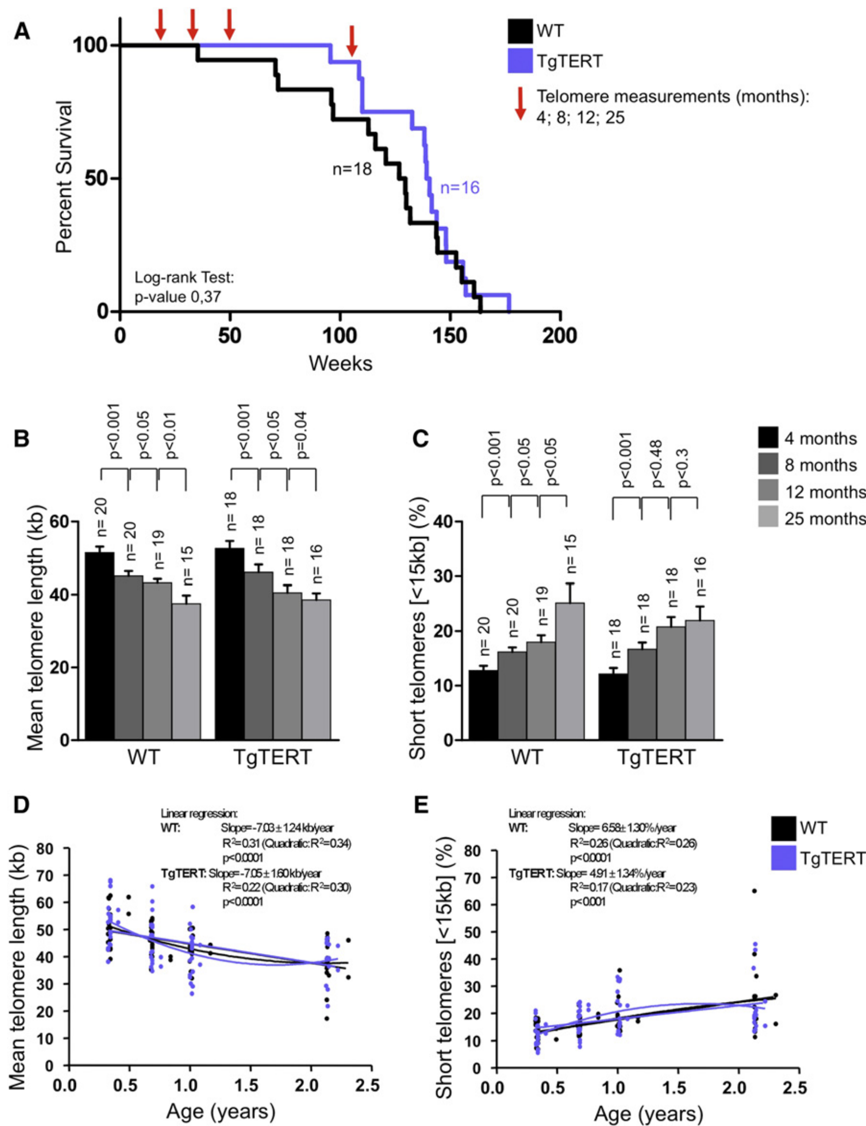

Figure 1. Mouse Telomeres Shorten 100-Fold Faster than Human Telomeres with Aging

(A) Kaplan-Meyer survival curves of the indicated cohorts. Arrows indicate time of blood extraction.

(B and C) The mean telomere length (B) and percentage of short telomeres (C) were determined by HT-QFISH on white blood cells (n, number of mice). Values are given as average ± SEM, and statistical significance was determined by one-tailed Student's t test. (B) From left to right (average ± SEM): 51.49 ± 1.633, 45.10 ± 1.322, 43.18 ± 1.118, 37.51 ± 2.241, 52.61 ± 2.049, 46.06 ± 2.142, 40.54 ± 2.053, 38.55 ± 1.801. (C) From left to right (average ± SEM): 12.78 ± 0.8693, 16.19 ± 0.8230, 17.99 ± 1.257, 25.14 ± 3.552, 12.16 ± 1.078, 16.68 ± 1.237, 20.77 ± 1.798, 21.93 ± 2.581.

(D and E) Adjustment of mean telomere length (D) and percentage of short telomeres (E) with aging to a linear or quadratic model. Second-order polynomial adjustment (quadratic) was used for the nonlinear fit model.

Here, we reasoned that the rate of increase in the percentage of short telomeres and the rate of telomere shortening throughout the lifespan of individuals might explain the different longevities of wild-type (WT) mice and humans, as well as the interindividual variations in longevity in mammals.

RESULTS AND DISCUSSION

With the aim of determining the rate of telomere shortening and the rate of increase in the percentage of short telomeres with aging in mice, we performed longitudinal telomere length determinations in the blood of individual mice by using an automated high-throughput quantitative fluorescence in situ hybridization (HT-QFISH) platform (Canela et al., 2007).

To correct for gender and genetic background, as well as to circumvent possible effects of reproduction on telomere length, we used only male mice of a 100% C57BL/6 background. In addition, the mice were individually housed at 3 months of age and fed a defined diet of 92.5 kcal per week (see Experimental Procedures). This caloric intake represents ~10% fewer calories than the average daily intake of C57BL/6 mice, and was used to avoid a possible impact of obesity on longevity. In parallel, we determined the same parameters in a transgenic mouse line overexpressing the mouse catalytic subunit of telomerase reverse transcriptase (TERT) in various epithelial tissues (hereafter termed transgenic TERT [TgTERT]; González-Suárez et al., 2001, 2002). This mouse line has a comparable 100% C57BL/6 background; however, it presents delayed signs of aging compared with WT mice (González-Suárez et al., 2005; this work), as well as a 40% increase in median longevity when combined with overexpression of tumor suppressor genes (Tomás-Loba et al., 2008). As shown in Figure 1A, the TgTERT mice used here show a delayed onset of the first deaths in the colony compared with the WT cohorts (see Figure 1A), in agreement with their increased healthspan. Thus, we set out to study whether any telomere-related parameter could predict longevity in these two independent mouse cohorts (TgTERT and WT) with slightly different longevities.

For cell analyses, we used peripheral blood leukocytes (PBLs), which are widely used for human telomere length studies because hematopoietic stems cells proliferate throughout life and thus reflect on cellular turnover associated with the aging process. Telomere length measurements in the blood have been shown to be representative of the general health status in humans (Canela et al., 2007; Cawthon et al., 2003; Collerton et al., 2007; Mainous et al., 2010; Valdes et al., 2005) and mice (Bernardes de Jesus et al., 2012), and provide an indicator of

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the general healthspan. In this regard, although the TERT transgenic mice used here do not target telomerase to blood cells, TERT expression in these mice has systemic beneficial effects that delay aging (Tomás-Loba et al., 2008), as also observed recently when a gene therapy strategy was used to overexpress TERT late in life (Bernardes de Jesus et al., 2012). We extracted 300 μl of blood from the facial vein of each mouse at 4, 8, 12, and 25 months of age (see Experimental Procedures) and the mice were left to age until the end of their lifespan, to obtain their complete longevity curve (Figure 1A). To avoid morbidity associated with this procedure in old mice, blood extractions were not performed after 25 months of age. Of note, we observed a delay in the age at onset of first death in the TgTERT cohort: the first mouse died at >100 weeks of age, compared with <50 weeks of age in the case of the WT cohort (Figure 1A). We determined both the average telomere length and the percentage of short telomeres at the indicated time points per individual mouse by using HT-QFISH (Canela et al., 2007). HT-QFISH is able to measure individual telomere signals at a single-cell level in interphasic nuclei. In this setting, each telomere signal corresponds to a clustering of few individual telomeres. The percentage used to represent short telomeres by HT-QFISH usually corresponds to the 10%–20% percentile in the reference population (in this case, we used 4-month-old WT mice as reference), which is 15 kb. We previously determined that telomere signals <15 kb are a good indicator of the presence of short telomeres in mouse blood cells and reflect differences in telomere length between different cohorts and treatments (Bernardes de Jesus et al., 2012). Therefore, here we set a cutoff of 15 kb to quantify the presence of short telomeres. In addition, we performed correlations with other cutoffs (<2, <5, and <10 kb), but we observed the strongest correlations at the 15 kb cutoff (not shown).

First, it is apparent that even though mouse telomeres are very long at young ages (>50 kb at 4 months of age), they shorten with age, as reflected by both a significant decrease in the mean telomere length and a significant increase in the percentage of short telomeres, detectable at 4 month intervals (Figures 1B and 1C). By adjusting the data to a linear regression model, we determined a rate of telomere shortening (as estimated by the slope of the regression line) of 7,000 bp per year (Figure 1D), which was comparable in both mouse cohorts and is 100 times faster than that obtained for human PBLs using the same technology (Canela et al., 2007). These results indicate that even though mouse telomeres are much longer than human telomeres at younger ages, they exhibit a much faster rate of shortening with age, highlighting the relevance of performing longitudinal telomere length studies rather than measuring telomeres at a single time point. The rate of increase in the percentage of short telomeres per year was 6.6% in WT mice and 4.9% in the TgTERT cohort (as estimated by the slope of the regression line; Figure 1E).

Next, we investigated whether the telomere length or the percentage of short telomeres at any of the studied timepoints is predictive of mouse longevity, as was recently reported for zebra finches (Heidinger et al., 2012). In the case of mice, however, we did not find a significant correlation between either the average telomere length or the percentage of short telomeres with longevity in any of the two mouse cohorts, with the exception of an inverse correlation between average telomere length and longevity at 12 months of age in the TgTERT mice (Figure S1).

Longitudinal studies allow one to examine telomere dynamics in a given individual over time. A given rate of telomere shortening or a rate of increase in the percentage of short telomeres is the result of a longitudinal trend in individual mice that may reflect very complex genetic traits associated with longevity, which cannot be inferred from a single time-point measurement. Therefore, we determined the rate of increase in the percentage of short telomeres per month and the rate of telomere shortening per month for every mouse under study. Strikingly, we found a significant correlation between the rate of increase in the percentage of short telomeres per month and longevity (Figure 2A), which could be observed in both mouse cohorts, and between the rate of telomere shortening per month and longevity (Figure 2B), which was only observed in WT mice. We also observed a significant correlation between the rates of increase in the percentage of short telomeres and mean telomere shortening (Figure 2C). Together, these results indicate that the rate of increase in the percentage of short telomeres is a reliable and robust predictor of mouse longevity in two different mouse strains with different TERT expression levels and different longevities. The mice with the higher rates of increase in the percentage of short telomeres were the ones that had shorter lives, while those with lower rates of increase in the percentage of short telomeres were the ones that enjoyed longer lives.

The fact that the TgTERT cohorts did not show a correlation between the rate of telomere shortening per month and longevity prompted us to address whether this was the consequence of TgTERT mice showing a delayed time of onset of the first death in the colony. In particular, to assess whether lifespan can influence the rate of telomere shortening or the rate of increase in the percentage of short telomeres, we plotted these two parameters at different time intervals of measurement (Figure S2). Indeed, age influenced the rate of mean telomere shortening per month in a discontinuous manner, so that we found higher rates of telomere shortening per month in young mice compared with older mice (Figure S2B). Of interest, this was not the case for the rate of increase in the percentage of short telomeres per month, which was quite constant at different age intervals in both mouse cohorts, with the exception of lower rates of increase in the percentage of short telomeres in old TgTERT mice (Figure S2A). The fact that the rate of telomere shortening per month was not constant with age in both mouse cohorts could account for the significant correlation between the rate of mean telomere shortening per month and longevity in the WT mice but not in the TgTERT mice (Figure 2B), because a significant fraction of WT mice (versus only one TgTERT mouse) died before measurement of the four time points. Indeed, even if we exclude the single TgTERT mouse that died before the fourth measurement, we observe a strong correlation between the rate of increase in the percentage of short telomeres and longevity (Figure S3). These findings indicate that the rate of increase in the percentage of short telomeres is a robust and reliable telomere marker for longevity studies.

It is of interest to note that differences in telomere length and longevity could not be attributed to differences in gender,

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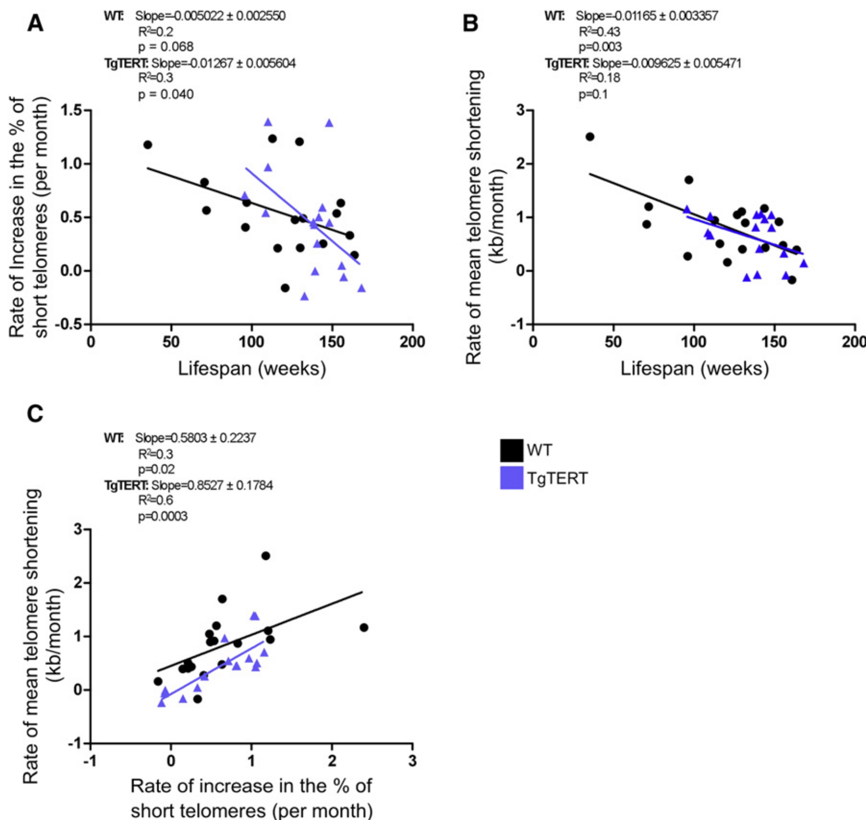

Figure 2. The Rate of Increase in the Percentage of Short Telomeres and the Rate of Telomere Shortening Predicts Mouse Longevity

(A–C) The percentage of short telomeres (<15 kb; A and C) and mean telomere length (B and C) were determined by HT-QFISH on white blood cells (see Figure 1). Linear regression lines were calculated for the values obtained for the rate of increase in the percentage of short telomeres and for the rate of mean telomere length shortening (kb/month) versus lifespan (weeks). (A) Rate of increase in the percentage of short telomeres (<15 kb) per month versus lifespan (weeks). (B) Rate of mean telomere length shortening (kb/month) versus the rate of increase in the percentage of short telomeres (per month). (C) Rate of mean telomere length shortening (kb/month) versus the rate of increase in the percentage of short telomeres (<15 kb) per month. The slope of each regression line is indicated.

See also Figures S1, S2, S3, S4, and S5.

reproduction, genetic background, or environmental factors, as only male mice of the C57BL/6 genetic background housed at our specific pathogen-free (SPF) facility were studied. In this regard, we did not find a correlation between the rate of increase in the percentage of short telomeres or rates of telomere shortening and weight gain with age (Figure S4), which together with the fact that the mice were fed a defined diet rules out a possible dietary contribution. These results suggest that differences in the rate of increase in the percentage of short telomeres or in the rate of telomere shortening, which have been shown to vary among individuals of the same age in different species (Canela et al., 2007; Heidinger et al., 2012; Monaghan, 2010), may be the result of small differences in modifier genes, or environmental factors, and offer an experimental platform to uncover new factors for modulation of aging and longevity.

Finally, we sought to determine whether variations in the rate of increase in the percentage of short telomeres had an impact on pathologies found at the time of death. To that end, we performed a full histopathological analysis of mice under study at their endpoint. The more prevalent pathologies found in C57BL/6 mice at their time of death are cancer (in ~50%–80% of mice) and age-associated pathologies (~50%–20%) (González-Suárez et al., 2002; Herranz et al., 2010; Tomás-Loba et al., 2008). We first determined whether the incidence of malignant cancers (lymphoma, sarcoma, and adenocarcinoma) showed any differential prevalence in mice belonging to either the higher or lower quartile of rate of increase in the percentage of short telomeres. We found a similar incidence of cancer or degenerative diseases in both cases, in spite of the fact that mice belonging to the higher quartile of rate of increase in the percentage of short telomeres showed a median age at death of 105 weeks, compared with 140 weeks in the case of mice belonging to the lower quartile (Figure S5). We observed the same tendency in the TgTERT cohort, and mice belonging to the higher quartile of rate of increase in the percentage of short telomeres showed a median age at death of 116 weeks, compared with 149 weeks in the case of mice belonging to the lower quartile.

Collectively, these findings demonstrate that the rate of increase in the percentage of short telomeres during an individual's lifetime, rather than the rate of telomere shortening over time, determines longevity in mice. These results bring into question the prevailing viewpoint that telomere shortening does not influence replicative aging in WT mice (Gomes et al., 2011; Wright and Shay, 2000), and highlight the importance of performing longitudinal telomere studies to predict traits as complex as longevity.

EXPERIMENTAL PROCEDURES

Mice

Male mice of a 100% C57BL/6 background were produced and stored at the SPF barrier area of the Spanish National Cancer Center, in accordance with the recommendations of the Federation of European Laboratory Animal Science Associations.

In this study we used a total of 38 mice (20 WT and 18 TgTERT; Figure 1B). Two mice from each cohort were sacrificed after the last telomere length measurement point for experimentation, and were excluded from the survival curves and other correlations involving final lifespan.

Experimental Setup

After weaning, five mice were housed per cage and fed a nonpurified diet (No. 2018; Harlan) ad libitum. For the aging study, 3-month-old mice were individually housed and were fed 92.5 kcal per week of chemically defined control

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diet (AIN-93M, diet No. F05312; Bio-Serv). This caloric intake was ~10% fewer calories than the average daily intake of C57BL/6 mice, to ensure that all food was consumed. Defined diets were cold-packed into 1 g pellets. The mice were fed two-sevenths of the weekly allotment of food on Monday and Wednesday, and three-sevenths of that amount of Friday (Dhahbi et al., 2004; Pugh et al., 1999).

Mice were inspected on a daily basis and sacrificed when they presented signs of illness or tumors, in accordance with the Guidelines for Humane Endpoints for Animals Used in Biomedical Research (Harrison et al., 2009). The date of euthanasia was considered as an estimation of the natural lifespan. The mice were subjected to necropsy and histopathological analysis.

Histological Analysis

Histopathology was performed as previously described (González-Suárez et al., 2001). Briefly, tissues and organs were fixed for 24 hr in a 10% neutral buffered formalin solution at room temperature, dehydrated through graded alcohols and xylene, and embedded in paraffin. Histological analysis was achieved on 4–5 μm sections according to standard procedures. Cancer-related pathologies and senile lesions were grouped as described previously (Tomás-Loba et al., 2008).

HT-QFISH on PBLs

Blood was extracted from the facial vein at the indicated time points. Upon erythrocyte lysis (buffer EL; Qiagen), PBLs were plated on a clear-bottom, black-walled, 96-well plate, and HT-QFISH was performed as previously described (Canela et al., 2007). The accuracy and sensitivity of HT-QFISH are similar to those of telomere QFISH on metaphasic chromosomes (Canela et al., 2007). We analyzed telomere length values using individual telomere spots (>5,000 per sample) corresponding to the specific binding of a Cy3-labeled telomeric probe. Fluorescence intensities were converted into kilobases as described previously (Canela et al., 2007; McIlrath et al., 2001).

Statistical Analysis

A log-rank test was used to calculate statistical differences in the survival curves. Student's t-test was used to calculate the statistical significance of the mean telomere and short telomere variation over time. To calculate the rate of increase in the percentage of short telomeres and the rate of mean telomere shortening, linear regression lines were calculated for each mouse with GraphPad Prism, representing the best-fit values of the four values calculated at the different time points (either percentage of short telomeres or mean telomere length). For mice that died before the acquisition of all time points, linear regression lines were determined from the available measurements. The p value was calculated by dividing the slope by its standard error, and represents the correlation between the axes (slope significantly different from zero). Pathological analysis was calculated with the χ^2 test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.08.023.

LICENSING INFORMATION

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