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# Normal mammalian cells negatively regulate telomere length by telomere trimming

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**In human cancer cells with telomeres that have been over-lengthened by exogenous telomerase activity, telomere shortening can occur by a process that generates circles of double-stranded telomeric DNA (t-circles). Here, we demonstrate that this telomere trimming process occurs in cells of the male germline and in normal lymphocytes following mitogen-stimulated upregulation of telomerase activity. Mouse tissues also contain abundant t-circles, suggesting that telomere trimming also contributes to telomere length regulation in mice. In cancer cells and stimulated lymphocytes, the mechanism involves the XRCC3 homologous recombination (HR) protein and generates single-stranded C-rich telomeric DNA. This suggests that, in addition to the well-documented gradual telomere attrition that accompanies cellular replication, there is also a more rapid form of negative telomere length control in normal mammalian cells, which most likely involves HR-mediated removal of telomere loops in the form of t-circles. We therefore propose that this telomere trimming mechanism is an additional factor in the balance between telomere lengthening and telomere shortening in normal human germline and somatic cells that may prevent excessive lengthening by processes such as telomerase activity.**

## INTRODUCTION

Mammalian telomeres are dynamic nucleoprotein structures at the ends of chromosomes, composed of tandem arrays of the 5′-TTAGGG-3′ repeat unit and the associated shelterin protein complex (1). Telomeric DNA terminates in a single-stranded (ss) G-rich 3′ overhang that is able to invade the double-stranded (ds) telomeric DNA by homologous recombination (HR), generating a Holliday junction (HJ), and forming an evolutionarily conserved stable secondary structure called a telomere-loop (t-loop) (2). An intact telomeric structure prevents the chromosome end from being identified as a site of DNA damage, and is vital for chromosome stability.

Telomere length is determined by the balance between telomere lengthening and shortening mechanisms. In some types of normal cells, including cells in the germline and in some highly proliferative somatic tissues, the ribonucleoprotein enzyme telomerase (3) uses its intrinsic RNA subunit as a template for the addition of telomeric DNA to the chromosome terminus by reverse transcription. Cancer cells activate either telomerase or an alternative lengthening of telomeres (ALT) pathway to prevent telomere shortening and thereby achieve immortalization (4,5); however, the level of telomerase activity in normal human somatic tissues is usually insufficient to completely prevent telomere shortening.

Two known mechanisms are responsible for telomere shortening: gradual telomere sequence attrition, which occurs with each cell cycle due to a combination of incomplete end-replication and nucleolytic degradation (6), and rapid loss of overlengthened telomeres by t-loop resolution, referred to as 'telomere rapid deletion' (TRD) (7) or 'telomere trimming' to avoid implying that the telomeres are completely deleted (8). The latter process is analogous to TRD in yeast cells, a RAD52-dependent mechanism that reduces over-elongated telomeres to the wild-type length (7), and has also been found to regulate telomere length in *Arabidopsis thaliana* (9).

Telomere trimming was identified in human cancer cells with telomeres that were artificially elongated by increased levels of telomerase (8), and can be detected by the presence

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of extrachromosomal telomeric repeat (ECTR) DNA predominantly in the form of t-circles. We deduced that telomere trimming occurs by HR-mediated resolution of the t-loop structure, and that this is a well-regulated process because it usually does not initiate a DNA damage response or result in telomere signal-free ends and chromosomal fusions (8). It seems likely that the increased abundance of t-circles in ALT cells (10) results from telomere trimming events counteracting excessive ALT-mediated telomere lengthening (11).

Here, we tested the hypothesis that telomere trimming occurs in normal mammalian cells. We analyzed two types of cells that are known to use telomerase-mediated telomere lengthening as a normal biological process. First, we analyzed cells of germline origin and, second, we investigated whether telomere trimming occurs in normal human somatic cells.

## RESULTS

### Extrachromosomal t-circles are present in the male germline

Telomerase is not detectable in mature human sperm (Fig. 1A); however, it has previously been detected in human testis, presumably in spermatogonial or spermatocyte precursor cells (12). We therefore reasoned that ECTR products arising from trimming of any telomeres overlengthened by telomerase may persist and be detectable in DNA isolated from mature sperm.

We analyzed telomere lengths in a panel of 10 matched human blood and sperm samples. Terminal restriction fragment (TRF) analysis demonstrated that telomere lengths were substantially greater in sperm, ranging from 10 to 14 kb, compared with matched blood leukocytes, which ranged from ~5 to 10 kb (Fig. 1B), consistent with the notion that telomere length is maintained in the germline. Telomere lengths in both blood and sperm varied among the individuals analyzed, presumably reflecting both inter-individual differences in the location of terminal restriction sites and the known variation of telomere length in the human population (13).

Two-dimensional (2D) gel electrophoresis enables the separation of TRFs by both size and structure, and was used to characterize ECTR DNA in cells following telomerase activation. Prominent t-circles were identified in sperm DNA from all 10 individuals and were notably absent from the matched blood DNA (Fig. 1C), even at longer exposure times (data not shown). This demonstrates that telomeres undergoing telomerase-mediated telomere lengthening in the germline are also subject to telomere deletion events. Consistent with the conclusion that telomere trimming occurs in the human germline, a previous study using single-telomere length analysis of sperm DNA detected a small number of telomeres that were severely truncated (14).

### Extrachromsomal t-circles are generated in stimulated lymphocytes, following telomerase-mediated telomere lengthening

Telomerase activity is repressed or tightly controlled in most normal human somatic cells, but is readily detectable in activated lymphocytes (15). The peripheral blood leukocyte population includes subsets of lymphocytes including T and B cells, which activate telomerase and undergo multiple cell divisions when stimulated. Using short-term cultures of whole blood from the same 10 human donors, we stimulated peripheral blood leukocytes using the mitogen phytohemagglutinin, which principally stimulates T cell division (16). Telomerase activity was not readily detected in leukocytes isolated from unstimulated blood, but was clearly present in stimulated lymphocytes from all 10 donors at 72 and 144 h (Fig. 2A).

TRF analysis demonstrated that, despite telomerase activation, there was no discernable increase in modal telomere length of lymphocytes after 72 or 144 h (Fig. 2B). However, at 72 h after stimulation, a distinct increase in the smear of telomeric DNA extending up into the wells of the gel was detected, indicating the presence of over-elongated telomeres at 72 h after mitotic stimulation. This upper smear of telomeric DNA is also likely to contain highly branched t-complex DNA, which is difficult to resolve by electrophoresis (17). Interestingly, the smear was no longer detected at 144 h after stimulation.

2D gel electrophoresis of DNA isolated from blood and stimulated lymphocytes identified t-circles 144 h after stimulation (Fig. 2C), coinciding with the disappearance of over-elongated and branched telomeres. T-circles were not detected in blood DNA. The disappearance of the telomeres that had been over-elongated by telomerase was therefore associated with the formation of t-circles, and is consistent with the notion that telomere trimming confers an upper limit to telomere length in normal somatic cells.

T-circle induction in stimulated lymphocytes is more subtle than that observed in the sperm DNA, reflecting the underlying extent of telomere lengthening, which is more substantial in the germline than in the stimulated lymphocyte immune response. In addition, Figure 2C highlights individual-specific variation in immune response, and the subsequent activation of telomerase and telomere lengthening, and thus telomere trimming. Specifically, the upper smears of extended telomeric fragments are less prominent in samples 9 and 10 than in samples 6–8 (Fig. 2B), demonstrating a direct link between the extent of telomere lengthening and the generation of telomere trimming-derived t-circles, which are correspondingly low in samples 9 and 10 (Fig. 2C).

### Telomere trimming requires the HR protein XRCC3

The shelterin component TRF2 is able to stimulate t-loop formation and its N-terminal basic domain is required to prevent aberrant t-loop deletion (10,18). Catastrophic deletion events associated with the formation of t-circles instigated by expression of a mutant TRF2, TRF2$^{\Delta B}$, that lacks the basic domain were dependent on XRCC3, a protein that forms a complex with RAD51C and is associated with HR (10,19,20). It has also been demonstrated that XRCC3 is required for the production of t-circles in ALT cells (21). In view of the data in these previous studies indicating that XRCC3 is involved in t-circle formation, we tested the hypothesis that XRCC3 is required for telomere trimming. Based on the hypothesis that

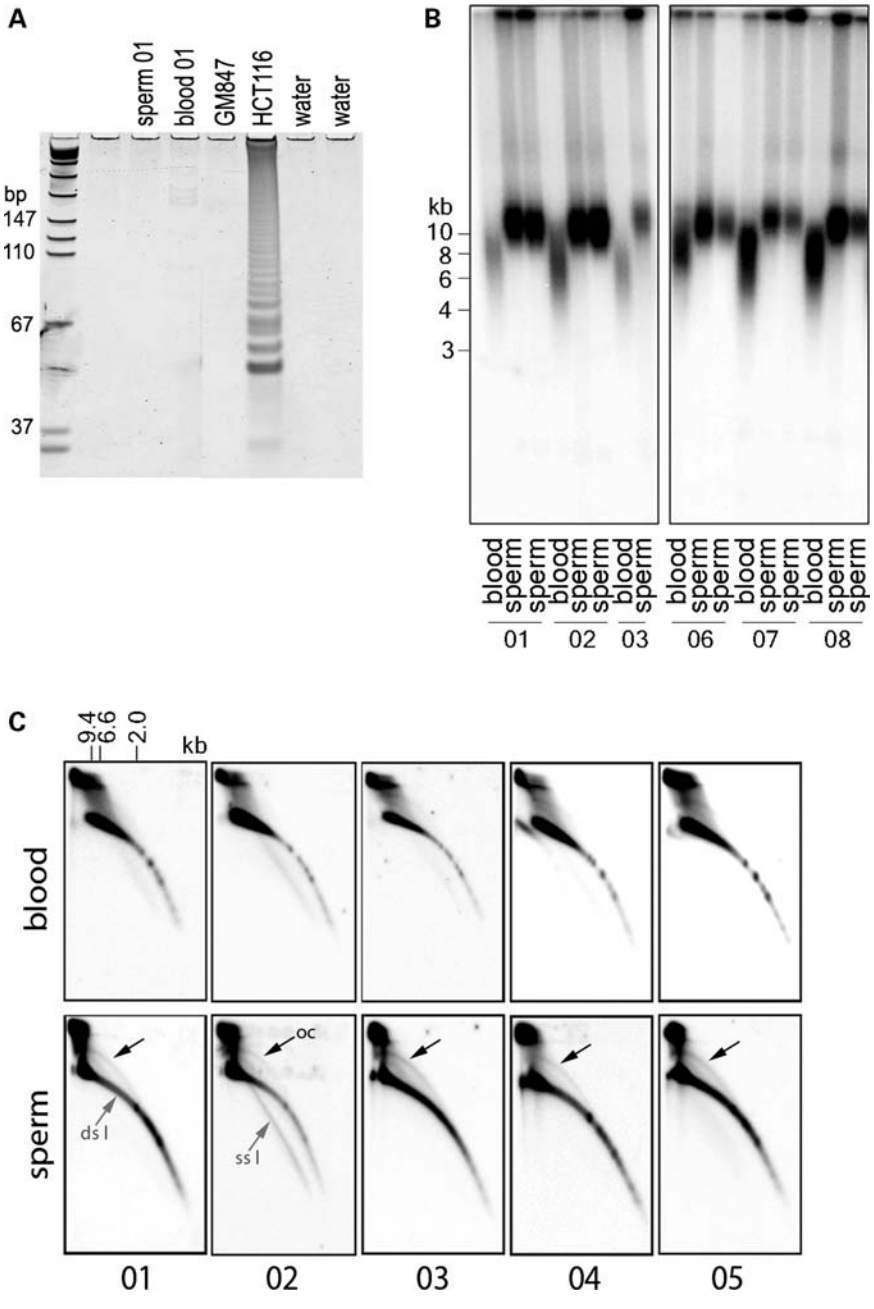


**Figure 1.** Extrachromosomal t-circles are present in sperm DNA. (**A**) Telomerase activity measured by immunoaffinity-purification (IP)-TRAP in blood and sperm whole-cell lysates from donor 01. GM847 and HCT116 cell lines were included as telomerase-negative and -positive controls, respectively. Water negative controls were also included. (**B**) TRF length analysis by PFGE of digested DNA from matched human blood and sperm samples (donors 01–03 and 06–08). Telomeric DNA was detected in-gel with a $\gamma$-$^{32}$P-labeled C-rich probe. (**C**) 2D gel electrophoresis of TRFs isolated from matched human blood and sperm samples (donors 01–05). Telomeric DNA was detected in-gel by hybridization with a $\gamma$-$^{32}$P-labeled C-rich probe. Black arrows indicate extrachromosomal open circular (oc) t-circles in sperm DNA. Ds linear (ds l) and ss linear (ss l) telomeric DNA are indicated with gray arrows. Representative samples are displayed in all cases.

t-circles might be generated by resolution of the HJ that occurs at the base of a t-loop, we also studied GEN1, a member of the RAD2/XPG nuclease family that was identified as an *in vivo* HJ resolvase (22).

Knockdown of GEN1 and XRCC3 was verified in hTR-overexpressing HT1080 cells (Fig. 3A), which undergo telomere trimming (8). Telomeric DNA was analyzed by 2D gel electrophoresis 144 h after siRNA-mediated knockdown. Knockdown of GEN1 had no detectable effect on t-circle levels and resulted in a t-circle signal comparable with the transfection and scrambled controls. Knockdown of XRCC3 resulted in an almost complete loss of detectable t-circles (confirmed by longer gel exposure times; data not shown), despite persistence of a strong linear telomeric DNA signal (Fig. 3B). TRF analysis demonstrated significant telomere lengthening 144 h after knockdown of XRCC3 (Fig. 3C and D),

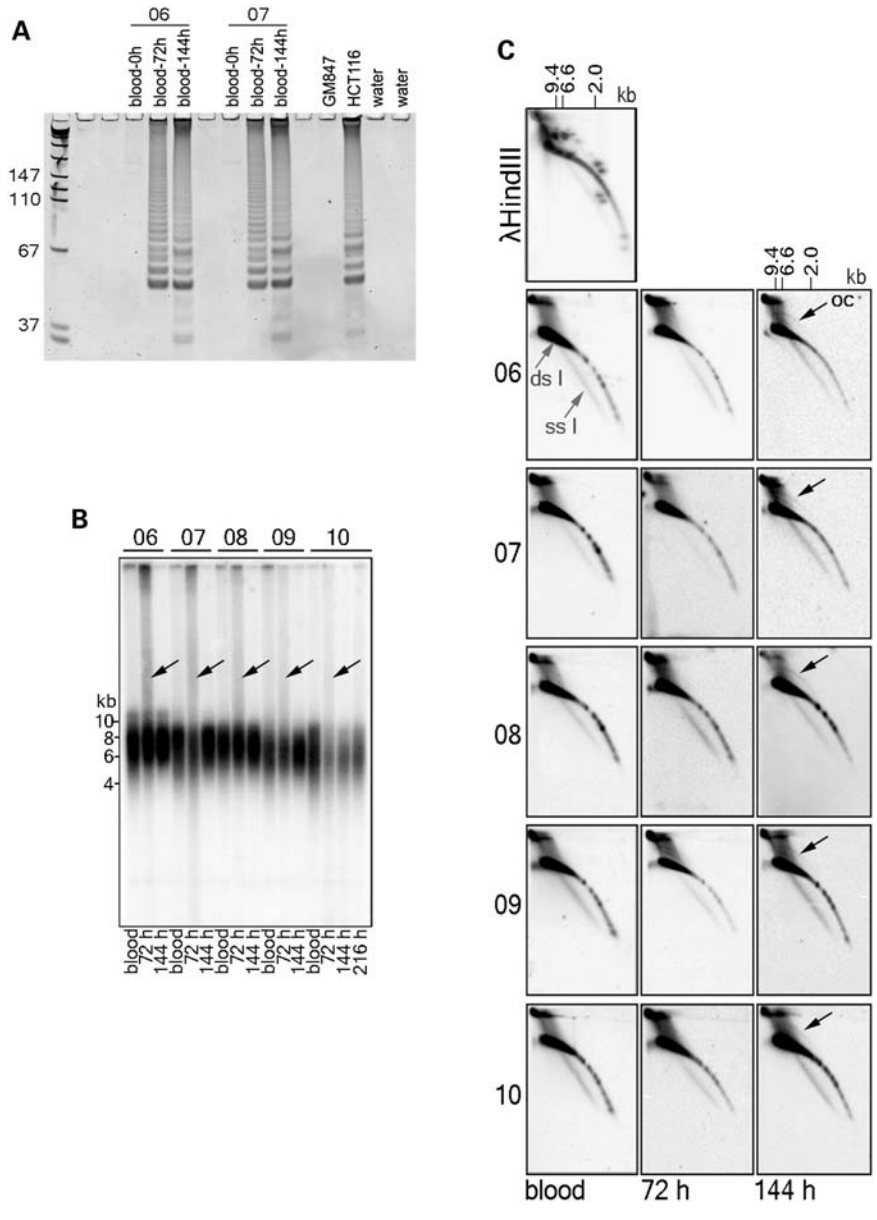


**Figure 2.** Extrachromosomal t-circles are present in stimulated lymphocytes following the activation of telomerase. (**A**) Telomerase activity measured by IP-TRAP on whole-cell lysates from unstimulated blood (0 h), and stimulated lymphocytes at 72 and 144 h after stimulation. GM847 and HCT116 cell lines were telomerase-negative and -positive controls, respectively. Water was an additional negative control. (**B**) TRF length analysis by PFGE of matched unstimulated blood and stimulated lymphocytes at 72 and 144 h. Arrows indicate telomeric smears. (**C**) 2D gel electrophoresis of TRFs isolated from matched unstimulated blood and stimulated lymphocytes at 72 and 144 h. Black arrows indicate extrachromosomal open circular (oc) t-circles in stimulated lymphocytes at 144 h. Ds linear (ds l) and ss linear (ss l) telomeric DNA are indicated with gray arrows. Representative samples from donors 06–10 are displayed in all cases. Circularized *Hin*dIII-digested bacteriophage lambda DNA was included as a marker for ds circular DNA.

consistent with telomere trimming being inhibited by depletion of this protein.

Similar knockdown experiments were carried out in stimulated lymphocytes. Consistent with the findings in HT1080 hTR cells, t-circles were generated in lymphocytes 144 h after stimulation in the presence of a scrambled control siRNA and despite knockdown of GEN1; however, levels of t-circles were substantially reduced following knockdown of XRCC3 (data not shown), confirming the specific requirement of XRCC3 for the generation of t-circles.

## Telomere trimming generates ss C-rich telomeric DNA

Human cells utilizing the ALT mechanism of telomere maintenance display prominent 5′ C-rich telomeric overhangs, which have recently been proposed to be an outcome of t-loop deletion (23). HT1080 hTR-overexpressing cells, which undergo telomere trimming in the absence of ALT, also have an abundance of ss C-rich linear telomeric DNA (8). In this study, we observed an elevated level of C-rich linear telomeric signal in sperm DNA and a more subtle

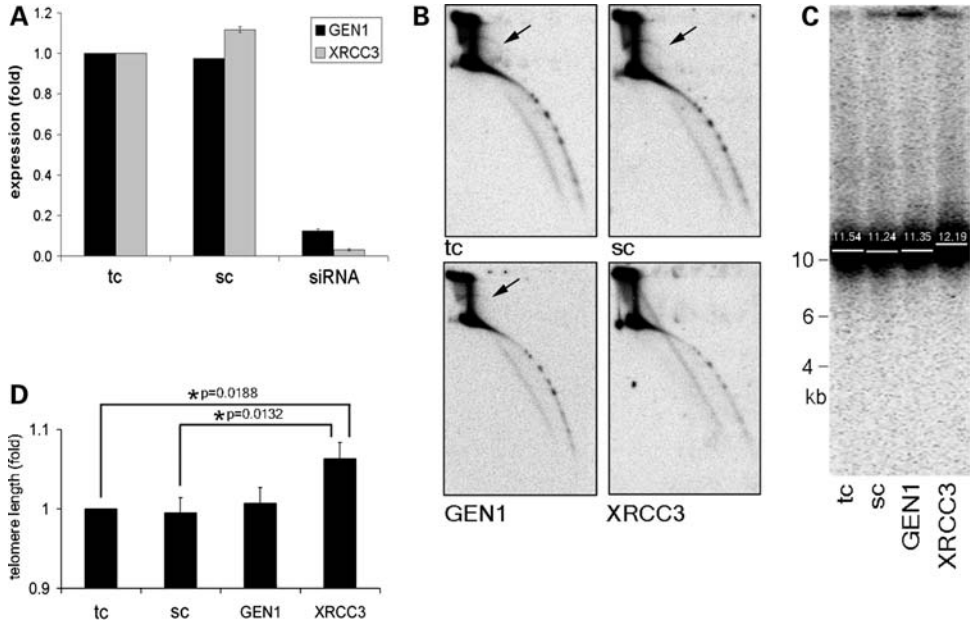


**Figure 3.** XRCC3 is required for telomere trimming. (**A**) Expression of GEN1 and XRCC3 as fold change relative to transfection control (tc), following siRNA transfection of HT1080 hTR-overexpressing cells, measured by quantitative RT-PCR (sc, scrambled siRNA control). Error bars represent standard deviation between three PCR experiments. (**B**) 2D gel electrophoresis of TRFs isolated from HT1080 hTR-overexpressing cells at 144 h following knockdown of GEN1 and XRCC3. Black arrows indicate extrachromosomal open circular t-circles. (**C**) TRF length analysis of HT1080 hTR-overexpressing cells at 144 h following knockdown of GEN1 and XRCC3, compared with control samples. A representative blot is displayed. (**D**) Telomere length as fold change relative to tc sample, following transfection of HT1080 hTR-overexpressing cells with tc, sc, GEN1 siRNA and XRCC3 siRNA. Error bars represent standard deviation between three separate knockdown experiments. *Statistical significance was determined using a two-tailed *t*-test.

increase in stimulated lymphocytes 144 h after stimulation, compared with blood DNA (Fig. 4A). Further characterization of the ss C-rich telomeric DNA was carried out by single telomere length analysis (STELA) of the Xp/Yp telomere using telorette oligos that anneal to either a 3′ G-rich overhang or a 5′ C-rich overhang. Telomeres >10 kb were not detectable by STELA in these analyses. Telomere amplification of 3′ G-rich overhangs detected a wide range of telomere lengths in blood, sperm and stimulated lymphocyte DNA. We detected 5′ C-rich telomeric overhangs in sperm DNA, and in stimulated lymphocytes at both 72 and, to a greater extent, 144 h after stimulation. No amplification of C-rich overhangs was observed in blood DNA (Fig. 4B and C). Our data indicate that telomere trimming generates ss C-rich linear telomeric DNA, consistent with similar observations in ALT cells which also employ telomeric HR (23).

## T-circles are detected in mouse tissues

To ascertain whether telomere trimming is conserved in mammalian cells, we used 2D gel electrophoresis to detect extrachromosomal t-circles in mouse tissues. It is well established that most laboratory mouse strains have long telomeres, in the range of 30–150 kb, in contrast to some wild-derived mouse species, which have considerably shorter telomeres, in the range of 10–25 kb (24). In addition, telomerase is active in the majority of mouse tissues (25) and there is no correlation between telomere length and longevity; mice are able to survive for several generations in the absence of telomerase and, depending on the mouse strain, it is only after four to six generations that shortened telomeres cause loss of cell proliferative capacity (26–28). We identified t-circles in DNA isolated from the kidney, liver, testes and brain from *Mus musculus castaneus* (CAST/EiJ) mice (with short telomeres) and *Mus musculus musculus* strain C57BL/6 (with long telomeres) (Fig. 5), demonstrating that telomere trimming occurs in mouse tissues, and suggesting the involvement of this mechanism in determining the species-specific telomere length set-point.

Next, we investigated the presence of telomere trimming in three consecutive generations of *Terc* knockout (KO) mice, which lack telomerase and consequently undergo telomere shortening with successive generations. T-circles were observed in DNA isolated from the liver and brain in all three generations of *Terc* KO mice (Fig. 5). In contrast, t-circles were reduced to low or undetectable levels in the kidney and testes.

Tissue-specific differences in telomere length and telomerase activity have previously been reported in mice (25), and this variation may underlie the tissue-specific differences in t-circles observed in the *Terc* KO mice. In addition, ALT activity has been detected in mouse cells and may contribute to telomere length maintenance in the absence of telomerase (23) (A.A.N., personal communication). ALT pathways in specific tissue progenitor cells, combined with the proliferative history of the tissue, might have a significant effect on telomere length and this will require further investigation. Nevertheless, the mouse data overall are consistent with the hypothesis that telomere trimming contributes to setting an upper limit on telomere length, which is likely to be of particular relevance for preventing excessive telomere lengthening in an organism with widespread telomerase expression.

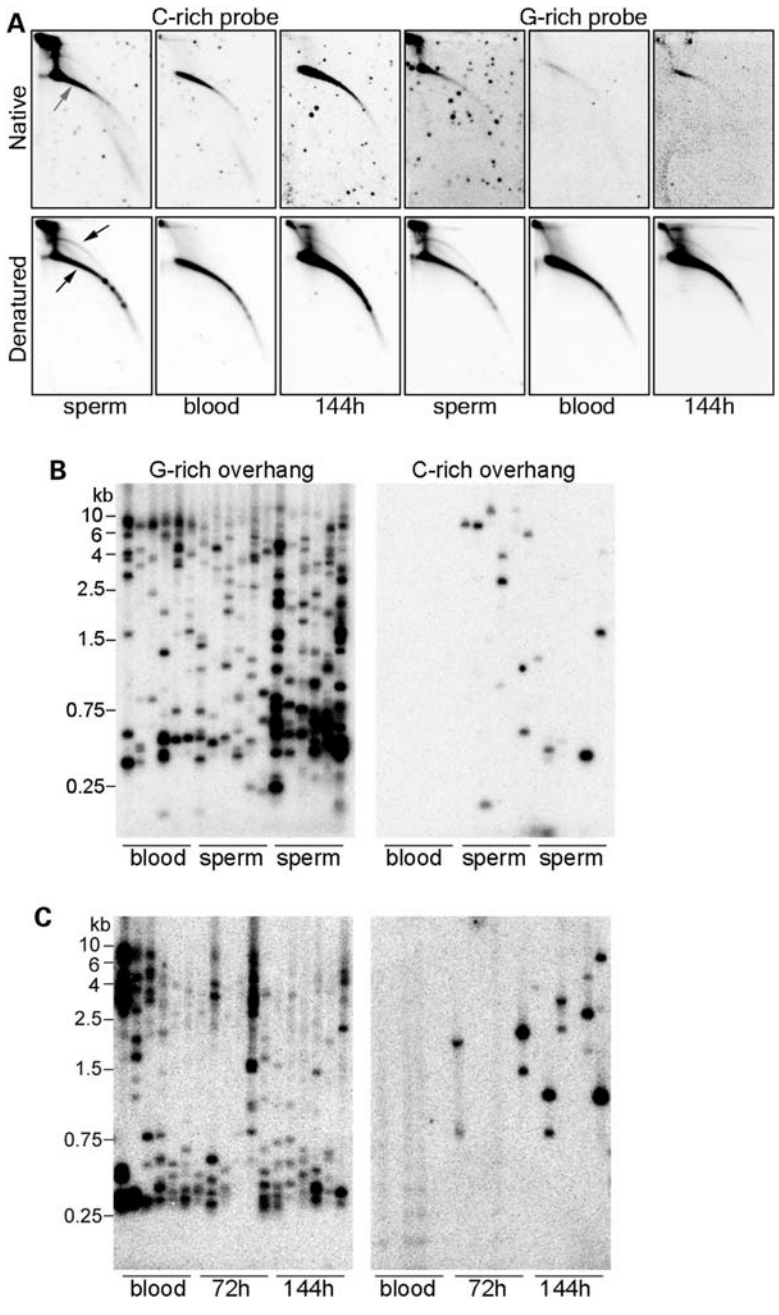


**Figure 4.** Telomere trimming results in an increase in ss C-rich linear telomeric DNA. (**A**) 2D gel electrophoresis of TRFs isolated from donor 08 blood, sperm and stimulated lymphocytes 144 h after stimulation. Hybridization was carried out in-gel with a γ-$^{32}$P-labeled C-rich or G-rich probe under native conditions to detect ss telomeric DNA (upper panel, gray arrow), and under denaturing conditions to detect total (ds plus ss) telomeric DNA (black arrows). (**B**) STELA of the XpYp telomere using genomic DNA isolated from donor 08 blood and sperm and (**C**) donor 08 blood and stimulated lymphocytes at 72 and 144 h after stimulation. Telorette oligonucleotides that anneal to either a 3′ G-rich overhang or a 5′ C-rich overhang were used in conjunction with the appropriate amplification primers. Blots were probed with a random-primed α-$^{32}$P-labeled XpYp telomere-adjacent probe.

## DISCUSSION

We have directly identified the production of t-circles as a response to telomerase activation and telomere lengthening in human stimulated lymphocytes. The appearance of t-circles coincided with a loss of elongated telomeric products. Furthermore, knockdown of XRCC3 not only resulted in a loss of t-circles, but also an increase in telomere length. T-circles were also detected in human sperm DNA following telomerase-mediated telomere lengthening in the germline, which is required to reconstitute telomere lengths prior to fertilization. The most likely conclusion is that excessive telomere elongation ultimately leads to the activation of an XRCC3-dependent rapid telomere length control mechanism, which generates t-circles. The mechanism whereby this occurs is speculative, but could include conversion of a

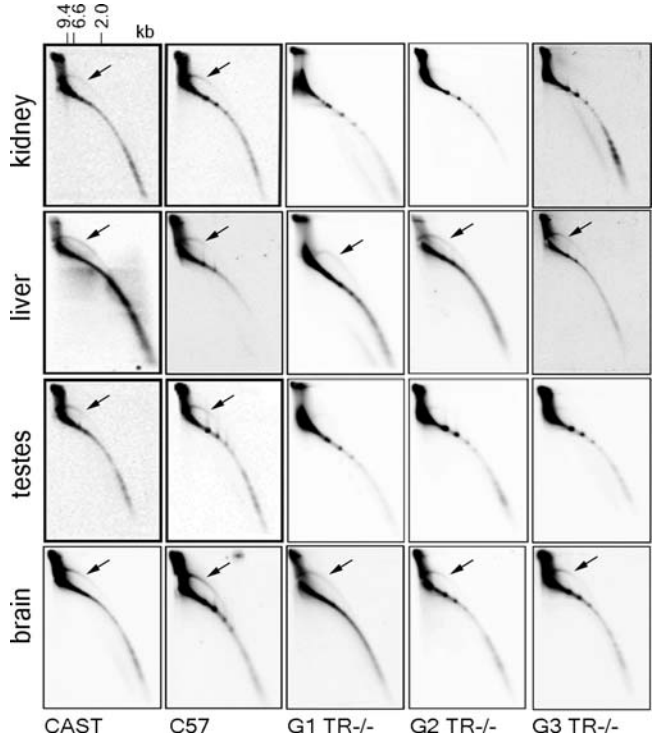


**Figure 5.** Telomere trimming occurs ubiquitously in mouse tissues. 2D gel electrophoresis of TRFs generated from the kidney, liver, testes and brain tissue from CAST/EiJ, C57BL/6 and generation (G) 1–3 *Terc* KO ($TR^{-/-}$) mice. Arrows indicate extrachromosomal open circular t-circles.

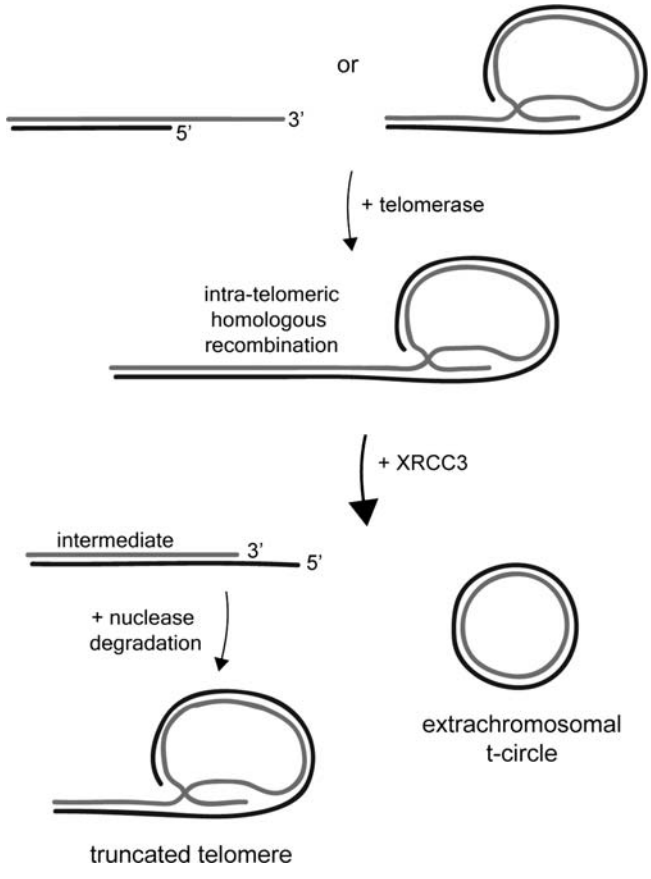


**Figure 6.** Proposed model of telomere trimming in mammalian cells. Telomere elongation beyond a threshold length initiates intra-telomeric HR through a t-loop intermediate. The mechanism is mediated by XRCC3 and generates a ss 5′ C-rich telomeric DNA intermediate, ultimately resulting in a truncated telomere and an extrachromosomal t-circle.

t-loop into a t-circle by HJ resolution (10), or excision of linear DNA that subsequently becomes circularized, for example, by an end-joining reaction. The presence of telomere trimming in both normal human cells and in mouse tissues indicates that this mechanism is a normal part of telomere biology.

The relevance of this mechanism is reflected in its conservation across many species, including yeast (7), plants (9) and mammals (this study). Over-lengthened telomeres are likely to be detrimental in several ways. First, cell division may be hindered by excessively long repetitive sequences that may become prone to replication slippage, stalled replication forks and the formation of secondary structures, including G-quadruplexes. It is also possible that G-quadruplex structures at the telomere may instigate t-loop formation and resolution. Second, long telomeres may present a replicative burden, slowing the cell cycle, which may account for the observation that most telomerase-positive cancer cells have a telomerase activity level that is only sufficient to maintain short, but stable telomere lengths. Finally, long telomeres delay the onset of senescence, resulting in increased risk of tumorigenesis, and may therefore have been selected against in human cells.

We propose that telomerase-mediated telomere lengthening beyond an individual-specific, and perhaps tissue-specific, threshold length triggers a shortening event via the resolution of the HJ present in a t-loop structure. This mechanism is mediated by the HR protein XRCC3, but not the GEN1 HJ resolvase, and generates both t-circles and ss 5′ C-rich telomeric DNA (Fig. 6). Further characterization of this mechanism may make it possible to manipulate telomere length by changing the telomere length set-point, and for cancer therapy, inducing rapid telomere deletion may be particularly useful in combination with telomerase inhibition.

## MATERIALS AND METHODS

### Sample collection and DNA extraction

Matched blood and semen samples were collected with informed consent from 10 adult male donors, with approval from the Children's Hospital at Westmead Human Research Ethics Committee. Whole blood was mixed with 25 U/ml of sodium heparin (Pfizer). Sperm heads were isolated and lyzed, and DNA was extracted as described previously (29). Leukocytes were isolated from whole-blood samples, lyzed and subjected to DNA extraction as described previously (30).

### Whole-blood culture

For whole-blood culture, blood was diluted 1:10 in PB-Max karyotyping medium (Gibco) and cultured in vertical flasks at 37°C, 5% $CO_2$. For siRNA transfection, red blood cells were lyzed in lysis buffer (10 mM $KHCO_3$, 150 mM $NH_4Cl$,

0.1 mM EDTA, 5% FCS) and centrifuged through 2 ml of fetal bovine serum. Cells were then cultured in PB-Max karyotyping medium. After 30 h, cells were transfected with siRNA, using siPORT neo-FX transfection reagent (Ambion), according to the manufacturer's instructions.

### TRF analysis

TRFs were prepared by digestion with *Hin*fI and *Rsa*I as described (8). Human TRFs were separated by 1D pulsed field gel electrophoresis (1D PFGE) and standard 2D gel electrophoresis. Mouse TRFs were separated by 2D PFGE. In-gel hybridization was carried out using a telomere-specific oligonucleotide probe (8). For hybridization under native conditions, agarose gels were dried at 50°C without denaturation and hybridized in-gel to $\gamma$-$^{32}$P-ATP-labeled $(CCCTAA)_4$ or $(TTAGGG)_4$ telomeric probes. After imaging, gels were denatured, neutralized and rehybridized to similar probes overnight, followed by subsequent washing and overnight exposure to a PhosphorImager screen.

### Immunoaffinity purification-telomere repeat amplification protocol

Immunoaffinity purification-telomere repeat amplification protocol (IP-TRAP) was carried out as described previously (31). Briefly, telomerase was immunoaffinity-purified from leukocyte, stimulated lymphocyte or sperm whole-cell lysates using the TERT HTCS2 antibody. The PCR-based TRAP assay was performed with modified PCR conditions [45 mM Tris–HCl, pH 8.8, 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 6.7 mM 2-mercaptoethanol, 4.4 mM EDTA, pH 8.0, 113 μg/ml bovine serum albumin, 1 mM each of dATP, dTTP, dGTP and dCTP] (32) and 10 U of *Taq* polymerase (Roche Diagnostics). IP-TRAP products were detected by staining with SYBR Green I (Sigma-Aldrich) after electrophoretic separation in a 10% polyacrylamide gel with 1× Tris-borate-EDTA buffer.

### STELA

STELA was performed as described (33), with some modifications. Briefly, 10 ng of genomic DNA was ligated to telorette 3 (5′-TGCTCCGTGCATCTGGCATCCCTAACC-3′) for G-rich telomeric overhangs and telorette 6 (5′-/5Phos/-GGTTAGGCTACGGTCTACGTGCCTCGT-3′) for C-rich telomeric overhangs (34) in a 10 μl reaction (1× ligase buffer, 0.5 U T4 ligase, 1 μM telorette 3) at 16°C for 1 h. Ligated DNA was diluted in water to 250 pg/μl. Multiple PCRs were carried out for each DNA sample in volumes of 20 μl containing 250 pg of DNA, 0.5 μM XpYpE2 (5′-GTTGTCTCAGGGTCCTAGTG-3′), 0.5 μM teltail (5′-TGCTCCGTGCATCTGGCATC-3′) to amplify G-rich telomeric overhangs and teltail rev (5′-ACGAGGCACGTAGACCGTAG-3′) to amplify C-rich telomeric overhangs in PCR buffer (32) and 1.5 U of a 25:1 mixture of *Taq* polymerase (Thermo Scientific) and *Pwo* polymerase (Roche). Reactions were cycled under the following conditions: 25 cycles of 94°C for 20 s, 64°C for 30 s, 68°C for 15 min. Products were resolved on a 0.8% agarose gel and detected by Southern hybridization with a random-primed $\alpha$-$^{32}$P-labeled telomere-adjacent probe generated by PCR using primers XpYpE2 and XpYpB2 (33). Hybridized fragments were detected by overnight exposure to a PhosphorImager screen.

### siRNA and qRT-PCR

HT1080 and HeLa vector control and hTR-overexpressing cells were cultured in DMEM + 10% FCS at 37°C, 5% $CO_2$. HT1080 cells stably overexpressing hTR that were passaged for approximately 250 population doublings (8) were transfected with three different GEN1 and XRCC3 siRNA sequences, scrambled control and transfection reagent control (Stealth siRNA, Invitrogen), using Lipofectamine RNAiMAX reagent according to the manufacturer's instructions (Invitrogen). Knockdown was verified by qRT-PCR, and the siRNA that produced the greatest knockdown of GEN1 or XRCC3 was used for all further experiments. RNA was extracted, cDNA synthesized and qRT-PCR performed as described previously (8) using F and R primers (GEN1F: 5′-GCCCAGACTGTTTACAGGAA-3′; GEN1R: 5′-CTTTGGGAGATAATCACAGC-3′; XRCC3F: 5′-TCCCCACGCAGCACCAGCGC-3′; XRCC3R: 5′-CGTCCGGCCAGCTCAGTGAT-3′; GAPDHF: 5′-ACCCACTCCTCCACCTTTG-3′; GAPDHR: 5′-CTCTTGTGCTCTTGCTGGG-3′) with SYBR green PCR mastermix (Applied Biosystems). RT minus controls were included. Matched PCR efficiencies for each primer set compared with the GAPDH primers were confirmed by standard curve comparison, and analysis was carried out by the comparative cycle threshold (Ct) method using GAPDH as the reference gene and expressed as fold change to the transfection reagent control.

### Mice

All animal work was carried out in accordance with the Australian Code of Practice for the care and use of animals for scientific purposes and was authorized by The Children's Hospital at Westmead/Children's Medical Research Institute Animal Care and Ethics Committee. Heterozygous male and female *Terc*$^{tm1Rdp}$/J (*Terc* KO) mice were obtained from The Jackson Laboratory (ME, USA) and were backcrossed with C57BL/6 mice for more than 10 generations. First-generation homozygous *Terc* KO mice were bred from heterozygous parents, and genotypes were verified by PCR of DNA isolated from mouse tails using the Wizard SV genomic DNA purification system (Promega), with forward and reverse primers for the mutant (MUTF: 5′-CTTGGGTGGAGAGGCTATTC-3′; MUTR: 5′-AGGTGAGATGACAGGAGATC-3′) and wild-type (WTF: 5′-CTCGGCACCTAACCCTGAT-3′; WTR: 5′-CGCTGACGTTTGTTTTTGAG-3′) alleles. Second- and third-generation homozygous *Terc* KO mice were bred from first- and second-generation *Terc* KO parents, respectively, and genotypes were confirmed by PCR. CAST/EiJ mice were obtained from The Jackson Laboratory. Mice were euthanized by $CO_2$ asphyxiation and tissues collected under aseptic conditions. In each case, tissues were analyzed from a minimum of two mice. Tissue samples were homogenized using a hand-held homogenizer with 1 ml of lysis solution (100 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA, 1% *N*-lauroylsarcosine).

Samples were treated with RNase A at a final concentration of 50 μg/ml for 1 h at 37°C. Proteins were digested using proteinase K at a final concentration of 100 μg/ml for 8 h at 55°C and subjected to phenol chloroform extraction, followed by ethanol precipitation. TRFs were prepared by DNA digestion with *Hin*fI and *Rsa*I, as described previously (8).

*Conflict of Interest statement*. None declared.

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## REFERENCES

1. de Lange, T. (2005) Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev.*, **19**, 2100–2110.
2. Griffith, J.D., Comeau, L., Rosenfield, S., Stansel, R.M., Bianchi, A., Moss, H. and de Lange, T. (1999) Mammalian telomeres end in a large duplex loop. *Cell*, **97**, 503–514.
3. Greider, C.W. and Blackburn, E.H. (1985) Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell*, **43**, 405–413.
4. Shay, J.W. and Bacchetti, S. (1997) A survey of telomerase activity in human cancer. *Eur. J. Cancer*, **33**, 787–791.
5. Bryan, T.M., Englezou, A., Dalla-Pozza, L., Dunham, M.A. and Reddel, R.R. (1997) Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nat. Med.*, **3**, 1271–1274.
6. Baird, D.M. (2008) Telomere dynamics in human cells. *Biochimie*, **90**, 116–121.
7. Lustig, A.J. (2003) Clues to catastrophic telomere loss in mammals from yeast telomere rapid deletion. *Nat. Rev. Genet.*, **4**, 916–923.
8. Pickett, H.A., Cesare, A.J., Johnstone, R.L., Neumann, A.A. and Reddel, R.R. (2009) Control of telomere length by a trimming mechanism that involves generation of t-circles. *EMBO J.*, **28**, 799–809.
9. Watson, J.M. and Shippen, D.E. (2007) Telomere rapid deletion regulates telomere length in *Arabidopsis thaliana*. *Mol. Cell. Biol.*, **27**, 1706–1715.
10. Wang, R.C., Smogorzewska, A. and de Lange, T. (2004) Homologous recombination generates T-loop-sized deletions at human telomeres. *Cell*, **119**, 355–368.
11. Cesare, A.J. and Reddel, R.R. (2010) Alternative lengthening of telomeres: models, mechanisms and implications. *Nat. Rev. Genet.*, **11**, 319–330.
12. Wright, W.E., Piatyszek, M.A., Rainey, W.E., Byrd, W. and Shay, J.W. (1996) Telomerase activity in human germline and embryonic tissues and cells. *Dev. Genet.*, **18**, 173–179.
13. de Lange, T., Shiue, L., Myers, R.M., Cox, D.R., Naylor, S.L., Killery, A.M. and Varmus, H.E. (1990) Structure and variability of human chromosome ends. *Mol. Cell. Biol.*, **10**, 518–527.
14. Baird, D.M., Britt-Compton, B., Rowson, J., Amso, N.N., Gregory, L. and Kipling, D. (2006) Telomere instability in the male germline. *Hum. Mol. Genet.*, **15**, 45–51.
15. Hiyama, K., Hirai, Y., Kyoizumi, S., Akiyama, M., Hiyama, E., Piatyszek, M.A., Shay, J.W., Ishioka, S. and Yamakido, M. (1995) Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J. Immunol.*, **155**, 3711–3715.
16. Knuutila, S. and Kovanen, P.E. (1987) Relative proportions of mitotic T and B cells in PHA-stimulated lymphocyte cultures. *Cancer Genet. Cytogenet.*, **29**, 151–154.
17. Nabetani, A. and Ishikawa, F. (2009) Unusual telomeric DNAs in human telomerase-negative immortalized cells. *Mol. Cell. Biol.*, **29**, 703–713.
18. Poulet, A., Buisson, R., Faivre-Moskalenko, C., Koelblen, M., Amiard, S., Montel, F., Cuesta-Lopez, S., Bornet, O., Guerlesquin, F., Godet, T. *et al.* (2009) TRF2 promotes, remodels and protects telomeric Holliday junctions. *EMBO J.*, **28**, 641–651.
19. Liu, Y., Tarsounas, M., O'Regan, P. and West, S.C. (2007) Role of RAD51C and XRCC3 in genetic recombination and DNA repair. *J. Biol. Chem.*, **282**, 1973–1979.
20. Liu, Y., Masson, J.Y., Shah, R., O'Regan, P. and West, S.C. (2004) RAD51C is required for Holliday junction processing in mammalian cells. *Science*, **303**, 243–246.
21. Compton, S.A., Choi, J.H., Cesare, A.J., Ozgur, S. and Griffith, J.D. (2007) Xrcc3 and Nbs1 are required for the production of extrachromosomal telomeric circles in human alternative lengthening of telomere cells. *Cancer Res.*, **67**, 1513–1519.
22. Ip, S.C., Rass, U., Blanco, M.G., Flynn, H.R., Skehel, J.M. and West, S.C. (2008) Identification of Holliday junction resolvases from humans and yeast. *Nature*, **456**, 357–361.
23. Oganesian, L. and Karlseder, J. (2011) Mammalian 5′ C-rich telomeric overhangs are a mark of recombination-dependent telomere maintenance. *Mol. Cell*, **42**, 224–236.
24. Hemann, M.T. and Greider, C.W. (2000) Wild-derived inbred mouse strains have short telomeres. *Nucleic Acids Res.*, **28**, 4474–4478.
25. Prowse, K.R. and Greider, C.W. (1995) Developmental and tissue-specific regulation of mouse telomerase and telomere length. *Proc. Natl Acad. Sci. USA*, **92**, 4818–4822.
26. Blasco, M.A., Lee, H.W., Hande, M.P., Samper, E., Lansdorp, P.M., DePinho, R.A. and Greider, C.W. (1997) Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell*, **91**, 25–34.
27. Lee, H.W., Blasco, M.A., Gottlieb, G.J., Horner, J.W., II, Greider, C.W. and DePinho, R.A. (1998) Essential role of mouse telomerase in highly proliferative organs. *Nature*, **392**, 569–574.
28. Hao, L.Y., Armanios, M., Strong, M.A., Karim, B., Feldser, D.M., Huso, D. and Greider, C.W. (2005) Short telomeres, even in the presence of telomerase, limit tissue renewal capacity. *Cell*, **123**, 1121–1131.
29. Jeffreys, A.J., Tamaki, K., MacLeod, A., Monckton, G., Neil, D.L. and Armour, J.A. (1994) Complex gene conversion events in germline mutation at human minisatellites. *Nat. Genet.*, **6**, 136–145.
30. Jeffreys, A.J., Neumann, R. and Wilson, V. (1990) Repeat unit sequence variation in minisatellites: a novel source of DNA polymorphism for studying variation and mutation by single molecule analysis. *Cell*, **60**, 473–485.
31. Au, A.Y., Hackl, T., Yeager, T.R., Cohen, S.B., Pass, H.I., Harris, C.C. and Reddel, R.R. (2011) Telomerase activity in pleural malignant mesotheliomas. *Lung Cancer*, **73**, 283–288.
32. Jeffreys, A.J., Wilson, V., Neumann, R. and Keyte, J. (1988) Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells. *Nucleic Acids Res.*, **16**, 10953–10972.
33. Baird, D.M., Rowson, J., Wynford-Thomas, D. and Kipling, D. (2003) Extensive allelic variation and ultrashort telomeres in senescent human cells. *Nat. Genet.*, **33**, 203–207.
34. Sfeir, A.J., Chai, W., Shay, J.W. and Wright, W.E. (2005) Telomere-end processing the terminal nucleotides of human chromosomes. *Mol. Cell*, **18**, 131–138.