Importance of TRF1 for Functional Telomere Structure*

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Telomeres are comprised of telomeric DNA sequences and associated binding molecules. Their structure functions to protect the ends of linear chromosomes and ensure chromosomal stability. One of the mammalian telomere-binding factors, TRF1, localizes telomeres by binding to double-stranded telomeric DNA arrays. Because the overexpression of wild-type and dominant-negative TRF1 induces progressive telomere shortening and elongation in human cells, respectively, a proposed major role of TRF1 is that of a negative regulator of telomere length. Here we report another crucial function of TRF1 in telomeres. In conditional mouse TRF1 null mutant embryonic stem cells, TRF1 deletion induced growth defect and chromosomal instability. Although no clear telomere shortening or elongation was observed in short term cultured TRF1-deficient cells, abnormal telomere signals were observed, and TRF1-interacting telomere-binding factor, TIN2, lost telomeric association. Furthermore, another double-stranded telomeric DNA-binding factor, TRF2, also showed decreased telomeric association. Importantly, end-to-end fusions with detectable telomere signals at fusion points accumulated in TRF1-deficient cells. These results strongly suggest that TRF1 interacts with other telomere-binding molecules and integrates into the functional telomere structure.

Telomeres are specialized DNA structures positioned at the termini of eukaryotic chromosomes (1). Since the original work of Muller (2) and McClintock (3), the telomere has been thought to protect chromosome ends from degradation and end-to-end fusions. Telomeres consist of repetitive G-rich DNA sequences and associated proteins that function to distinguish natural chromosome ends from damage-induced DNA breaks. During the proliferation of mammalian cells, telomeres shorten after each round of DNA replication, because DNA polymerase fails to synthesize the distal ends of chromosomal DNA (4). This “end replication problem” limits the growth potential of normal cells. However, in cells that divide indefinitely (e.g. germ and tumor cells), telomere length is maintained by telomerase, a reverse transcriptase that adds telomeric TTAGGG repeats onto the 3’ ends of chromosomes (5–7).

The length of telomeric DNA repeats has been believed to be crucial for the proper function of telomeres. Indeed, chromosomal stability has been shown to require a minimum telomere length. However, more recent studies indicate that telomere function critically depends on structural aspects of the telomere rather than length alone (8). Consistent with this hypothesis, the formation of a functional telomere structure requires the expression of several telomere-interacting molecules.

In mammals, three telomeric DNA-binding proteins, termed POT1, TRF1, and TRF2, have been identified (9–11). POT1 binds to single-stranded TTAGGG repeats. In contrast, TRF1 and TRF2 homodimerize and bind specifically to double-stranded TTAGGG repeats through their Myb-related DNA-binding domains. Although TRF1 and TRF2 possess similar biochemical characteristics, their roles in telomere function are quite distinct. Based on in vitro and in vivo studies, TRF2 promotes T-loop formation in which the 3’ telomeric overhang is invaded into the preceding duplex telomeric repeat array (12). Overexpression of dominant-negative TRF2 activates the ATM/p53 or p16/RB pathways and induces cellular senescence or apoptosis (13, 14). Moreover, inhibition of TRF2 induces end-to-end fusions and chromosomal instabilities (15, 16). These data strongly suggest that TRF2 is a key player in “functional telomere structure” and protects chromosome ends. In contrast, current evidence indicates that TRF1 functions to negatively regulate telomere length (17). Overproduction of wild-type or dominant-negative TRF1 induces progressive telomere shortening or elongation, respectively. However, TRF1 expression appears to play no role in cell growth in humans as demonstrated by its dominant-negative form. The TRF1 protein has been shown to associate with additional factors, called tankyrase 1 and 2, which also modulate telomere length (18, 19). Tankyrase 1 and 2 are a poly(ADP-ribose) polymerase that targets TRF1 and induces progressive telomere lengthening via the dissociation of TRF1 from telomeric structures in humans (19, 20). On the other hand, TIN2 mimics TRF1 function to negatively regulate telomere length (18).

Dominant inhibition studies suggest that the primary function of TRF1 is to regulate telomere length (17). However, these overexpression studies do not rule out the possibility that some endogenous TRF1 remains bound to telomeres in cells expressing the dominant-negative mutant (17, 21). Even low levels of wild-type TRF1 may be sufficient to mediate a subset of telomere functions. To directly test the role of TRF1 in telomere function, we generated conditional TRF1-deficient embryonic
pCLC-mTRF1, a 1.6-kb mTRF1 min in media containing 20% retargeted with the targeting plasmid.

C57BL/6 mouse thymocyte cDNA phage library.

buffer for 20 min. After several washes, the cells were incubated for 20 min with fluorescein isothiocyanate-conjugated anti-mouse IgG Ab (e-bioscience) diluted 1:200 in the blocking washes, the cells were incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG Ab (e-bioscience) diluted 1:200 in the blocking buffer for 20 min. After several washes, the cells were incubated for 20 min with 50 μg/ml RNase A. Propidium iodide (PI) (10 μg/ml) was added just before analysis. For TUNEL analysis of ES clones summarized in B, Genomic DNA from ES cells was digested with BamHI (C) and EcoRV (D) and probed with the 3′ RIB probe (C) or the exon 1 probe (D). The bands corresponding to the endogenous (8.1 kb) and the targeted (KO) allele (6.6 kb) in the endogenous (4.5 kb) and mTRF1 cDNA-containing (5.5, 10, and 20 kb) fragments in D are indicated. − and + indicate the cells cultured for 2 days in the absence and presence of 0.8 μM OHT. E, the expressions of mTRF1 or Mer-Cre-Mer in various cells were analyzed by Western blot analysis using anti-mTRF1 or anti-Ero (Santa Cruz) Abs.

TRF1 Targeting and Expression Constructs—To generate mTRF1+/− ES cells, 20 μg of the linearized mTRF1 targeting plasmid was electroporated into 2 × 10^5 E14 ES cells as described (22). To generate mTRF1+/− ES cells that carry the mTRF1 transgene, the neo gene flanked by loxP sites in the targeted locus was removed by infection with adenovirus expressing the site-specific Cre recombinase (23). One of the mTRF1+/−, conTG+, Mer-Cre-Mer+ ES cell clones, HB, was retargeted with the targeting plasmid.

Flow Cytometric Analysis of Cell Cycle—ES cells were labeled for 30 min in media containing 20 μg/ml BrdUrd and fixed in 70% ethanol at −20 °C. After rehydration in phosphate-buffered saline, fixed cells were treated with 2 N HCl, 0.5% Triton X-100 for 20 min, washed extensively in blocking buffer (1% bovine serum albumin, 0.5% Tween 20 in phosphate-buffered saline), and stained with anti-BrdUrd Ab (Becton Dickinson) and fluorescein isothiocyanate-conjugated anti-rabbit IgG conjugated with Zenon Alexa 568 (Molecular Probes). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 4-9 diamino-2-phenylindole, DAPI, 4-8 diamino-2-phenylindole, DAPI, 4-8 diamino-2-phenylindole) and analyzed with AxioVision software (Zeiss).

Immunofluorescence Analysis—ES cells were treated with colcemid (0.1 μg/ml) for 2 h and allowed to swell in a hypotonic buffer (10 mM Tris-HCl, pH 7.4, 40 mM glycerol, 20 mM NaCl, 5 mM KCl, 1 mM CaCl_2, 0.5 mM MgCl_2). Cytospun cells were fixed with 4% paraformaldehyde for 10 min and washed with 0.5% Triton X-100 for 20 min, and incubated with rabbit anti-mTRF1, anti-mTRF2, or anti-mTIN2 Abs at 37 °C for 40 min. Anti-rabbit IgG conjugated with Zenon Alexa 568 Fluor (Molecular Probes) was used for detection of single staining. The nuclei were counterstained with DAPI, observed under fluorescence microscopy, and analyzed with AxioVision software (Zeiss).

Fluorescence in Situ Hybridization (FISH)—ES cells were harvested after colcemid treatment (0.1 μg/ml) for 2 h. The cells were washed with phosphate-buffered saline and fixed in methanol: acetic acid (3:1). The cell suspensions were placed on wet, clean slides and dried overnight. FISH with Cy3-labeled (C3TA2)4 peptide nucleic acid was performed as described (24).

Chromatin Immunoprecipitation (ChIP) and Slot Blot Analysis—Preparation of fixed chromatin and ChIP were performed as described (22). Fixed and solubilized chromatin before immunoprecipitation (for input DNA) and immunoprecipitated chromatin bound to protein A beads were digested with 100 μg/ml proteinase K for 90 min at 56 °C and extracted once with phenol/chloroform. The precipitated DNA was un-cross-linked for 8 h at 70 °C, denatured in 0.4 n NaOH for 5 min, neutralized with a 0.5 M Tris-HCl pH 8.0 solution containing 1.5 M NaCl, and slot-blotted onto a nylon membrane (Paul) with Hybri-slot hybridization; ChIP, chromatin immunoprecipitation; MBN, mammalian bean nuclease; OHT, 4-hydroxytamoxifen; PI, propidium iodide.

3 The abbreviations used are: ES, embryonic stem; mTRF1, murine TRF1; BrdUrd, bromodeoxyuridine; Ab, antibody; TUNEL, TdT-mediated dUTP nick-end labeling; DAPI, 4,6-diamino-2-phenylindole; DAPI, 4-8 diamino-2-phenylindole, DAPI, 4-8 diamino-2-phenylindole; DAPI, 4-8 diamino-2-phenylindole; FISH, fluorescence in situ hybridization; ChIP, chromatin immunoprecipitation; MBN, mammalian bean nuclease; OHT, 4-hydroxytamoxifen; PI, propidium iodide.

FIG. 1. Establishment of the conditional mTRF1-deficient ES cells. A, the partial restriction map of the endogenous mTRF1 locus (exons 1–3 of Terf1), the TRF1-targeting plasmid and the targeted locus are shown. RI, EcoRI, RV, EcoRV, B, BamHI, S, SpeI. B, the genotypes of cells analyzed in this study are shown. C and D, Southern blot analysis of ES clones summarized in B. Genomic DNA from ES cells was digested with BamHI (C) or EcoRV (D) and probed with the 3′ RIB probe (C) or the exon 1 probe (D). The bands corresponding to the endogenous (8.1 kb) and the targeted (KO) allele (6.6 kb) in the endogenous (4.5 kb) and mTRF1 cDNA-containing (5.5, 10, and 20 kb) fragments in D are indicated. − and + indicate the cells cultured for 2 days in the absence and presence of 0.8 μM OHT. E, the expressions of mTRF1 or Mer-Cre-Mer in various cells were analyzed by Western blot analysis using anti-mTRF1 or anti-Ero (Santa Cruz) Abs.
FIG. 2. Growth characteristics of the conditional mTRF1-deficient ES cells. A, the mouse TRF1 expression in ES cells at indicated days after 2 days of OHT treatment was analyzed with Western blot analysis using anti-mTRF1 Ab. Because these protein samples were electrophoresed on 15% SDS-PAGE, endogenous and exogenous TRF1 were not completely separated, and the bands overlapped in Western blot analysis. Consequently in HB cells treated with OHT, TRF1 bands show more reduced intensities than that in untreated HB cells that are expressing exogenous mTRF1 cDNA flanked by loxP sites. The asterisks indicate nonspecific bands. B, the growth characteristics of control ES cells, HB and M21, and TRF1−/−, conTG+ ES cells, LR8 and LR50, treated without (−) or with (+) 0.8 μM OHT for 2 days. The population doublings (PDLs) of control cells and that of conditional KO cells are plotted. C–E, the flow cytometric analyses of the conditional mTRF1-deficient ES cells are shown. The examined cells were pretreated without/with OHT 2 days and further cultured 2 days without OHT. The relative DNA contents were based on the staining with PI. The percentages indicate the apoptotic sub-G1 populations (C). Apoptotic cells were labeled with TUNEL and analyzed by flow cytometry (D). BrdUrd incorporation, together with PI staining to determine DNA content, was measured by flow cytometry (E). Representative flow cytometric plots are shown.

FIG. 3. Immunostaining analysis of mTRF2 and mTIN2 in the conditional mTRF1-deficient ES cells. A and B, nuclear localization and metaphase telomere association of mTRF1, mTRF2, and mTIN2 were analyzed in control ES cells, M21 (A) and TRF1−/−, conTG+ ES cell, LR8-1 (B) pretreated without (−) or with (+) OHT 2 days and further cultured 3 days without OHT. Nuclei or condensed chromosomes are visualized by DAPI staining (blue). C, LR8-1 pretreated with OHT was dual stained with anti-mTRF1 (TRF1 panel) and anti-mTRF2 (TRF2 panel). Intensities of the mTRF2 signals were reproducibly less extensive in TRF1-negative cells (right cell) than those in TRF1-positive (mTRF1 transgene undeleted) cells (left cell). The merged signals are visualized as yellow in the merged panel.
of 118 clones screened). These data suggested that mTRF1 may be essential for cellular growth or viability. Therefore, we next introduced a mTRF1 expression plasmid into 14-2C2 and then retargeted. Of 34 clones screened, four clones were targeted to the mTRF1 locus, and two (R11 and R35) were mTRF1−/− (data not shown). Western blot analysis confirmed no endogenous TRF1 expression in these mTRF1−/− ES cells expressing exogenous mTRF1 molecules (Fig. 1E, lane R35, and not shown). Because six amino acid substitutions exist between TRF1 derived from the 129 strain (E14) cDNA (DBJ data bases with accession number AB1058693) and the C57BL/6 strain (exogenous transgene) cDNA (25), it is possible to distinguish endogenous and exogenous TRF1 in terms of size in SDS-PAGE.

To further confirm that TRF1 is critical for cellular growth or viability and to examine how TRF1 is involved in these cellular events and the functional telomere structure, we established conditional mTRF1-deficient ES cells. We introduced a mTRF1 expression plasmid containing exogenous mTRF1 cDNA flanked by loxP sites and a plasmid expressing a Mer-Cre-Mer fusion molecule into 14-2C2. Mer-Cre-Mer is hormone-dependently (OHT) imported into the nucleus and functions as a site-specific Cre recombinase on the nuclear DNA substrate (26). From the mTRF1−/− ES cell clone HB, which contains these two plasmids (mTRF1−/−, conTG+), we established two clones of the conditional mTRF1 null mutant ES cells (mTRF1−/−, conTG+), LR8 and LR50, by retargeting with the mTRF1 targeting vector (Fig. 1, C and E, lanes LR8 and LR50). Genotypes of the cells used in this study are summarized in Fig. 1B.

**Growth Defect of mTRF1-deficient ES Cells**—We assayed the growth potential of the conditional mTRF1-deficient ES cells. Each of three sublines of LR8, LR50, control clones M21 (mTRF1−/−, TG+), or HB were cultured in the absence or presence of OHT (0.8 μM) for 2 days and examined for mTRF1 expression. Most of the exogenous mTRF1 in all LR8 and LR50 sublines and in HB disappeared by 2 days of OHT treatment, but not in M21 (Fig. 2A, row for 2 days of OHT treatment (−/+), and data not shown). Southern blot analysis also confirmed that most of the exogenous mTRF1 transgene flanked by loxP sites were OHT-dependently deleted (Fig. 1D, lanes HB, LR8, and LR50). OHT was washed out after 2 days of incubation, and the treated ES cells were further cultured. As shown in Fig. 2B (left panel), control ES cells treated with OHT did not show any growth defect. On the other hand, OHT pretreatment clearly induced growth delay to the conditional mTRF1-deficient ES cells LR8 and LR50, and the doubling time was extended to about twice that of OHT-untreated cells (Fig. 2B, right panel, +/−). This growth defect gradually recovered after 6 days of OHT (−) culture. Correlating with the growth recovery, re-expression of mTRF1 was observed in the OHT pretreated LR8 and LR50 (Fig. 2A, rows for 6 and 8 days low LR8 (+), and data not shown). These data indicated that a small percentage of the cells treated with OHT for 2 days still contained the mTRF1 transgene, and this minor but transgene-positive population expands because of a predicted advantage in growth over the mTRF1-negative cells. Furthermore, the mTRF1-deficient ES cell clones established from the LR8 and LR50 sublines treated with OHT also displayed continuous growth retardation phenotypes (data not shown).

Because TRF1 deletion induced growth defects, we examined p53 activation, p16 accumulation, and apoptosis induction in the conditional mTRF1 mutant ES cells treated with OHT. No clear accumulation of p16, p21, or p53 was observed by Western blot analysis (data not shown). However, PI and TUNEL staining showed a small (10–15%) but consistent induction of the sub-G1, and TUNEL-positive apoptotic population in the TRF1-deficient ES cells (Fig. 2, C and D). Furthermore, we performed cell cycle analysis using BrdUrd incorporation and PI staining. Flow cytometry analysis showed that the efficiency of BrdUrd incorporation into the cells and the S phase transition of the cell cycle were not affected in the growth-retarded conditional TRF1-deficient ES cells, but the relative percentage of the S phase population reproducibly decreased (average percentage ± standard deviation for three independent experiments; 59.15 ± 3.13% in 8-1 (+) against 71.81 ± 1.75% in 8-1 (−) cells; represented one of the three analyses in Fig. 2E). Because ES cells are difficult to arrest at any particular stage of the cell cycle, and these analyses could not be conducted under a synchronous condition, we were not able to determine the cell cycle stage that was affected by the TRF1 deficiency.

**Dissociation of TIN2 and TRF2 from Telomeres in mTRF1-deficient ES Cells**—Because the majority of the conditional TRF1-deficient ES cells survived in culture, we next studied how TRF1 deficiency affects telomere structure. Interphase
and metaphase spreads were prepared from control M21 ES cells or conditional LR8-1 mTRF1-deficient ES cells pretreated with or without OHT or mTRF1-deficient ES cells, and the nuclear and telomeric localization of the telomere-associated factors, mTRF1, mTRF2, and mTIN2, were examined. As shown in Fig. 3, without OHT pretreatment, mTRF1, mTRF2, and mTIN2 all formed telomere-associated nuclear speckles in interphase and localized in the telomeres of metaphase spreads in M21 and LR8-1 (Fig. 3, A and B; data not shown). In OHT-treated M21 cells, localization of these telomere factors was not affected (Fig. 3A). On the other hand, OHT-pretreated LR8-1 showed distinct staining profiles. In correlation with Western blot analyses (Fig. 2A), more than 90% of OHT-treated LR8-1 cells lost their mTRF1 foci (Fig. 3, B and C). Furthermore, in mTRF1-negative LR8-1 cells, mTIN2 lost telomeric association in both interphase and metaphase spreads, but mTRF2 remained associated with telomeres (Fig. 3, B and C, TIN2 and TRF2 panels; and data not shown). However, this telomeric association of mTRF2 was consistently and reproducibly less extensive (Fig. 3C, TRF1 and TRF2 panels). LR50 behaved identically to LR8-1 after OHT treatment, and such phenotypes were maintained in the TRF1-deficient clones (data not shown). These data demonstrated that mTIN2 is indeed an mTRF1-dependent telomere-associating factor, as in humans (18). It is now suggested that there is some functional interaction, direct or indirect, between mTRF1 and mTRF2 for the telomere association of mTRF2.

To further quantitate the effect of mTRF1 deletion on the telomeric association of these telomere-associated factors, we performed ChIP analyses. Fixed and solubilized chromatin fractions were prepared from control M21 and LR8-1 cells with or without OHT pretreatment and incubated with anti-mTRF1, anti-mTRF2, anti-mTIN2, or control Abs (rabbit preimmune serum and anti-CENP-A Ab). Because centromeres are physically adjacent to the p-arm telomeres in mice, we used CENP-A, one of the centromere-binding molecules, as a control...
target molecule for ChIP. As illustrated in Fig. 4A, OHT pretreatment induced mTRF1 deletion but had no effect on the expressions of mTRF2, mTIN2, and CENP-A in LR8-1 cells. Slot blot analysis demonstrated that telomeric DNA arrays were enriched by anti-mTRF1 and anti-mTIN2 Abs but not by preimmune serum and anti-CENP-A Ab from either M21 or LR8-1 cells (Fig. 4B and data not shown). In M21 cells, OHT treatment did not greatly affect the recovery of telomeric DNA arrays (Fig. 4, B and C, upper panels). However, in LR8-1 cells pretreated with OHT, the recovery of telomeric DNA arrays by anti-mTRF1 and anti-mTIN2 was reduced to less than and about 25%, respectively (Fig. 4, B and C, lower panels). Furthermore, telomeric signals of the OHT-treated LR8-1 cells immunoprecipitated with anti-mTRF2 were also reduced to about 40%. Importantly, anti-CENP-A specifically precipitated minor satellite DNA arrays from both cell lines, and the amount of precipitation was not changed despite OHT pretreatment, suggesting that the phenotypes of the telomere-associated factors in the OHT-treated LR8-1 are specific. The same findings were also obtained in other control and conditional mTRF1-deficient cell lines (data not shown). These results were consistent with our immunohistochemical staining data and further strengthened the notion that the telomeric localization of mTIN2 is dependent on that of mTRF1. Moreover, these data supported the supposition that there is no competition for telomeric binding between TRF1 and TRF2, as was proposed by de Lange and co-workers (12) and further suggested that mTRF1 is, to some extent, involved in the telomere association of mTRF2. In the *in vitro* studies, human TRF1 showed bending and parallel pairing activities against double-stranded DNA containing the telomeric repeats (27, 28). Therefore, it was speculated that these activities could facilitate or stabilize T-loop formation mediated by TRF2 *in vitro* (12). According to the previous data (29), TRF2 preferentially localizes to the junction where double-stranded telomeric repeats are invaded by the 3′ telomeric overhang in the T-loop structure. If complete deletion of TRF1 induces reduction of T-loop formation, it is possible that mTRF2 reduction associated with the telomere in the mTRF1-deficient ES cells may occur.

### Table I

| Endogenous TRF1 | TRF1 cDNA | 5 days after 2 days of OHT treatment |
|-----------------|-----------|-----------------------------------|
|                 |          | HB –OHT | HB +OHT | LR8-1 –OHT | LR8-1 +OHT | LR50–7 –OHT | LR50–7 +OHT |
| No. of metaphases analyzed | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| No. of chromosomes/metaphase | 40.1 | 40.3 | 41.2 | 40.4 | 41.2 | 40 | 41 |
| No. of abnormal telomere signalsa,b | 6 (0.3) | 7 (0.4) | 4 (0.2) | 39 (2.4) | 2 (0.1) | 205 (12.7) |
| No. of end-to-end fusions containing TTAGGGc | 0 | 0 | 0 | 6 (0.15) | 0 | 2 (0.05) |

**a** "Abnormal" denotes the presence of more than one telomere signal at the end of one sister chromatid.  
**b** The numbers in parentheses indicate the percentages of total telomeres detected. (Number of telomeres is four times the number of chromosomes.)  
**c** The numbers in parentheses indicate frequency per metaphase.

### Table II

| End-to-end fusions observed in mTRF1 KO cells |
|-----------------------------------------------|
| Clone 81–8 TRF1a | Clone 81–26 TRF1a | Clone 81–7 TRF1b | Clone 81–24 TRF1c |
| No. of metaphases analyzed | 10 | 10 | 12 | 12 |
| No. of chromosomes/metaphase | 41.2 | 41.1 | 39.4 | 37.5 |
| No. of end-to-end fusionsa | 11 (2.7) | 10 (2.4) | 55 (11.6) | 68 (15.1) |
| Containing TTAGGG | 1 | 0 | 34 | 53 |
| Not containing TTAGGG | 10 | 10 | 21 | 15 |
| No. of chromosome fragmentsb | 2 | 0 | 1 (0.2) | 1 (2.7) |
| No. of ring chromosomes (chromatid-type fusion)c | 0 | 0 | 1 (0.2) | 4 (0.9) |

**a** The numbers in parentheses indicate percentages of metaphase chromosomes detected.

### Abnormal Telomere Structure and Chromosomal Instability in mTRF1-deficient ES Cells—Finally, we examined the telomeric DNA arrays, telomere length, and chromosomal stability in the mTRF1-deficient ES cells. Metaphase spreads prepared from M21, LR8-1, and LR50-7 cells with or without OHT pretreatment were hybridized with the peptide nucleic acid-telomere probe. As shown in Fig. 5A, typical 4 spot telomere signals on one metaphase chromosome were observed in M21 cells with or without OHT treatment and LR8-1 and LR50-7 cells without OHT treatment. However, in LR8-1 and LR50-7 cells pretreated with OHT and cultured for 3 more days, metaphase chromosomes showed abnormal and diffused telomere signals. Approximately 12% (11.7% for LR8-1 and 12.7% for LR50-7) of the metaphase chromosomes examined displayed more than one telomere signal at the end of one sister chromatid (*inset* in Fig. 5A, LR50-7 (1+), and Table I). Furthermore, we frequently observed unequal intensities of two telomere signals between one arm of sister chromatid ends, and about 2% of the chromatid ends completely lost telomere signals (Fig. 5A, *inset*, LR8-1 (1+), and Table I). Chromosome instability in the form of end-to-end fusions was barely detected in the short term cultured mTRF1-deficient ES cells as revealed by DAPI staining (Table I).

Induction of replicative senescence or overexpression of dominant-negative TRF2 induces 3′ overhang of single-stranded telomeric DNA erosion (15, 30) and overexpression of dominant-negative TRF1 induces progressive telomere elongation (17). Therefore, we examined the telomere length and status of the 3′ overhang of the telomeric arrays in the conditional mTRF1-deficient ES cells treated with OHT by Southern blot analysis. Although we have seen abnormal telomere signals in the metaphase spreads prepared from the short term cultured mTRF1-deficient ES cells (Fig. 5A), by means of TRF assay, there were no clear changes in their size, distribution, and signal intensity (Fig. 5B). Furthermore, the single-stranded 3′ overhang of the telomeric signals was not considerably affected in LR8-1 or LR50-7 cells pretreated with OHT (Fig. 5C). The present data suggest that the frequently observed abnormal telomere signals, detected by peptide nucleic acid-telomere FISH, in the short term

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cultured TRF1-deficient ES cells, hardly ever result from dynamic telomere DNA rearrangements. We also examined telomeric arrays and chromosomal stabilities in the mTRF1-deficient ES cell clones prepared after about 50 cell divisions. We found accumulation of abnormal chromosomes in mTRF1-deficient cells in addition to the abnormal telomere signals as seen in the short term cultured mTRF1-deficient cells, and as many as 11–15% of metaphase chromosomes showed end-to-end fusions (Fig. 5E and Table II, clones 81-7 and 81-24). Furthermore, many chromosome anomalies might exist as minute segments of chromosome aberrations (Fig. 5F, arrow). Intensities of telomeric DNA signals on the ends of mTRF1-deficient metaphase chromosomes became highly divergent in contrast to those of the mTRF1-positive clones (Fig. 5D), potentially indicating the induction of inter-telomeric recombinations. Supporting this possibility, at least some population of the TRFs detected by the telomere probe clearly elongated in these mTRF1-deficient ES cell clones (Fig. 5F). Of course, the reduction of telomeric association of mTRF2 in mTRF1-deficient cells may have contributed to this chromosomal instability phenotype, in addition to the growth defect.

There was no growth defect in the human cell lines overexpressing dominant-negative TRF1 (17). The reason why two different TRF1 knock-out systems gave rise to different results is not clear, but this issue is probably important for dissecting the roles of TRF1 in cell growth. One possibility is that the cells overexpressing dominant-negative TRF1 sustained some endogenous TRF1 at their telomeres, which might be sufficient for their normal cell growth. Another possibility is that the growth potential associated with TRF1 might be independent from telomere-bound TRF1. Furthermore, we could not evaluate the proposed function of TRF1 on telomere length regulation because of the much longer telomeric DNA arrays in mouse (Fig. 5B). However, the current data strongly suggested that mTRF1 integrates into the “functional telomere structure,” and therefore the dysfunction of TRF1 is potentially involved in cellular senescence related with telomere erosion and chromosomal instabilities. So far, a number of TRF1 interacting molecules such as TIN2, Tankyrase, RUV70/80, and PINX1 have been shown to mimic or modulate some functions of TRF1 (18, 19, 31, 32). Furthermore, recent findings demonstrated that association of POT1 with telomeres is partly mediated by TRF1 (33). To further address the issue of how TRF1 is integrated into the functional telomere structure, it is important to clarify how each TRF1-associated molecule and other telomere-binding factors are involved with the phenotypes of the mTRF1 null cells described in this study.

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Addendum—While this manuscript was under review, Karlseder et al. (34) reported that deletion of the first exon of mouse TRF1 (Terf1) results in growth defects and promotes embryonic death, consistent with our results.

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