FANCC suppresses short telomere-initiated telomere sister chromatid exchange

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Telomere shortening has been linked to rare human disorders that present with bone marrow failure including Fanconi anemia (FA). FANCC is one of the most commonly mutated FA genes in FA patients and the FANCC subtype tends to have a relatively early onset of bone marrow failure and hematologic malignancies. Here, we studied the role of Fancc in telomere length regulation in mice. Deletion of Fancc (Fancc²⁻/⁻) did not affect telomerase activity, telomere length or telomeric end-capping in a mouse strain possessing intrinsically long telomeres. However, ablation of Fancc did exacerbate telomere attrition when murine bone marrow cells experienced high cell turnover after serial transplantation. When Fancc²⁻/⁻ mice were crossed into a telomerase reverse transcriptase heterozygous or null background (Tert¹/² or Tert²/²) with short telomeres, Fancc deficiency led to an increase in the incidence of telomere sister chromatid exchange. In contrast, these phenotypes were not observed in Tert mutant mice with long telomeres. Our data indicate that Fancc deficiency accelerates telomere shortening during high turnover of hematopoietic cells and promotes telomere recombination initiated by short telomeres.

INTRODUCTION

Telomeres are specialized structures consisting of tandem repeats, TTAGGG in human and mouse, together with telomere-associated proteins to form caps at the ends of linear chromosomes (1,2). Telomeres prevent the recognition of chromosome termini as broken DNA ends and are critical to maintaining genomic stability. Telomere dysfunction, resulting from loss of telomere repeats or loss of protection by telomere-associated proteins, can trigger DNA damage responses, cell apoptosis, cell proliferation defects or genome instability. Telomere dysfunction has also been linked to bone marrow failure syndromes and tumor formation (3,4). Telomerase is essential in telomere length maintenance by replenishing telomere loss due to incomplete DNA replication (1). In mice, deficiency in either telomerase core component, telomerase RNA (Terc) or telomerase reverse transcriptase (Tert) leads to progressive telomere shortening (5–8), which is accompanied by cell proliferation defects and apoptosis in highly proliferating organs including the bone marrow (9–11). Furthermore, Terc or Tert heterozygous mice bred for increasing generations also exhibit progressive telomere shortening and loss of tissue renewal capacity (8,12,13). In humans, mutations in the telomerase components are associated with accelerated telomere shortening and the development of bone marrow failure syndromes, such as dyskeratosis congenita, acquired aplastic anemia and idiopathic pulmonary fibrosis (14). Previous reports show that peripheral blood cells derived from Fanconi anemia (FA) patients have shorter telomeres compared with age-matched healthy donors (15–19), but it is unclear whether telomere attrition in hematopoietic cells from FA patients contributes to the pathogenesis of bone marrow failure in FA.

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Telomere shortening is considered a biological clock counting down cellular replicative senescence (20). Cells may overcome this barrier and become immortalized by maintaining telomere length through activation of telomerase and homologous recombination (HR)-mediated pathways (20,21). In human and murine telomerase-deficient cells, short telomeres can initiate HR between telomere sister chromatids, or telomere sister chromatid exchange (T-SCE), by which telomere length is maintained (22–26). Furthermore, short telomeres are also capable of initiating telomere recombination in the presence of telomerase (27). Although it is not entirely clear what molecules regulate telomere recombination, a loss of function in the pathways controlling telomere length maintenance, telomere capping or telomere chromatin can affect telomere recombination. For example, inactivation of Werner (Wrn) protein promotes T-SCEs in spontaneously immortalized telomerase-null mouse embryonic fibroblasts (28). Alterations in telomere capping or epigenetic modifications due to disruption of murine telomere capping proteins (e.g. Pot1 or Trt2 in combination with Ku70) or histone methyltransferases (e.g. Suv4-20h or Suv39h) can also contribute to elevation of T-SCEs (29–32).

FA is an autosomal recessive disorder characterized by cancer susceptibility, bone marrow failure and cellular sensitivity to DNA inter-strand cross-linking agents. To date, 13 FA proteins (FANCA, B, C, D1, D2, E, F, G, I, J, L, M and N) have been identified. Increasing evidence demonstrates that FA proteins play an important role in genome integrity via DNA replication-dependent repair (33,34). Several FA proteins form the FA nuclear core complex, which is required for the monoubiquitination of FANCD2 and FANCI and the localization of FA proteins to chromatin, possibly at the sites of DNA repair. FANCD2 and FANCI function as signal transducers and DNA-processing molecules in a DNA damage response network consisting of ATR, BRCA1 and a RecQ helicase, BLM. Abrogation in any of FA core components disrupts the monoubiquitination of FANCD2 and FANCI. FA proteins may respond to endogenous DNA damage, such as DNA inter-strand cross-links or oxidative DNA damage (35). Whether the FA pathway can respond to dysfunctional telomeres is yet to be determined.

FANCC is one of the most commonly mutated genes in FA patients and is conserved among vertebrates (36). Its encoded protein, FANCC, is 63 kDa in size with no discernable motifs or domains (33,34). FANCC is a member of the FA core complex where it interacts with FANCE and FANCJ, and it also associates with the Bloom syndrome protein complex in a supercomplex called BRAFT (33,34,37). In addition to participating in monoubiquitination of FANCD2 and FANCI as part of the core complex, FANCC is also involved in HR pathways (38–40). These observations, together with prior reports showing telomere shortening in FA patients, led us to investigate whether FANCC regulates telomere length and telomere recombination in vivo. In this study, we employed Fancc-deficient mouse models in the strain background with long (C57BL/6 strain) or short telomeres (telomerase reverse transcriptase mutant strain after successive breedings) and wild-type recipient mice that had undergone serial transplantsations with Fancc−/− bone marrow cells. Using these genetically modified murine models, we demonstrate that Fancc plays a role in stress-induced telomere attrition and short telomere-initiated recombination in primary murine hematopoietic cells.

RESULTS

Fancc deficiency does not compromise telomeres in a mouse strain with intrinsically long telomeres

FA patients were reported to harbor short telomeres (15–19). To investigate whether Fancc plays a direct role in telomere length maintenance in vivo, we examined telomere length of wild-type and Fancc−/− mice in the C57BL/6 genetic background, known to have several-fold longer telomeres than humans (41). Hematopoietic cells were isolated from 2- to 4-month-old mice. The mean and median telomere signal intensities as well as the distribution of individual telomere signal intensities were similar between wild-type and Fancc−/− mouse bone marrow cells by Q-FISH analysis (Fig. 1). In addition, the average telomere signal intensity was comparable between wild-type and Fancc−/− bone marrow, spleen and thymus cells via Flow-FISH measurements (Fig. 2). No
significant differences in telomere activity and the expression of the telomerase core components, Tert and Terc, were observed between wild-type and Fancc−/− mouse bone marrow cells (Fig. 3).

Accumulating evidence suggest that telomere integrity depends not only on telomere length but also on the proper capping of chromosome ends by telomere-associated proteins and telomere special structures (3,4,42). Therefore, telomeres with normal length do not necessarily reflect that they are functionally capped. To explore whether Fancc plays a direct role in telomeric end-capping in vivo, we examined Fancc−/− bone marrow cells for evidence of telomeric end-capping defects, i.e. chromosome end-to-end fusion and telomere signal-free end (SFE). Fancc−/− bone marrow cells did not exhibit chromosome end-to-end fusions and SFEs (Fig. 1 and Table 1). Furthermore, Fancc−/− bone marrow cells did not display spontaneous chromosomal abnormalities, e.g. chromosome breakages and fragments (Table 1). Together, these results suggest that FANCC does not play a direct role in regulating telomere length, telomerase activity and telomeric end-capping, when telomeres are long and functional.

**Inactivation of Fancc accelerates telomere attrition in serially transplanted bone marrow cells**

In both humans and mice, telomere dysfunction leads to cell proliferation defects and apoptosis in highly proliferating organs, especially in the bone marrow (9–11,14). Fancc−/− mice do not have obvious bone marrow abnormalities (43). Thus, it is not surprising that the mutant mice do not have any detectable telomere defects. On the other hand, Fancc−/− bone marrow cells display decreased hematopoietic stem cell repopulating ability after primary and secondary transplantations (44,45). It is unclear whether telomere length is altered in Fancc−/− hematopoietic cells during serial bone marrow transplantation and consequently contributes to the decreased repopulating ability of these cells. Utilizing Q-FISH analysis, we examined telomere length of wild-type and Fancc−/− bone marrow cells, which had previously undergone serial transplantation in lethally irradiated secondary recipient mice. Wild-type bone marrow cells from secondary transplant recipients had a decrease in mean and median telomere signal intensity compared with wild-type bone marrow cells from untransplanted mice (mean telomere signal intensity was 34 780 and 82 930, after and before transplantation, respectively) (Figs 1 and 4). Interestingly, Fancc−/− bone marrow cells from secondary transplant recipients had an additional reduction in telomere signal intensity compared with transplanted wild-type cells (Fig. 4). The appearance of chromosome ends with greatly reduced or no telomeric end-capping, when telomeres are long and functional, bone marrow cells derived from C57BL/6 mice. Flow-FISH analysis of average telomere length is altered in Fancc−/− mice (44,45). It is unclear whether telomere length, telomerase activity and telomeric end-capping, when telomeres are long and functional.
Fancc deficiency promotes short telomere-initiated T-SCE in late generation Tert mutant mice

Although these data suggest that FANCC does not directly control long telomeres, it is unclear whether FANCC regulates short telomeres. A body of evidence suggests that short telomeres can initiate recombinogenic events, including T-SCEs (22–27), even in the presence of telomerase (27). However, the molecular mechanism of short telomere-initiated telomere recombination is not well known. Given that FANCC is involved in HR pathways, we questioned whether FANCC may be involved in HR to resolve endogenous DNA damage, such as critically shortened telomeres. To test this hypothesis, we introduced Fancc+/+ into Tert+/+ mice (Supplementary Material, Fig. S1). After successive breedings (Supplementary Material, Fig. S1), bone marrow cells from Fancc+/+ and Fancc−/− (HG5 in Tert+/+ background and G2 in Tert−/− background) exhibited decrease in overall telomere length and increases in the appearance of chromosome ends with greatly reduced, or non-existent, telomere signals as detected by telomere restriction fragment and Q-FISH analysis (Supplementary Material, Fig. S1, Fig. 6 and Table 1). These mutant mice with short telomeres allowed us to investigate the impact of Fancc deficiency on short telomere-initiated T-SCEs in the presence or absence of telomerase.

We examined the frequencies of T-SCEs in heterozygous generation 1 (HG1) and HG5 Tert+/+ Fancc+/+ and Tert−/− Fancc−/− bone marrow cells via CO-FISH analysis. In HG1 mice with long telomeres (Supplementary Material, Fig. S2), T-SCEs were nearly undetectable in both Tert+/+ Fancc+/+ and Tert−/− Fancc−/− mice (Fig. 7). In HG5 mice with short telomeres (Supplementary Material, Fig. S2), higher frequencies of T-SCEs were observed in Tert−/− Fancc−/−, compared with Tert+/+ Fancc−/− (average 5.5% T-SCEs/chromosome in Tert+/+ Fancc−/− and 3.5% T-SCEs/chromosome in Tert+/− Fancc+/+) may be involved in HR to resolve endogenous DNA damage, such as critically shortened telomeres.

### Table 2. Frequencies of chromosomal abnormalities in wild-type and Fancc−/− bone marrow cells after two series of transplantations

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Aneuploidy</th>
<th>Fragment and breaks</th>
<th>SFEs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>17 ± 6%</td>
<td>0.77 ± 0.32%</td>
<td>2.7 ± 1%</td>
</tr>
<tr>
<td>Fancc−/−</td>
<td>20.7 ± 4%</td>
<td>0.48 ± 0.27%</td>
<td>4.2 ± 0.8%</td>
</tr>
</tbody>
</table>

The data were obtained from wild-type and Fancc−/− mice (n = 4), and more than 50 bone marrow cells from each mouse were scored.

- Percentage of abnormal cells.
- Percentage of abnormal events per chromosome.

Figure 4. Accelerated telomere attrition is observed in Fancc−/− bone marrow cells after two serial bone marrow transplantations. Q-FISH analysis of wild-type and Fancc−/− bone marrow cells after two serial bone marrow transplantations (n = 4). (A) Representative metaphase spreads of wild-type and Fancc−/− bone marrow cells showing DAPI staining (upper panel, blue) and telomere fluorescence signals (upper panel, red; lower panel, black). There was a decrease in telomere signal intensities in Fancc−/− cells (Bii) in comparison to wild-type cells (Bi), shown here as shift of the dynamic range in overlapping histogram (Biii) and box-plot (C).

Figure 5. Telomerase activity in wild-type and Fancc−/− bone marrow cells after two serial bone marrow transplantations. qT-PCR analysis indicates a comparable telomerase activity in serially transplanted wild-type and Fancc−/− mouse bone marrow cells (n = 4). Error bars represent the standard error from different mice of each genotype.

Figure 6. Telomere length measurement in wild-type and Fancc−/− bone marrow cells after two serial bone marrow transplantations. Q-FISH analysis of wild-type and Fancc−/− bone marrow cells showing DAPI staining (upper panel, blue) and telomere fluorescence signals (upper panel, red; lower panel, black). There was a decrease in telomere signal intensities in Fancc−/− cells (Bii) in comparison to wild-type cells (Bi), shown here as shift of the dynamic range in overlapping histogram (Biii) and box-plot (C).
T-SCEs further increased in G2 Tert\(-/-\) Fancc\(+/-\) and Tert\(-/-\) Fancc\(-/-\) mutant mice. Q-FISH analysis of bone marrow cells derived from G2 Tert\(-/-\) Fancc\(+/-\) and Tert\(-/-\) Fancc\(-/-\) mice (n = 6). Representative metaphase spreads of G2 Tert\(-/-\) Fancc\(+/-\) and Tert\(-/-\) Fancc\(-/-\) mouse bone marrow cells showing DAPI staining (upper panel, blue) and telomere fluorescence signals (upper panel, red; lower panel, black). SFEs are detectable in G2 Tert\(-/-\) Fancc\(+/-\) and Tert\(-/-\) Fancc\(-/-\) mice (see arrows).

(Fig. 7), T-SCEs further increased in G2 Tert\(-/-\) Fancc\(+/-\) and Tert\(-/-\) Fancc\(-/-\) bone marrow cells harboring critically short telomeres (see SFEs in Fig. 6 and Table 1), and the latter displayed more T-SCE events (average 24% T-SCEs/chromosome in Tert\(-/-\) Fancc\(+/-\) and 13% T-SCEs/chromosome in Tert\(-/-\) Fancc\(-/-\)) (Fig. 7). Thus, deletion of Fancc leads to elevated T-SCEs in late generation telomerase mutant mice with short telomeres. These observations suggest that short telomeres in late generation telomerase mutant mice become prone to T-SCEs and that inactivation of Fancc promotes T-SCEs.

Inactivation of FANCC in chicken DT40 cells results in elevated genome SCEs (40). We hypothesized that inactivation of Fancc in mice would cause spontaneous genome SCEs that in turn contribute to T-SCE events. We examined the frequencies of genome SCEs in HG5 Tert\(+/-\) Fancc\(+/-\) and Tert\(-/-\) Fancc\(-/-\) mouse bone marrow cells, and our data showed that the frequencies of genome SCEs in these mice were comparable (average 12% SCEs/chromosome in Tert\(+/-\) Fancc\(+/-\) and 10.7% SCEs/chromosome in Tert\(-/-\) Fancc\(-/-\)) (Fig. 8). Thus, deletion of Fancc does not affect
it is possible that higher turnover of Fancc−/− bone marrow cells may accelerate telomere shortening, which may, in turn, contribute to decreased Fancc−/− hematopoietic stem cell repopulation ability (44). Thus, telomere shortening may be an indirect consequence of Fancc deficiency.

Late generation Tert−/− mice displayed short telomeres and increased T-SCE events. When Fancc was deleted, the incidence of T-SCEs was exacerbated. These observations suggest that inactivation of FANCC promotes short telomere-initiated T-SCEs. It is possible that Fancc inhibits short telomere-initiated telomere recombination in primary murine hematopoietic cells, but inactivation of Fancc leads to loss of this suppressive mechanism. The exact mechanism of how FANCC suppresses telomere recombination is unclear. FANCC does not directly bind to DNA (49), and it may thus regulate proteins or pathways that are involved in telomere recombination. As a component of the FA core complex, FANCC regulates the monoubiquitination of FANCD2. A previous report demonstrates that the modified FANCD2 localizes to telomeres in an immortalized cell line that maintains telomeres through HR-based mechanisms; however, depletion of FANCD2 causes increased short telomeres and decreased T-SCEs in this line (50). These telomere phenotypes are in contrast to the observations in HG5 Tert−/− Fancc−/− and G2 Tert−/− Fancc−/− mice. A possible explanation for these apparent inconsistencies could be that FA proteins may not only serve as checkpoint proteins that prevent primary cells from the engagement of illegitimate telomere recombination, but also regulate the maintenance of telomere recombination to keep the shortest telomeres intact in immortalized cells. Alternatively, these discrepant findings may be because the former study employed an immortalized cell line that had already escaped checkpoints for the illegitimate telomere recombination, whereas our studies utilized primary hematopoietic cells with intact checkpoints for illegitimate telomere recombination. FANCC also associates with the Bloom syndrome protein complex (37). It has been shown that the frequencies of spontaneous SCEs in fancc and blm double-mutant chicken DT40 cells are similar to those in blm single mutant (40). These studies suggest a functional linkage between FANCC and BLM in a common pathway to suppress SCE. Deletion of Fancc leads to reduced levels of Blm in mice (A.N. Suhasini and R.M. Brosh, personal communication). Decreased Blm levels may thus serve a role in promoting telomere recombination in Fancc and Tert double-mutant mice. Although ablation of another member of RecQ helicase proteins, Wrn, also results in elevated T-SCEs in late generation telomerase-null mice (28), the Wrn and telomerase double null cells display elevated incidence of critically short telomeres (28). In contrast, Fancc deficiency reduces the incidence of critically short telomeres in late generation telomerase-null mice (Table 1). These observations do not support the involvement of Wrn in Fancc-regulated telomere recombination in mice. However, deletion of fancc in chicken DT40 cells can lead to elevated spontaneous SCEs that depend upon a key HR protein, the RAD51 paralog, XRCC3 (40). In addition, ablation of murine telomere capping proteins (e.g. Pot1 or Trf2 in combination with Ku70) and histone methyltransferases (e.g. Suv4-20h or Suv39h) can cause elevated T-SCEs (29–32). It is unknown whether FANCC regulates XRCC3,

**DISCUSSION**

In this study, we examined the role of FANCC in telomere length regulation and telomere recombination in vivo. Although deletion of Fancc did not directly affect telomere length or end-capping in a strain with long telomeres, it led to an increase in the incidence of T-SCEs in late generation telomerase mutant mice with short telomeres. These genetic data support the notion that FANCC does not directly regulate long telomeres, but does regulate short telomere-initiated telomere recombination. Thus, ablation of Fancc function may promote telomere recombination. To our knowledge, this is the first study to demonstrate a molecular event that regulates short telomere-initiated telomere recombination in non-transformed murine tissues.

Fancc−/− mice did not show telomere attrition or telomere defects in the C57BL/6 genetic background that has exceedingly longer telomeres than humans. A similar observation was reported in a mouse model deficient in Fancc (46). Interestingly, in an experimental system that dramatically increases the hematopoietic stem and progenitor cell turnover (i.e. serial bone marrow transplantation), Fancc−/− bone marrow cells showed elevated telomere shortening compared with wild-type bone marrow cells, even though telomerase activity was comparable in these cell types. Since Fancc−/− hematopoietic stem and progenitor cells exhibit increased cycling compared with wild-type cells (47,48), it is possible that higher turnover
telomere-binding proteins or histone methyltransferases in telomere recombination.

FA patients without functional FA proteins are predisposed to bone marrow failure and malignancies (33,34). FANCC may therefore control cell viability and immortalization of primary cells, including bone marrow cells, by safeguarding genome stability. Telomere dysfunction triggers cell apoptosis and genomic instability preferentially in highly proliferating organs, e.g. bone marrow. In rare events, cells may overcome this barrier and become immortalized by activating the pathway involved in maintaining telomere length (21). Increasing evidence suggests that short telomeres initiate telomere recombination likely due to a dysfunction in the highly regulated mechanisms controlling HR. We have shown that inactivation of FANCC facilitates short telomere-initiated telomere recombination. In addition, Fance deficiency can accelerate telomere shortening during high hematopoietic cell turnover. It is possible that FANCC may function to control cell viability and immortalization of primary cells by influencing telomere attrition and telomere recombination.

MATERIALS AND METHODS

Mice

Fance and Tert knockout mice (Fance<sup>−/−</sup> and Tert<sup>−/−</sup>) in C57BL/6 genetic background were produced as described previously (7,8,43,44). Mouse breeding strategy is illustrated in Supplementary Material, Figure S1. In brief, Tert and Fance heterozygous mice (Tert<sup>+/−</sup> Fance<sup>+/−</sup>) were generated by interbreeding Tert<sup>+/−</sup> and Fance<sup>+/−</sup> mice and were named as HG1 mice. HG1 mice from separate mating events were mated to obtain HG2 mice, which were crossed again until generation 5 (HG5). Tert<sup>−/−</sup> Fance<sup>−/−</sup> mice were generated from HG5 Tert<sup>+/−</sup> Fance<sup>+/−</sup> breeders and were named as G1 mice. G1 mice from separate mating events were mated to obtain G2. All animal experiments were carried out according to the ‘Guide for the Care and Use of Laboratory Animals’ (National Academy Press, USA, 1996) and were approved by the NIA IACUC.

Serial mouse bone marrow transplantation

Bone marrow was flushed from tibias and femurs of experimental mice and low-density mononuclear cells were prepared by density centrifugation (ficoll-hypaque density 1.119, Sigma, St Louis, MO, USA). Primary and secondary transplants were conducted as described previously with slight modification (44,45). Briefly, low-density mononuclear bone marrow cells (2 × 10<sup>6</sup> cells) were resuspended in 200 μl IMDM supplemented with 20% fetal bovine serum (Biowhittaker, Walkersville, MD, USA). Cells were transplanted into congeneric lethally irradiated B6.SJL-PtcrcaPep3b/BoyJ recipient mice obtained from the Stem Cell Transplant Mouse Core in the Indiana University Cancer Center. Bone marrow from primary recipients was harvested 12 months after transplantation and prepared for secondary transplantation. Secondary transplants were conducted exactly the same as primary transplants. Four months after secondary transplantation, bone marrow was harvested for telomere studies.

Telomere length measurements

Flow-FISH. The average telomere fluorescence in splenocytes, thymocytes and bone marrow cells was scored for each mouse and the data were pooled from the indicated number of mice in each genotype and measured according to the previously published protocol with minor modifications (51). A telomere-specific FITC-conjugated (CCCTAA)<sub>3</sub> PNA probe (0.3 μg/ml, Panagene) was used.

Q-FISH. Mice were injected with 100 μl of 0.5% colchicine intraperitoneally for ~30 min before being sacrificed. Bone marrow cells were then collected by flushing 1 ml of phosphate-buffered saline from femurs. Collected bone marrow cells were immediately incubated in 0.075 m KCl for 15 min in 37°C, followed by fixation in ice-cold (3:1) methanol and glacial acetic acid. Metaphase spreads were then hybridized with a Cy3-labeled PNA (CCCTAA)<sub>3</sub> probe (0.3 μg/ml, Panagene) and counterstained with 4,6-diamidino-2-phenylindole as described previously (52). Images were captured using Cytovision™ software (Applied Imaging Corp.) on a fluorescence microscope (Axio2; Carl Zeiss, Germany), followed by quantification of telomere fluorescence signals using the TFL-Telo software (a kind gift from P. Lansdorp, Vancouver, BC, USA). For histograms and box-plots, data from different mice of each genotype were pooled and scored and R statistical package (http://www.r-project.org) along with R.-utils package and Biobase package (http://www.brajul.com/R) was used. The frequencies of telomeres within a given range of telomere signal intensities were plotted against the telomere signal intensity using arbitrary units. Metaphases from different mice of each genotype were scored for chromosomal and telomeric abnormalities (i.e. frequencies of cells with more or less than 40 chromosomes, frequencies of chromosomal fragmentations and breakages, and frequencies of chromosome ends with no detectable telomere signals) as described previously (26,53).

Telomere restriction fragment analysis. The analysis was carried out as described by Hemann and Greider (54). Approximately 1 × 10<sup>6</sup> mouse bone marrow cells were embedded in agarose plugs. DNA was digested with DpnII (BioLabs) and electrophoresed through 1% w/v pulsed-field grade agarose (Bio-Rad, CA, USA) in 1 x Tris–acetate–EDTA buffer. Electrophoresis was carried out in a CHEF DR-III pulsed-field apparatus (Bio-Rad) at 14°C with 3 V/cm and a switch time of 10 s for 48 h. The gel was denatured, dried, probed with 32P-labeled (AATCCC)<sub>4</sub> probe and visualized by autoradiography.

Measurement of T-SCE and genome SCE

Chromosome orientation FISH (CO-FISH) was used to measure the frequency of T-SCE (22). The measurement for genome SCEs was carried out as described previously (26). Briefly, bone marrow cells were flushed from femurs and tibias and cultured with Iscove’s modified Dulbecco’s...
medium (IBCO-BRL, Gaithersburg, MD, USA) supplemented with 20% fetal calf serum (Hyclone, Logan, UT, USA) in the presence of interleukin 6 (200 U/ml) and stem cell factor (100 ng/ml: Peprotech, Rocky Hill, NJ, USA) (44). Bone marrow cells were subcultured in medium containing a 3:1 ratio of BrdU/BrdC (Sigma) at a final concentration of 1 × 10^{-5} \text{ M} and collected around 12 or 24 h for detecting T-SCE or SCE, respectively. Colcemid (0.1 \mu\text{g}/ml) was added 2 h before harvest. Metaphase spreads were then stained with Hoechst 33258, exposed to UV light and digested with exonuclease III to remove newly synthesized DNA strands. Hybridization and wash conditions were identical to those described for telomere FISH (52). A chromosome with more than two telomeric DNA signals by FITC-labeled (CCCTAA)_3 or Cy3-labeled (TTAGGG)_3 PNA probes (0.3 \mu\text{g}/ml, Panagene) was scored as T-SCE-positive. A SCE was scored each time a color switch between dark or light sister chromatids occurred. The frequencies of T-SCEs and SCEs were obtained from different mice of each genotype.

Analysis of telomerase activity and Tert and Terc levels
Telomerase activity was measured by using Biomax Telomerase detection kit (Biomax, Inc., MD, USA) according to the manufacturer’s recommendations. Briefly, freshly isolated bone marrow cells were lysed, and the cell extracts were then added to a pre-mix for quantitative telomerase activity in a real-time PCR. For detecting Tert and Terc levels, total RNA was extracted from mouse bone marrow using RNeasy kit (Qiagen) and then reverse transcribed with random hexamers by the SuperScript III first-strand synthesis system for RT–PCR (Invitrogen Life Technologies). The PCR included cDNA reaction, 2 × Sybr master mix (Bio-Rad), and a set of primers for mouse Tert, Terc or Beta-Actin (Supplementary Material, Fig. S3). The levels of Tert and Terc were normalized to that of Beta-Actin. MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) was used to conduct the reaction where each sample was done in triplicates and performed according to the manufacturer’s instructions. Tert-deficient mouse bone marrow cell extracts or cDNA were used as a control. Relative telomerase activity was expressed as log of C_T value. Relative Tert and Terc RNA levels were normalized to Beta-Actin and expressed using the comparative C_T method according to the manufacturer’s instructions.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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