The telomerase reverse transcriptase is limiting and necessary for telomerase function *in vivo*

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Mammalian telomerase is essential for the maintenance of telomere length [1–5]. Its catalytic core comprises a reverse transcriptase component (TERT) and an RNA component. While the biochemical role of mammalian TERT is well established [6–11], it is unknown whether it is sufficient for telomere-length maintenance, chromosome stability or other cellular processes. Cells from mice in which the *mTert* gene had been disrupted showed progressive loss of telomere DNA, a phenotype similar to cells in which the gene encoding the telomerase RNA component (*mTR*) has been disrupted [1,12]. On prolonged growth, *mTert*-deficient embryonic stem (ES) cells exhibited genomic instability, aneuploidy and telomeric fusions. ES cells heterozygous for the *mTert* disruption also showed telomere attrition, a phenotype that differs from heterozygous *mTR* cells [12]. Thus, telomere maintenance in mammals is carried out by a single, limiting TERT.

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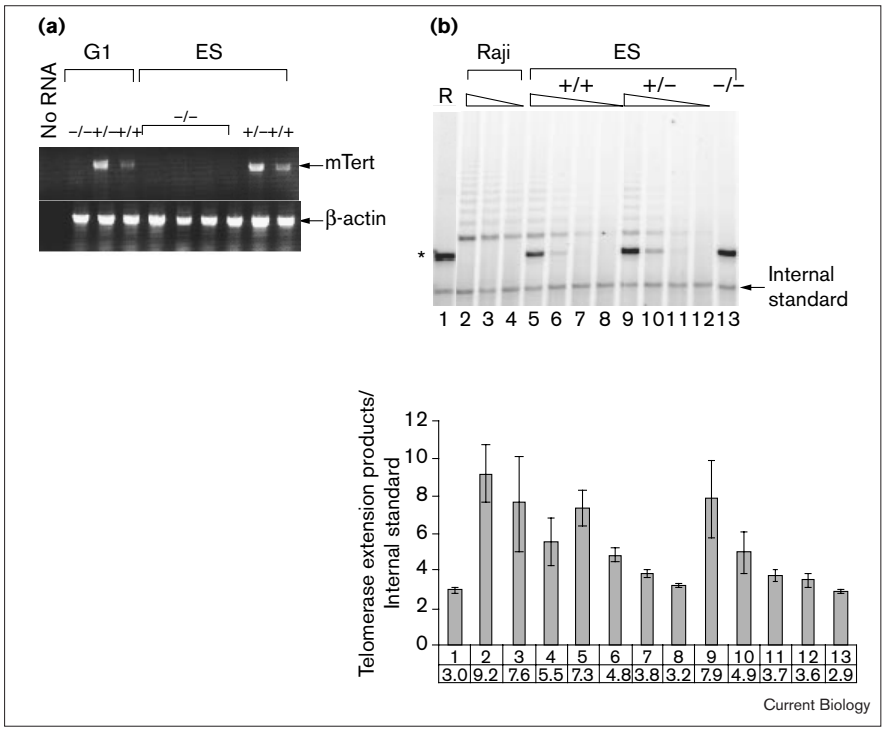
Results and discussion

We disrupted the telomerase-specific motif and the first three of the seven reverse transcriptase motifs of *mTert* by gene targeting (Figure S1a in the Supplementary material). ES cell clones and mice disrupted for one ($mTert^{+/-}$) or both ($mTert^{-/-}$) alleles of *mTert* were confirmed by PCR and Southern blot analysis (Figure S1b in the Supplementary material, and data not shown). The $mTert^{-/-}$ ES cell clones and G_1 mouse tissues did not possess any detectable *mTert* transcript (Figure 1a and data not shown), which differs from the *mTert* knockout mice produced by Yuan *et al.* [13]. The latter knockout mice produce a shorter *mTert* transcript missing the targeted exons. The *mTert*-deficient mice generated by us were viable for at least four generations.

To determine the requirement of mTERT for telomerase catalysis *in vivo*, the telomere repeat amplification protocol (TRAP) [14] was performed on four independently derived $mTert^{-/-}$ ES cell clones and several mouse tissues. No telomerase activity was detected in all $mTert^{-/-}$ ES cell clones, but both the $mTert^{+/-}$ and $mTert^{+/+}$ ES cell clones possessed telomerase activity (Figure 1b upper panel and data not shown). Using semi-quantitative measurements in which twofold changes in telomerase activity would be detectable, there was no difference in telomerase activity between $mTert^{+/+}$ and $mTert^{+/-}$ ES cell lysates (Figure 1b, lower panel). Telomerase activity was restored by transfection of *mTert* into $mTert^{-/-}$ ES cells (Figure 2 in the Supplementary material). In tissue extracts from testes, liver, thymus, lung and kidney of $mTert^{+/+}$ and $mTert^{+/-}$ mice, telomerase activity was readily detected, but in $mTert^{-/-}$ mice telomerase activity was undetectable (Figure S2 in the Supplementary material and data not shown). Similar results were obtained when telomerase activity was measured using the conventional telomerase elongation assay, which does not rely on PCR amplification [15,16] (data not shown).

Mice and ES cells disrupted for the telomerase RNA component undergo telomere shortening and eventually show an increase in end-to-end chromosome fusions [1,3,4,12]. To examine the consequences of *mTert* deficiency on telomere-length maintenance, we analyzed telomere lengths in four cultured $mTert^{-/-}$ ES cell clones upon prolonged culture. As $mTert^{+/-}$ and $mTert^{-/-}$ clones have undergone more population doublings during G418 selection than the parental line, the early passages (referred to as P1) from each genotype were not strictly equivalent (Figure 2a). Using fluorescence *in situ* hybridization (FISH) combined with flow cytometry (Flow–FISH) [17], we observed that all four *mTert*-deficient ES cell clones progressively lost telomeric DNA with increasing passage in culture (Figures 2a and 3b). In contrast, wild-type ES cells did not show any significant alteration in telomere fluorescence, regardless of the total number of population doublings (Figures 2a and 3b). Interestingly, in later passages of $mTert^{+/-}$ ES cells, we also observed a loss of telomeric DNA with successive growth in culture

Figure 1

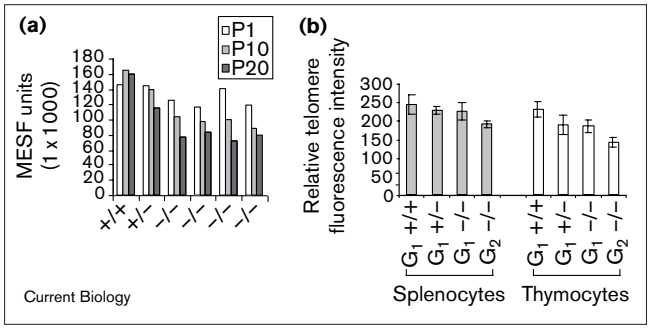


Disruption of the *mTert* gene in ES cells and mice. **(a)** Detection of *mTert* transcript by reverse transcription (RT)–PCR in G_1 mice (testis) and ES cells. The upper panel shows the *mTert* cDNA product encompassing the targeted exons in each respective genetic background. The same results were also obtained from the PCR primer sets derived from the 5′ and 3′ regions of the *mTert* cDNA (data not shown). A control β-actin RT–PCR product is shown in the bottom panel. **(b)** Telomerase activity in *mTert*-deficient ES cells and tissues. Upper panel, TRAP was performed for 20 PCR cycles on cell extracts (1.0, 0.5, 0.25 and 0.125 μg) prepared from wild-type, $mTert^{+/-}$, and $mTert^{-/-}$ ES cell extracts, and on 10, 5 and 2.5 μl of partially purified human Raji cell lysate. Arrow, internal PCR standard; asterisk, a non-specific product from mouse extracts that is resistant to RNase A treatment. Lower panel, the telomerase extension products were normalized to the internal standard using NIH Image Quant analysis. In each sample, data were pooled from three individual experiments (error bars represent the SD).

(Figures 2a and 3b). Similar results were also obtained with another $mTert^{+/-}$ ES cell line (data not shown). Therefore, removal of one allele of *mTert* was sufficient to confer a telomere-shortening phenotype in ES cells.

G_1 $mTert^{-/-}$ and $mTert^{+/-}$ mice possessed a similar telomere signal intensity as wild-type G_1 littermates (Figure 2b).

Figure 2

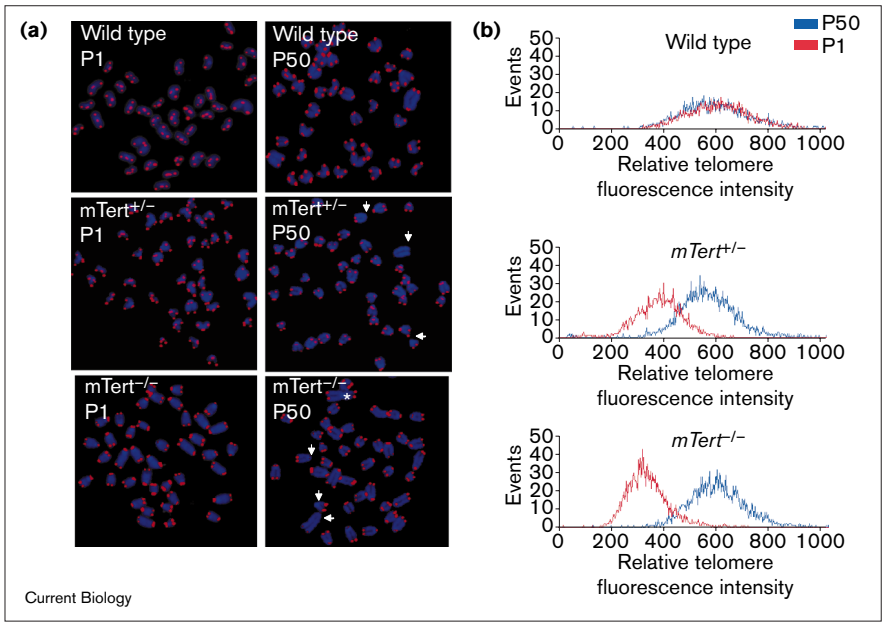


Analysis of telomere length in ES cells, splenocytes and thymocytes by Flow–FISH. **(a)** Average telomere fluorescence in early and late passages of wild-type, $mTert^{+/-}$, and four $mTert^{-/-}$ ES cell clones. The MESF (molecules of equivalent soluble fluorochrome) value represents one individual ES clone at the indicated passage; thus, there are no error bars. **(b)** The average telomere fluorescence in splenocytes and thymocytes derived from G_1 and G_2 $mTert^{-/-}$ mice. In each set, data were pooled from at least five individual mice (error bars represent the SD).

However, G_2 *mTert*-deficient mice showed a significant reduction in relative telomere length compared with wild-type and G_1 *mTert*-deficient mice (Figure 2b). The telomeric restriction fragments (TRFs) in $mTert^{+/-}$, $mTert^{-/-}$ and wild-type ES cells from early (P1) and later passages (P25 and 50) were also monitored by pulsed-field gel electrophoresis. Although differences in telomere length are typically less evident by this method [1], nonetheless a modest reduction in TRF length was observed in the late passages of $mTert^{+/-}$ and $mTert^{-/-}$ ES cells, but not in the wild-type ES cells (Figure S3 in the Supplementary material).

To examine chromosome stability in late passage $mTert^{+/-}$ and $mTert^{-/-}$ ES cells, we performed FISH analysis on metaphase spreads (Figure 3). All passages of wild-type ES cells and early passages of $mTert^{+/-}$ and $mTert^{-/-}$ ES cells showed no overall changes in telomere signal intensity. However, late passages of $mTert^{+/-}$ and $mTert^{-/-}$ ES cells possessed decreased telomere signal intensity at some chromosome ends (Figure 3). Aneuploidy was elevated in late passages of $mTert^{+/-}$ and $mTert^{-/-}$ ES cells compared with wild-type ES cells. However, only in $mTert^{-/-}$ ES cells at P50 (~300 population doublings) was a significant increase in end-to-end chromosome fusions observed (Figure 3, Table 1). Although later passages of $mTert^{+/-}$ ES cells possessed shortened telomeres, no end-to-end fusions were evident. Mitogen-stimulated cells from spleen of G_1 and G_2 $mTert^{-/-}$ mice also did not exhibit any increase in

Figure 3



FISH analysis from early (P1) or late passage (P50) ES cells. **(a)** Representative metaphase preparations of P1 or P50 wild-type, *mTert*$^{+/-}$ and *mTert*$^{-/-}$ ES cells. Note the reduced telomeric signal on some chromosome ends in late passage *mTert*$^{+/-}$ and *mTert*$^{-/-}$ ES cells (arrows), and an overall heterogeneity in the telomere fluorescence, which is also observed in mice and ES cells lacking the telomerase RNA component [1,12]. End-to-end fusions were visible in *mTert*$^{-/-}$ ES cells at P50 (asterisk). Metaphase spreads, FISH and image analyses were performed as described previously [1,27]. The Cy-3-labeled $(CCCTAA)_3$ PNA was used as a probe. **(b)** Histogram of Flow-FISH analysis of early and late passage ES cells. The data is shown for the same clones as in (a). The horizontal axis represents the relative telomere fluorescence intensity of individual cells, and the vertical axis shows the distribution of signal intensity in the population (see Supplementary material for methodological detail).

chromosomal aberrations compared with wild-type mice (Table 1, and data not shown). This phenotype is similar to *mTR*-deficient mice, in that the appearance of end-to-end fusions occurs only in later generations [1,3,4,12].

The first genetic evidence to establish the importance of TERT in telomere-length maintenance was obtained in the yeasts *Saccharomyces cerevisiae* [18,19] and *Schizosaccharomyces pombe* [20]. Recent evidence for a single TERT in multicellular organisms has been demonstrated in *Arabidopsis thaliana* [21]. We have shown that murine TERT, like the telomerase RNA, is not immediately essential for normal development and cell viability but is required for telomere-length maintenance *in vivo* [1,12]. While telomere length and chromosome stability was not analyzed in the previously reported *mTert*$^{-/-}$ mice, the presence of 3′ telomere single-stranded DNA (‘G-strand tails’) was preserved [13]. It remains to be determined whether late generations of *mTert*$^{-/-}$ mice (G_4 or later) show a similar phenotype as *mTR*-deficient mice, such as end-to-end chromosome fusions, apoptosis, and defects in cellular proliferation. Telomere shortening rates between ES cells and mice would be difficult to equate, and it will be necessary to compare the telomere shortening phenotypes of *mTR*- and *mTert*-deficient mice in the same genetic background. In *S. cerevisiae*, deletion of the telomerase RNA component (*TLC1*) and the TERT *EST2* are phenotypically indistinguishable [18,19,22].

Interestingly, ES cells deficient in one allele of *mTert* lost telomeric DNA in successive passages. These results contrast with *mTR*$^{+/-}$ ES cells, in which the average TRF length was virtually unchanged on prolonged culture [12]. These results suggest that TERT is a limiting component for telomere-length maintenance, at least in this strain of ES cells. However, we were unable to detect a reproducible difference in telomerase activity *in vitro* between wild-type and heterozygous ES cells or testis. These results differ from the *mTert*$^{+/-}$ mice generated by Yuan *et al.* in which telomerase activity was decreased in heterozygous animals (as measured by the stretch-PCR telomerase assay) [13]. In our studies, despite the obvious loss of telomere DNA *in vivo*, assiduous efforts to detect even a twofold difference in telomerase activity were unsuccessful (Figure 1b, data not shown). This discrepancy could be the result of different genetic backgrounds or differences in telomerase extension conditions *in vitro*. It is also possible that a truncated mTERT protein, if present in the *mTert*$^{+/-}$ mice generated by Yuan *et al.*, could decrease telomerase activity through a ‘dominant-negative’ mechanism. *Arabidopsis* tissues heterozygous for *AtTERT* also showed no detectable difference in telomerase activity, but telomere length in successive generations of *AtTERT*$^{+/-}$ tissues was not examined [21]. There may therefore be limitations to the *in vitro* telomerase assay in assessing subtle differences in the dosage of telomerase components *in vivo*.

It will be important to determine whether the *mTert* haploinsufficiency can be extended to other murine genetic backgrounds and other organisms. Haploinsufficiency of individual telomerase components has not been noted previously, but in *S. cerevisiae* multiple heterozygous combinations of different telomerase components do appear to exhibit telomere shortening [19,23]. It was suggested that

Table 1

Chromosomal abnormalities in *mTert*$^{-/-}$ ES cells or mice.

	Metaphases analyzed	Aneuploidy (%)	End-to-end fusions (fusions per metaphase)
Splenocytes*			
Wild type	25	4 (16%)	0
G_1 *mTert*$^{-/-}$	25	2 (8%)	0
G_2 *mTert*$^{-/-}$	20	3 (15%)	0
ES cells			
Wild type P1	25	2 (8%)	0
Wild type P30	25	3 (12%)	0
Wild type P50	25	9 (36%)	0
mTert$^{+/-}$ P1	23	2 (9%)	0
mTert$^{+/-}$ P30	24	5 (24%)	0
mTert$^{+/-}$ P50	21	9 (43%)	0
mTert$^{-/-}$ P1	25	3 (12%)	0
mTert$^{-/-}$ P30	24	10 (41%)	0.04
mTert$^{-/-}$ P50	50	29 (58%)	0.58

*Splenocytes were selected using anti-CD3 antibody and interleukin-2.

'additive haploinsufficiency' could result from perturbations in the stoichiometry of individual telomerase components within the telomerase complex [19,23]. Mice heterozygous for *mTert* may thus provide a useful genetic model system in which to test whether deficiencies in other telomerase-associated proteins exacerbate a partial loss of mTERT function. Human telomerase activity can be virtually abolished in tumor cell lines by the overexpression of catalytically inactive hTERT, leading to telomere shortening and cell death [24–26]. The haploinsufficiency of *mTert* in ES cells raises the intriguing possibility that TERT may also be limiting in immortalized human cells, and that even a modest reduction of endogenous telomerase activity in some human tumor cell types may be sufficient to induce telomere shortening and cell death.

Supplementary material

Supplementary material including additional methodological detail, telomerase activity and three figures showing the targeting strategy and TRF analysis of ES cells is available at http://current-biology.com/supmat/supmatin.htm.

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