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Short Telomeres, even in the Presence of Telomerase, Limit Tissue Renewal Capacity

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SUMMARY

Autosomal-dominant dyskeratosis congenita is associated with heterozygous mutations in telomerase. To examine the dosage effect of telomerase, we generated a line of $mTR^{+/-}$ mice on the CAST/EiJ background, which has short telomeres. Interbreeding of heterozygotes resulted in progressive telomere shortening, indicating that limiting telomerase compromises telomere maintenance. In later-generation heterozygotes, we observed a decrease in tissue renewal capacity in the bone marrow, intestines, and testes that resembled defects seen in dyskeratosis congenita patients. The progressive worsening of disease with decreasing telomere length suggests that short telomeres, not telomerase level, cause stem cell failure. Further, wild-type mice derived from the late-generation heterozygous parents, termed wt*, also had short telomeres and displayed a germ cell defect, indicating that telomere length determines these phenotypes. We propose that short telomeres in mice that have normal telomerase levels can cause an occult form of genetic disease.

INTRODUCTION

Autosomal-dominant dyskeratosis congenita is a disease of stem cell failure due to telomere shortening. Affected individuals exhibit symptoms that appear to be due to limited cellular reserves in tissues of high turnover. The primary cause of death is due to complications of aplastic anemia or loss of bone marrow stem cells (Dokal, 2000). Additionally, patients develop abnormal skin pigmentation, premature graying, enteropathy, hypogonadism, and an increased cancer predisposition. Families with autosomal-dominant dyskeratosis congenita have mutations in telomerase components (Vulliamy et al., 2001; Armanios et al., 2005) and display anticipation, an earlier onset, and increasing severity of phenotypes with each generation (Dokal, 2000; Vulliamy et al., 2004). Telomere length is normally maintained by the enzyme telomerase. In the absence of telomerase, telomeres shorten progressively due to the end replication problem and telomere end processing (Reviewed in Greider, 1996; Lingner and Cech, 1998). Telomerase adds a simple tandem repeated sequence onto chromosome ends to balance this shortening (Greider and Blackburn, 1985). The telomerase core enzyme consists of two essential components: telomerase RNA, TR, which contains the template for repeat addition, and the catalytic component telomerase reverse transcriptase or *TERT* (Lingner et al., 1997). In addition to families with dyskeratosis congenita, mutations in both *hTR* and *hTERT* have been described in familial and apparently sporadic cases of aplastic anemia where only hematologic phenotypes have been described (Vulliamy et al., 2002; Yamaguchi et al., 2003, 2005). Since these phenotypes likely represent a milder spectrum of dyskeratosis congenita, we will refer to individuals with these phenotypes as having dyskeratosis congenita. The loss of stem cells in these individuals with mutations in telomerase components has implicated telomere length as a determinant of stem cell maintenance (Dokal, 2000).

In the absence of telomerase, mice show progressive telomere shortening with increased generations of interbreeding (Blasco et al., 1997). In the first generation of telomerase deficiency, $mTR^{-/-}$ G1 mice on the C57BL/6J genetic background show no overt phenotypes. However with further interbreeding, a series of phenotypes appear and worsen as telomere shortening continues. This increased severity of disease resembles the anticipation seen in autosomal-dominant dyskeratosis congenita (Vulliamy et al., 2004).

Short telomeres in mice that lack telomerase cause apoptosis. This apoptosis is due to short dysfunctional telomeres triggering a DNA-damage checkpoint response (Hemann et al., 2001a; d'Adda di Fagagna et al., 2003; IJpma and Greider, 2003; Hao et al., 2004). In mice, the apoptosis leads to loss of germ cells and consequently decreased fertility with each generation (Lee et al., 1998; Hemann et al., 2001b).

Evidence from both human and mouse suggests that haploinsufficiency for the telomerase RNA may cause telomere shortening. A telomerase RNA truncation in an autosomal-dominant dyskeratosis congenita family results in half of the level of *hTR* and causes telomere shortening (Vulliamy et al., 2001). Families with autosomal-dominant dyskeratosis congenita show progressive telomere shortening with each generation and a more severe and earlier onset of disease (Vulliamy et al., 2004). Further, point mutations in *hTR* found in dyskeratosis congenita and aplastic anemia patients show reduced telomerase activity in vitro (Marrone et al., 2004; Cerone et al., 2005; Ly et al., 2005). Similarly, haploinsufficiency is also observed when the protein component of telomerase, *hTERT*, is deficient (Armanios et al., 2005; Yamaguchi et al., 2005). In mice that are heterozygous for *mTR* there is reduced ability to elongate short telomeres in interspecies crosses between strains with short telomeres and those with long telomeres (Hathcock et al., 2002). Finally the *mTERT* knockout mouse shows progressive telomere shortening and an increased severity of phenotypes as seen in the $mTR^{-/-}$ mice (Erdmann et al., 2004). These data argue that the level of telomerase activity, altered by either perturbation in *TR* or *TERT*, affects the maintenance of telomere length in vivo.

Telomere lengths show a wide variation in the human population (Harley et al., 1990; Cawthon et al., 2003; Valdes et al., 2005; Yamaguchi et al., 2005). Individual variation in telomerase activity may account for part of this variability. Such variability, even in cells that express telomerase, may limit tissue renewal in normal individuals. For example, although mature B and T cells, as well as $CD34^+$ cells, express telomerase (Morrison et al., 1996), telomere shortening occurs in white blood cells with increasing age (Wynn et al., 1998; Rufer et al., 1999; Hodes et al., 2002). This suggests that the level of telomerase expressed in hematopoietic progenitor cells is not sufficient to maintain telomere length. Recent evidence also suggests an association between telomere shortening and the risk of death due to age-related diseases such as heart disease and infection (Cawthon et al., 2003; Valdes et al., 2005).

To examine directly whether telomerase dosage affects telomere length and disease phenotypes, we created mice heterozygous for a null allele of telomerase RNA in a strain that already has short telomeres. The telomere length distribution in this CAST/EiJ strain is short and more homogeneous similar to human telomere lengths (Hemann and Greider, 2000). Successive breeding of CAST/EiJ $mTR^{+/-}$ heterozygous mice resulted in progressive telomere shortening that was seen as organ failure in tissues of high turnover similar to dyskeratosis congenita patients. Further, we find that the wild-type littermates, wt*, derived from later-generation heterozygotes that inherit short telomeres also displayed phenotypes associated with telomere dysfunction. We consider this phenomenon a form of occult genetic disease. We refer to these mice as wt* since the genotype at the responsible locus, *mTR*, is wild-type in the affected individuals who inherited short telomeres from $mTR^{+/-}$ heterozygous parents. The presence of disease in wt* animals indicates that loss of telomere function can occur in the presence of normal levels of telomerase.

RESULTS

Progressive Telomere Shortening of CAST/EiJ $mTR^{-/-}$ and $mTR^{+/-}$ Animals

To critically examine whether haploinsufficiency for telomerase causes telomere dysfunction, we crossed the *mTR* null allele onto the CAST/EiJ genetic background that has a short homogeneous telomere length distribution (see Experimental Procedures). After establishing a line of CAST/EiJ $mTR^{+/-}$ mice, these heterozygotes were intercrossed to obtain $mTR^{+/+}$, $mTR^{+/-}$, and $mTR^{-/-}$ animals. The heterozygous mice from this initial CAST/EiJ cross, designated $mTR^{+/-}$ HG1, were then intercrossed to generate $mTR^{+/-}$ HG2 animals, which were further intercrossed to generate $mTR^{+/-}$ HG3, HG4, HG5, and HG6 mice (Figure 1A). We have previously shown that $mTR^{+/-}$ mice express half of the steady-state level of telomerase RNA (Hathcock et al., 2002). To investigate whether haploinsufficiency for telomerase RNA leads to telomere shortening, we examined telomere length by quantitative fluorescence in situ hybridization (Q-FISH). CAST/EiJ $mTR^{-/-}$ mice had significantly shorter telomeres than wild-type. Furthermore, successive generations of CAST/EiJ $mTR^{+/-}$ heterozygous mice showed progressive telomere shortening (Figure 1B). The fact that the telomeres in the heterozygotes were intermediate between wild-type and $mTR^{-/-}$ mice indicates that telomerase is present and elongates some telomeres but the level is not sufficient to maintain telomeres at wild-type lengths. The decrease in the mean telomere length was associated with an increase in signal free ends (SFE) (Figure 1C). These signal free ends represent dysfunctional telomeres and correlate with cell death and chromosome rearrangements (Hemann et al., 2001b). The progressive telomere shortening with later generations of $mTR^{+/-}$ mice is similar to the phenomenon of genetic anticipation that is seen in autosomal-dominant dyskeratosis congenita (Vulliamy et al., 2004; Armanios et al., 2005). This telomere shortening and the increase in signal free ends further confirms that the CAST/EiJ $mTR^{+/-}$ mice are haploinsufficient for telomere-length maintenance.

CAST/EiJ $mTR^{-/-}$ and CAST/EiJ $mTR^{+/-}$ Mice Show Tissue Renewal Defects

The CAST/EiJ $mTR^{-/-}$ mice showed a dramatic decrease in survival compared to wild-type animals (Figure 2A). Such a reduction in survival was not seen in C57BL/6J $mTR^{-/-}$ mice (Rudolph et al., 1999). Pathology examination revealed intestinal lesions, which included severe depletion of the intestinal epithelial crypts and villus atrophy/loss in the small

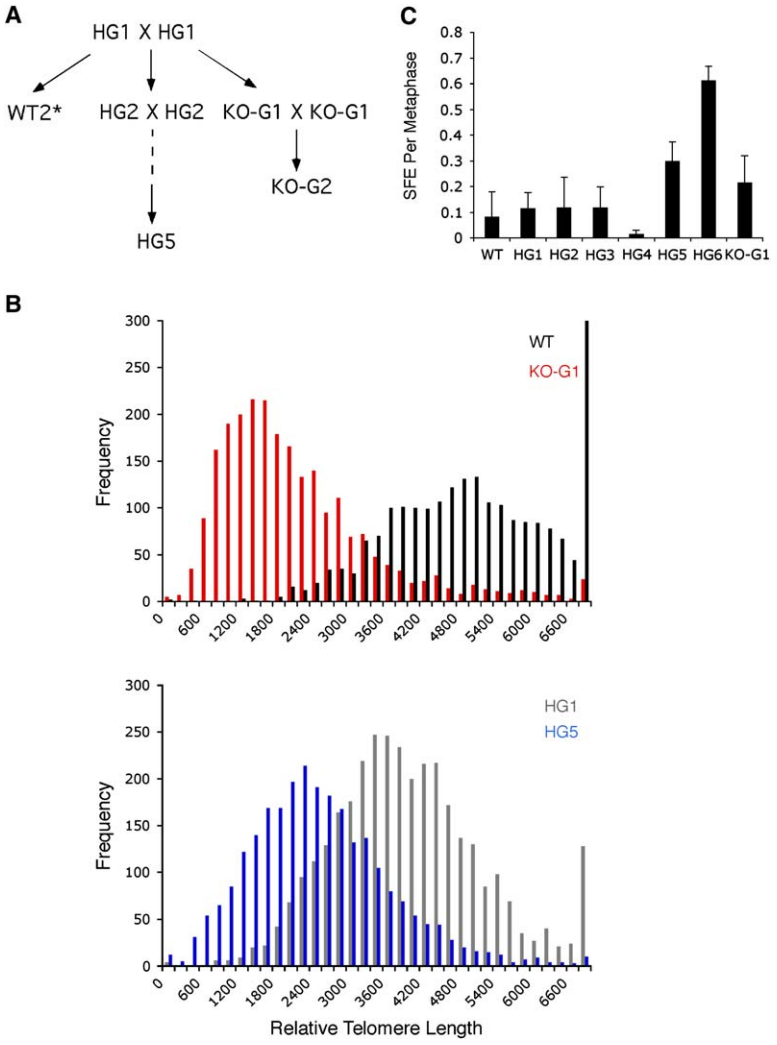


Figure 1. Breeding Scheme and Telomere-Length Analysis

(A) Heterozygous generation 1 $mTR^{+/-}$ (HG1) animals were interbred to generate HG2, wt2*, and $mTR^{-/-}$ KO-G1. KO-G1 animals were intercrossed to generate KO-G2 offspring. Heterozygous animals of the same generation were interbred for five generations.

(B) Top panel: Q-FISH telomere-length analysis of wild-type (black, n = 3) and KO-G1 (red, n = 3) animals. The bottom panel shows telomere length of HG1 (gray, n = 4) and HG5 (blue, n = 4) animals. The histogram is truncated at 6600 fluorescence units for clarity; the number of telomeres above this value is plotted at the end on the far right. A Two-sample Wilcoxon rank-sum test showed KO-G1 telomeres are significantly shorter than wild-type ($p < 0.0001$) and HG1 telomeres are significantly shorter than HG5 telomeres ($p < 0.0001$).

(C) Histogram of the average number of signal free ends (SFE) per metaphase from different animals. Error bars are mean ± SEM. More than seven metaphases were analyzed for each animal and results from multiple animals were pooled for each genotype shown (n = 3 for wild-type, n = 4 for HG1, n = 2 for HG2, n = 3 for HG3, n = 5 for HG4, n = 2 for HG6, and n = 6 for KO-G1). The Q-FISH analysis was scored in a blinded fashion.

intestine. Histological sections of the large and small intestines in $mTR^{-/-}$ G1 and G2 animals showed multifocal to coalescing areas of crypt depletion. Villus atrophy was present in the small intestine in 7 of 7 $mTR^{-/-}$ mice (Figure 2C). The atrophy occupied 19% of the small intestine (range 9%–27%). In 4 of 7 $mTR^{-/-}$ mice (57%), we also saw villus atrophy in the colon occupying 6% (range 5%–12%) (Figure 2F). Microadenomas were present concurrently with the atrophic lesion in all the mice examined (7 of 7). Villous atrophy and microadenomas were not seen in wild-type mice (n = 9). This degree of crypt depletion and small intestinal villus atrophy was significantly greater than seen in C57BL/6J $mTR^{-/-}$ G5 mice (Herrera et al., 1999; Rudolph et al., 1999). The microadenomas of the intestinal epithelium are reminiscent of the premalignant lesions of the oral epithelium, known as leukoplakia, that occur in dyskeratosis congenita patients and may represent crypt dropout and compensatory proliferative lesions initiated by short telomeres. Finally, The CAST/EiJ $mTR^{-/-}$ mice also showed hypocellular seminiferous tubules and a consequent decrease in testes weight due to germ cell apoptosis as seen in C57BL/6J $mTR^{-/-}$ mice (Hemann et al., 2001a). The reduced viability together with the reduction in testes weight and increased aberrant seminiferous tubules resulted in small litter sizes and the inability to generate $mTR^{-/-}$ G3 mice.

The telomere shortening was substantial in late-generation CAST/EiJ $mTR^{+/-}$ heterozygous mice, and there were a large number of signal free ends. To examine whether the short telomeres in the heterozygotes were dysfunctional, we examined testes sections from these mice. We found germ cell hypoplasia/degeneration with empty seminiferous tubules in the HG3 and HG4 mice (Figure 2D). Further significant crypt depletion and hyperplasia accompanied by villus atrophy/loss were also seen in an HG5 mouse that showed similar signs of illness to the $mTR^{-/-}$ mice (Figure 2E) and had significant telomere shortening (data not shown), suggesting that the short telomeres also cause disease in these animals. These phenotypic manifestations of telomere dysfunction in heterozygotes provide genetic evidence for haploinsufficiency. Furthermore, the correlation of phenotypes with short telomeres indicates that it is telomere length, not a reduction in telomerase activity, that causes disease.

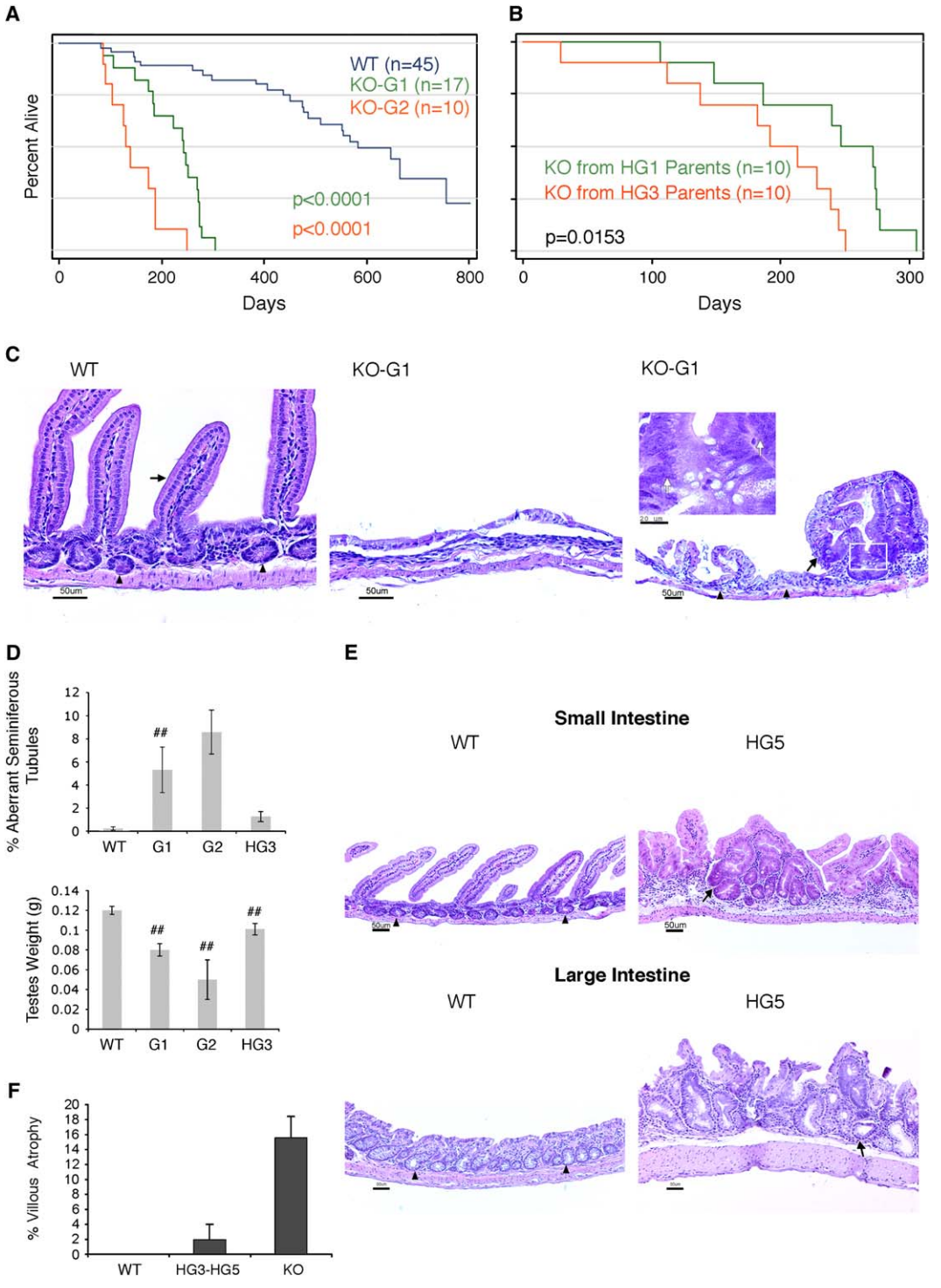


Figure 2. Phenotype Analysis of *mTR*$^{-/-}$ and *mTR*$^{+/-}$ Animals

(A) Kaplan-Meier survival curve of the KO-G1, KO-G2, and wild-type animals shows a statistically significant decrease in survival in the null animals compared to wild-type ($p < 0.0001$). The median survival for wild-type was 627 days, for KO-G1 it was 242 days, and for the KO-G2 it was 129 days.

(B) Kaplan-Meier survival curve of 10 *mTR*$^{-/-}$ KO-G1 animals that were derived from HG1 cross (green) and 10 *mTR*$^{-/-}$ KO-G1 animals that were derived from HG3 cross (orange) shows significantly different survival ($p = 0.0153$). The median survival for KO-G1 from HG1 crosses was 247 days and for the KO-G1 animals from HG3 crosses it was 192 days.

(C) H&E section of small intestine. Normal crypts (arrowheads) and villi (arrow) in the wild-type control in the left panel. The middle panel shows an area of severe crypt depletion and villus atrophy. The right panel shows a microadenoma (arrow) with an adjacent area of small intestine with crypt depletion and

The Reduced Survival of CAST/EiJ *mTR*$^{-/-}$ Mice Shows Anticipation

Autosomal-dominant dyskeratosis congenita shows genetic anticipation, presumably due to the inheritance of short telomeres that get shorter with each generation (Vulliamy et al., 2004). This anticipation implies that CAST/EiJ *mTR*$^{-/-}$ null animals that are derived from late-generation heterozygotes may have more severe phenotypes than those from early generations. To examine this, we looked at the survival of CAST/EiJ *mTR*$^{-/-}$ mice that came from crosses of either HG1 or HG3 parents (Figure 2B). We found a significant reduction in the life span of *mTR*$^{-/-}$ animals whose parents were HG3 compared to the *mTR*$^{-/-}$ animals whose parents were HG1. Thus, the inheritance of short telomeres from heterozygous parents contributes to the worsening of phenotypes in the offspring, which leads to premature death. This is the hallmark of genetic anticipation.

Hematopoietic Dysfunction in CAST/EiJ *mTR*$^{+/-}$ Mice Correlates with Short Telomeres

Autosomal-dominant dyskeratosis congenita patients have impaired hematopoietic function that often leads to death due to bone marrow failure. Histological examination of the bone marrow in the *mTR*$^{-/-}$ G1 and G2 mice showed normal cellularity and morphology but with a skewed myeloid to erythroid ratio of 5:1 (normal 2.5:1; Sanderson and Phillips, 1981). There was also extensive extramedullary hematopoiesis in the liver, spleen, and kidney (data not shown) in the *mTR*$^{-/-}$ animals, indicating that the mice had ineffective bone marrow hematopoiesis. To further evaluate bone marrow function in *mTR*$^{-/-}$ and *mTR*$^{+/-}$ mice, we examined peripheral blood through complete blood counts (CBC). There was a reduction in peripheral white blood cell, platelet, and red blood cell numbers in each successive generation of heterozygous mice, and these counts were significantly below the lower normal limits in *mTR*$^{+/-}$ HG4 (Figure 3B).

Telomere-length heterogeneity leads to significant mouse-to-mouse variation in mean telomere length (Herrera et al., 1999; Hemann et al., 2001b). To more directly examine the role of telomere shortening in the reduced blood counts, we examined telomere length by Q-FISH in individual mice that were used for the peripheral blood counts described above. Short telomeres were tightly correlated with reduced white cell, platelet, and red cell counts (Figure 3A). In fact, the correlation of reduced CBC with telomere length was better than the correlation with increasing heterozygous generation (Figure 3A). Thus it is the short telomeres, not the absence, or lower levels of telomerase that cause the reduction in hematopoietic function.

CAST/EiJ Heterozygotes with Short Telomeres Are Sensitive to Chemotherapy

A decrease in marrow reserves can manifest as an increased sensitivity to cytotoxic agents. We thus examined the response to the chemotherapeutic agent 5-fluorouracil (5-FU). 5-FU is cytotoxic to cells in S phase, and efficient recovery from treatment reflects the number and function of progenitor cells in the affected tissues. Total white blood cell counts were measured in wild-type, *mTR*$^{+/-}$ HG5, and, *mTR*$^{-/-}$ G1 mice before injection with 5-FU. White blood cell counts were then monitored on day 6 and day 13 after injection (see Experimental Procedures). All mice showed a significant reduction in white counts 6 days after injection, as expected. However, *mTR*$^{+/-}$ HG5 mice had lower white counts on day 13 than the wild-type mice (Figure 3B). *mTR*$^{-/-}$ animals had the lowest white counts on day 6 and day 13 as compared to heterozygous and wild-type animals. This indicates that the capacity to sustain and recover from the stress of 5-FU treatment is compromised by limited bone marrow reserves. This general decrease in progenitor reserves due to short telomeres may reflect the sensitivity seen in dyskeratosis congenita patients to chemotherapy and to bone marrow transplant preparative regimens (Yabe et al., 1997; Rocha et al., 1998).

Bone Marrow from Mice with Short Telomeres Is Inefficient at Hematopoietic Reconstitution

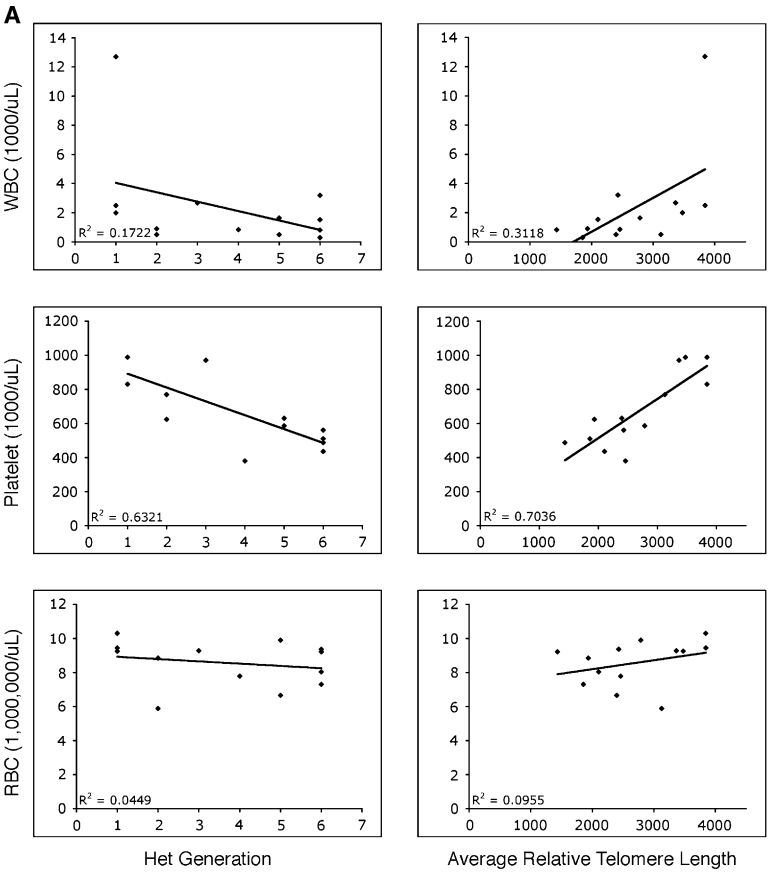
To examine the function of the hematopoietic precursor pool directly, we used a hematopoietic reconstitution assay (Morrison et al., 1995) (Figure 4). Donor bone marrow, isolated from CAST/EiJ wild-type, *mTR*$^{-/-}$ G1, and *mTR*$^{+/-}$ HG5 mice, was injected into lethally irradiated CAST/EiJ wild-type mice (see Experimental Procedures). Irradiated mice that received no donor cells died within 20 days after irradiation. All mice reconstituted with marrow from wild-type donors lived for over 150 days. Mice reconstituted with marrow from CAST/EiJ *mTR*$^{-/-}$ G1 donors had a median survival of 53 days, and those reconstituted with CAST/EiJ *mTR*$^{+/-}$ HG5 donor marrow had a median survival of 64 days. This diminished capacity of both the *mTR*$^{-/-}$ and *mTR*$^{+/-}$ marrow to reconstitute effectively the hematopoietic system of irradiated mice indicates a functional reduction in the hematopoietic cell precursor pool.

villus atrophy/loss (arrowheads). The microadenoma had areas with mild dysplasia of intestinal epithelial cells (inset, arrows). All animals were age matched. The slides were read in a blinded fashion.

(D) Decreased testes weight and increased aberrant testicular tubules found in the KO and heterozygous animals. Top panel shows the percent aberrant tubules found in each genotype (n = 5 for wild-type, 7 for G1, 2 for G2, and 8 for HG3). Bottom panel shows the testes weight for each animal (n = 9 for wild-type, 4 for G1, 2 for G2, and 9 for HG3). Error bars are mean ± SEM. ## indicates p value of less than 0.05 using Student's t test.

(E) GI tract defect in *mTR*$^{+/-}$ HG5 animal observed in the small intestine (top panels) and large intestine (bottom panels). The wild-type controls are shown to the left. The top right shows crypt proliferation (arrow) with adjacent areas of crypt depletion in the small intestine. The bottom left panel shows normal colon (left) with colonic crypts (arrowheads) and flat intercrypt table surface epithelium. The bottom right image shows an irregular intercrypt table epithelial surface and hyperplasia of colonic crypts (arrow) in the HG5 mouse. Scale bars represent 50 μm.

(F) Quantitation of the percent of villous atrophy from large and small intestines from wild-type (n = 9), HG3-HG5 (n = 10), and KO-G1 (n = 7) animals from H&E sections shows more severe atrophy in the heterozygous as well as the KO-G1 animals compared to wild-type controls.



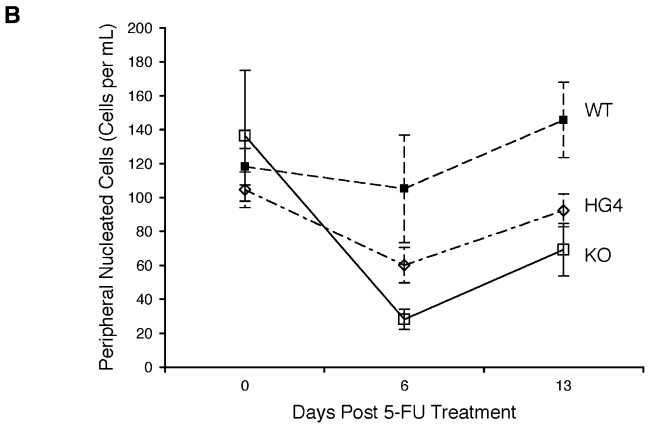


Figure 3. Hematological Defects

(A) White blood cell, platelet, and red blood cell counts were analyzed in HG1, HG2, HG3, HG4, HG5, and HG6 mice. A total of 13 animals were analyzed. The counts in each mouse are shown as a dot and the decrease with each generation is shown as a regression line (left panels). The p value for white blood cells, platelet, and red blood cells are 0.1585, 0.0012, and 0.487, respectively. The panels on the right show the blood cell counts plotted relative to the mean telomere length of the individual animal. Due to animal-to-animal variation there is a better correlation of short telomeres with reduced blood counts than the correlation with heterozygous generation. The p value for white blood cells, platelet, and red blood cells are 0.0473, 0.0003, and 0.3041, respectively.

(B) The peripheral white blood cell count from wild-type (wt) and HG4 and $mTR^{-/-}$ G1 (KO) were analyzed before 5-FU injection and 6 and 13 days after injection. Error bars are mean ± SEM.

wt* Animals Derived from $mTR^{+/-}$ Parents Have Short Telomeres and Occult Disease

The genetic anticipation and progressive telomere shortening in the CAST/EiJ $mTR^{+/-}$ mice suggested that wild-type mice might also inherit short telomeres. To examine this directly, we measured by Q-FISH telomere length from all genotypes derived from intercrossing CAST/EiJ $mTR^{+/-}$ HG4 mice. Surprisingly, we found that the telomere length of the wild-type pups was shorter than from wild-type animals whose ancestors had never been deficient for telomerase. This suggests that one generation in the presence of wild-type levels of telomerase is not sufficient to restore telomeres to wild-type length. Since these mice were not identical to wild-type, we designated these mice as wt*. Wt* mice that were generated from CAST/EiJ $mTR^{+/-}$ HG4 parents were designated wt5* to correspond with their $mTR^{+/-}$ HG5 littermates and to indicate the number of generations the parents were heterozygous. Correspondingly, wild-type mice from HG3 parents were designated wt4* (Figure 5A). We examined three independent mouse families from HG3 and HG4 parents to compare telomere lengths in the littermate pups of different genotypes. In each of the families studied, the telomeres in the wt* mice were shorter than those of the true wild-type CAST/EiJ animals obtained from The Jackson

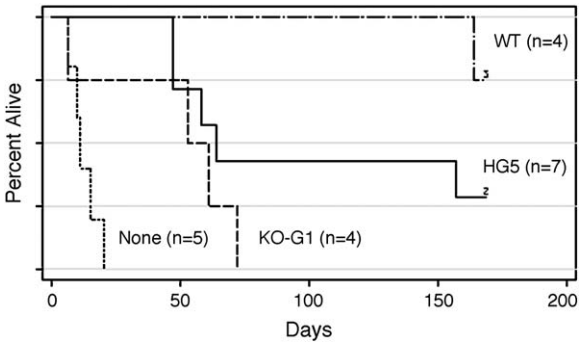


Figure 4. Bone Marrow Transplantation

CAST/EiJ recipient mice were irradiated and injected with marrow from HG5, KO-G1, and wild-type animals. Wild-type bone marrow fully rescued viability in recipients for more than 100 days (n = 4) whereas the knockout donor cells was deficient in rescuing viability (n = 4, p = 0.0067 when compared to wild-type using Log-rank test). The heterozygous donor cells partially rescued (n = 7, p = 0.1041 when compared to wild-type using Log-rank test). The unreconstituted control animals (labeled as None) died within 3 weeks after irradiation (n = 5).

Laboratory (Figure 5B). Telomere lengths of the heterozygous littermates were shorter still, and finally the null animals showed the shortest telomeres. Thus the level of telomerase present had some effect on telomere length, indicating that telomerase is limiting, but having a full complement of telomerase for one generation was not sufficient to restore telomeres to the proper length.

The fact that the wt5* had short telomeres suggested that there may be telomere dysfunction in these animals. To test this directly, we examined testes weight and found a significant reduction in the wt4* mice compared to wild-type mice (Figure 5D). Histological examination showed evidence of germ cell hypoplasia/degeneration with empty seminiferous tubules in the wt4* mice (Figure 5C). This loss of germ cells in the wt4* mice indicates that short telomeres, which were inherited from the heterozygous parents, can exhibit telomere dysfunction even in the presence of wild-type levels of telomerase. Further, it indicates that genetic disease can be present in animals that are genetically wild-type. Thus, we refer to this surprising result as hidden, or occult, genetic disease.

DISCUSSION

Patients with autosomal-dominant dyskeratosis congenita and aplastic anemia, caused by heterozygous mutations in telomerase components, exhibit bone marrow failure due to the loss of hematopoietic stem cells. In a formal, genetic sense, haploinsufficiency for telomerase causes the disease. However, the CAST/EiJ $mTR^{+/-}$ mouse model described here shows that although the ability of telomerase to maintain telomere length depends on telomerase dosage, it is not the amount of telomerase but rather the short telomeres themselves that cause stem cell failure. The fact that telomere length is the prime determinant of phenotype is demonstrated by the fact that short telomeres can cause phenotypes even in wild-type animals. The ability of short telomeres to limit cellular renewal has implications for patients with dyskeratosis congenita. In addition, the fact that short telomere can cause phenotypes even in wild-type animals has implications for normal individuals who inherit short telomeres; it suggests that short telomeres can contribute to stem cell loss and can limit tissue renewal capacity.

Possible Mechanisms for Loss of Function at Short Telomeres

There are a number of mechanisms that have been proposed for how telomeres become dysfunctional in cells that lack telomerase. Two models suggest that the telomerase enzyme may function directly in telomere end protection. First the ability of yeast telomerase to remain bound to telomeric primers in vitro led to the proposal that telomerase binds to and protects telomeres (Prescott and Blackburn, 1997; Blackburn, 2000). More recently it has been proposed that telomerase is required to synthesize the G-strand overhang at telomeres that is essential for telomere end protection. It has also been suggested that the loss of G-strand overhangs is responsible for the telomere-mediated senescence in human fibroblasts (Masutomi et al., 2003; Stewart et al., 2003). However, a G-strand overhang is still present in C57BL/6J $mTR^{-/-}$ G6 mice with very short telomeres (Hemann and Greider, 1999). Further, we show here that telomere function is lost on short telomeres even in the presence of telomerase in both the CAST/EiJ $mTR^{+/-}$ and the wt* mice. This indicates that that telomerase itself does not play a direct role in the protection of telomeres; short telomeres are dysfunctional even when normal telomerase activity is present.

Understanding that it is short telomeres, not the absence of telomerase, that causes telomere dysfunction limits the possible mechanisms that may cause the dysfunction. The simple loss of all telomeric repeats may initiate the damage response that leads to cell death or senescence. Alternatively, very short tracts of telomeric repeats may fail to bind the essential telomere binding proteins that protect telomeres and distinguish them from DNA breaks (Smogorzewska and de Lange, 2004). Finally, telomeres in many organisms form T loops in which the single-stranded G-strand 3′ tail is looped around and base paired in the double-stranded telomere sequence (Griffith et al., 1999). Very short telomeres are predicted not to form these T loops and may thus signal DNA damage by failing to form this protective end structure (de Lange, 2002).

Telomerase Dosage Affects the Maintenance of Telomere Length

The short telomeres and the genetic anticipation seen in autosomal-dominant dyskeratosis congenita families indicate that insufficient telomerase activity causes progressive telomere shortening. Autosomal-dominant dyskeratosis congenita can result from null alleles of *hTR* or *hTERT* that show loss of function in vitro, suggesting that a simple decreased dosage of functional telomerase causes telomere shortening (Armanios et al., 2005; Marrone et al., 2005). In our studies, the CAST/EiJ $mTR^{+/-}$ mice show progressive telomere

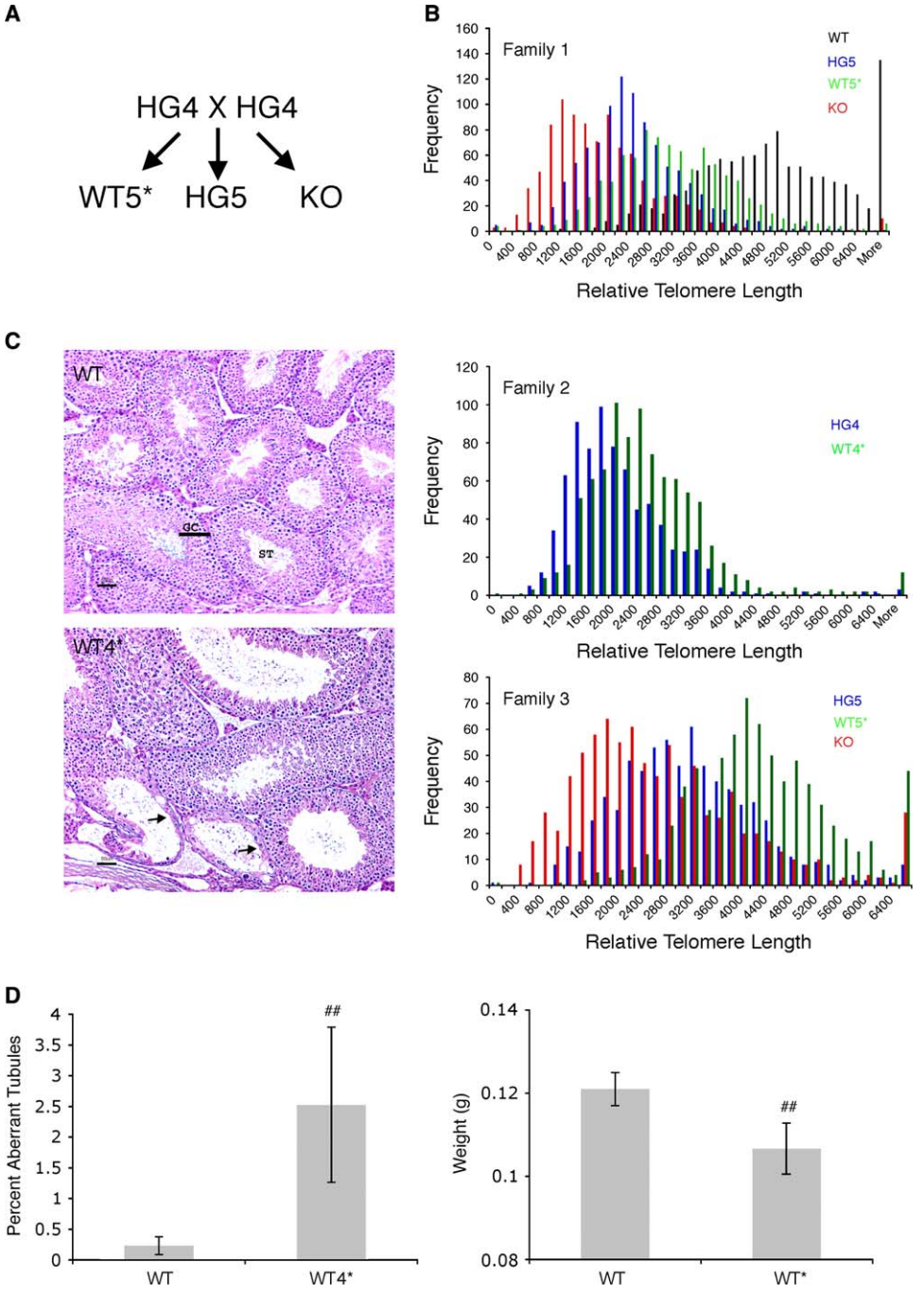


Figure 5. Occult Genetic Disease in wt*

(A) The breeding scheme to derive wt5* animals.

(B) Telomere-length analysis using Q-FISH in three mouse families. Top panel: wild-type telomere lengths are from animals obtained directly from The Jackson Laboratory. The HG5, wt5*, and KO are three littermates from a HG4 cross. Middle panel: the HG4 and wt4* littermates from a HG3 cross are shown. Bottom panel: the HG5, wt5*, and KO are three littermates from a HG4 cross.

(C) H&E section of control wt (upper) and wt4* (lower) testes showing aberrant tubules with degeneration/hypoplasia of germ cell epithelium (arrows). GC = germ cell epithelium; ST = seminiferous tubule.

(D) Left panel: the percent aberrant tubules seen in histological sections from wt* animals (n = 4) and wt (n = 5). Right panel: the testes weight of wt* (n = 9) and wt control animals (n = 6). Error bars are mean ± SEM. ## indicates a p value of less than 0.05 using Student's t test. The analysis was scored in a blinded fashion.

shortening with each generation of interbreeding. This demonstrates that half of the level of telomerase is not sufficient to maintain the telomere distribution at the wild-type equilibrium length. While the breeding scheme we used in crossing $mTR^{+/-}$ mice to each other simplifies the telomere-length analysis, in human families, only one parent may carry the *hTR*/*hTERT* hypomorphic or null allele. Thus the rate of telomere shortening across generations in autosomal-dominant dyskeratosis congenita families is expected to be slower. The level of the catalytic TERT protein also shows a dosage effect on telomere-length maintenance in mice (Erdmann et al., 2004). This inability of reduced levels of telomerase to maintain the telomere-length equilibrium is surprising. In vitro telomerase is very efficient, and in yeast in vivo elongation of only a few telomeres at each division is sufficient to maintain homeostasis of the telomere-length distribution (Teixeira et al., 2004). Further, the shortest telomeres in the length distribution are preferentially elongated by telomerase (Hemann et al., 2001b; Teixeira et al., 2004). This implies that, for disease to arise, the number of short dysfunctional telomeres must overwhelm the limiting amount of telomerase. This requirement for many short telomeres for disease to be manifest may explain why phenotypes were not seen in the C57BL/6J $mTERT^{+/-}$ mice despite telomere shortening (Erdmann et al., 2004). The very broad distribution of telomere lengths in C57BL/6J mice means that, at any given time, there are very few short telomeres that may not be sufficient to signal dysfunction. In contrast, the CAST/EiJ mouse has a much narrower telomere-length distribution, so shortening of this distribution results in a significantly higher fraction of short telomeres. The number of short dysfunctional telomeres eventually exceeds the number that can be elongated by telomerase during each cycle.

Short Telomeres Limit Tissue Renewal

Although telomerase dosage affects telomere-length distribution, it is the short telomeres that determine disease phenotype. This is evident from the wt* mice that inherit short telomeres and show telomere dysfunction in the presence of wild-type levels of telomerase. This form of occult genetic disease has not been documented with any other type of genetic change. Since short telomeres are inherited in humans (Graakjaer et al., 2004), this phenotypic manifestation of telomere dysfunction, in the absence of any overt mutation, may occur in families with autosomal-dominant dyskeratosis congenita, or even in individuals with short telomeres without a family history of disease (see below). This fact has important clinical implications; for example, aplastic anemia is sometimes treated with bone marrow transplant, often from an unaffected relative. In families with telomerase mutations however, a relative may not have a mutation but may have short telomeres. Such wt* individuals may not be a good choice for a bone marrow donor as their stem cell pool may be limited. Our data also suggest that bone marrow donors with long telomeres may be preferable for transplant where it is possible. The availability of reliable clinical assays for telomere length would greatly facilitate the classification of donor telomere lengths. Second, patients with dyskeratosis congenita are often exquisitely sensitive to certain cytotoxic bone marrow preparative regimens. Knowing whether an individual has short telomeres and possibly decreased bone marrow reserves may predict toxicities to treatment and therefore be relevant to treatment decisions. Finally, in diagnosing dyskeratosis congenita, telomere length should be examined even if mutations in *hTR* or *hTERT* are not present because some individuals may be wt*. Given that it is telomere length, not the presence or level of telomerase, that causes disease, it is possible that mutations in other genes that affect telomere length lead to disease. There are a large number of proteins that are involved in the maintenance of telomere length (Smogorzewska and de Lange, 2004). It will be interesting to determine if mutations in these genes play a role in other forms of dyskeratosis congenita for which no genetic changes have yet been identified (Marrone et al., 2005).

Implications of wt* Mice for Telomere Effects on Human Age-Related Disease

The loss of telomere function on short telomeres in wt* mice has implications for the role of short telomeres in disease states in normal individuals. The variation in telomere length within the population and possibly also individual variation in the expression of telomerase components may lead to telomere dysfunction in normal individuals. The particular organ system that is affected may depend on environmental factors that increase turnover in a particular tissue in one individual. Short telomeres do in fact correlate with an increased risk of mortality due to age-related disease (Cawthon et al., 2003; Valdes et al., 2005). The evidence presented here that, irrespective of telomerase levels, it is the short telomeres that are the primary determinant of disease implies that individuals with short telomeres but no family history of mutations in telomerase may be predisposed to defects in tissue renewal capacity. These effects are more likely to appear in tissues of high turnover such as the bone marrow or gastrointestinal tract where stem cell function may be limiting. Since stem cell failure has been linked to aging (Tyner et al., 2002), subtle defects in telomere function that affect stem cell renewal potential may predispose to age-related disease.

EXPERIMENTAL PROCEDURES

Mouse Breeding and Colony

CAST/EiJ mice were purchased from The Jackson Laboratory (Maine). We initially backcrossed the null *mTR* allele from the C57BL/6 background onto the CAST/EiJ background for five generations. These heterozygous animals after five backcrosses were designated CAST/EiJ HG1 for heterozygous generation 1. Interbreeding of HG1 generated KO-G1, HG2, and wt2* as indicated in Figure 1. HG2 crosses generated KO-G1, HG3, and wt3*. Interbreeding of the $mTR^{-/-}$ KO-G1 gave $mTR^{-/-}$ KO-G2 animals. $mTR^{-/-}$ KO-G2 animals were always generated from $mTR^{-/-}$ KO-G1 that came out of the same heterozygous generation breeding. To generate more HG1 animals, HG1 animals were bred to wild-type mice purchased from The Jackson Laboratory and the heterozygous pups were treated as HG1 animals. All animals were housed and bred in the pathogen-free environment at The Johns Hopkins University. All procedures were approved by the Institutional Animal Care and Use Committee at The Johns Hopkins University.

Quantitative FISH

Splenocytes were used to generate metaphases for Q-FISH as described (Hemann et al., 2001b). In short, the spleen was isolated and a single-cell suspension obtained using a cell strainer (BD Falcon) and cultured in RPMI Medium 1640 (Invitrogen) with 10% fetal bovine serum (Invitrogen), 1% Pen/Strep/Glu (Invitrogen), 1% HEPES (Invitrogen), and 1% MEM Sodium Pyruvate (Invitrogen). Cells were stimulated with 1 mg LPS (Sigma), 25 Cu/ml IL2 (Roche), and 5 μg/ml Con A (Sigma) for 40–48 hr at 37ºC with 5% CO_2 prior to colcemid arrest (10 μg/ml, Invitrogen) for 30 min at 37ºC. Cells were treated with hypotonic solution containing 0.4% KCl for 4 min at 37ºC and then fixed with 3:1 Methanol:Acetic Acid fixative. Cells were washed with fixative six times and then metaphases were dropped. Slides were chilled at 4ºC overnight and then hybridized for Q-FISH with Cy3-labeled PNA telomere probe (Applied Biosystems). All the Q-FISH analysis was done as a blind study in which the genotype of the animals was not known to the person performing the analysis.

Statistical Tests and Survival Curves

The Wilcoxon rank sum test for the telomere-length analysis and the linear regression of CBC were calculated in the STATA 8.0 program. Animals used for Kaplan-Meier survival curve were set aside at weaning age according to their genotype. Animals were closely monitored daily and sacrificed when signs typical of terminal illness in this colony appeared. Kaplan-Meier curves were generated using STATA 8.0 program and p values were obtained using log-rank test.

Bone Marrow Transplantation

CAST/EiJ recipient wild-type mice (Jackson Laboratory) were irradiated with 10Gy using GammaCell 40 (Cs^{137} source) irradiator. To prepare donor cells, bone marrow was isolated from femurs and tibias from donor animals by flushing with PBS using a 23G needle (BD Falcon). Cell were counted with a hemocytometer and resuspended to 5×10^6 per ml of PBS. 1×10^6 cells were injected into the tail vein of irradiated recipients. Unreconstituted animals died within 2–3 weeks after irradiation. Reconstituted animals were monitored on a daily basis to generate the Kaplan-Meier survival curve shown in Figure 2.

Histology

Organs were fixed in 10% buffered formalin as soon as animals were sacrificed. GI tract and airway were infused with 10% formalin to preserve the architecture of the organs. Fixed organs were then embedded in paraffin, sectioned at 5 μm, and stained with H&E. For testes analysis, testes were fixed in Bouin's fixative for 3 days and similarly processed and sectioned. Slides were evaluated for aberrant tubules in blinded fashion where the genotype of the animal was not known to the person doing the analysis.

Complete Blood Count

Animals were anesthetized and heart puncture was performed immediately. Blood was drawn into EDTA coated tubes (BD) and sent to Antech Diagnostics for complete blood count on the same day.

5-FU and Peripheral Blood Counts

Age-matched animals were used for 5-FU experiment. 5-FU was purchased from Sigma and resuspended into 20 mg/ml PBS. Animals were weighed and 150 mg of 5-FU per kg of body weight was given intraperitoneally (Rudolph et al., 1999). Fifty microliters of blood was collected through tail vein every week into EDTA containing tubes (BD). Peripheral white blood cell counts were obtained by diluting 10 μl of whole blood into 190 μl of red blood cell lysing reagent (388 mM NH4Cl, 29.7 mM $NaHCO_3$, 25 μM Na2EDTA), and the number of white cells was counted using a hemocytometer.

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