Supplemental Data

Mammalian Telomeres Resemble Fragile Sites and Require TRF1 for Efficient Replication

Agnel Sfeir, Settapong T. Kosiyatrakul, Dirk Hockemeyer, Sheila L. MacRae, Jan Karlseder, Carl L. Schildkraut, and Titia de Lange

Supplemental Experimental Procedures

Cell culture and retroviral infection

MEFs were isolated from E13.5 embryos and cultured in DMEM supplemented with 1 mM Na-pyruvate, 100 U of penicillin per ml, 0.1 µg of streptomycin per ml, 0.2 mM L-glutamine, 0.1 mM nonessential amino acids, and 15% (vol/vol) fetal calf serum (FCS). Immortalized MEFs were cultured in media with 10% FCS without sodium pyruvate. Cre recombinase was introduced using Hit&Run-Cre (Silver and Livingston, 2001), Ad5 CMV Cre (Resource Center, The University of Iowa, Iowa City, IA), or pWZL-Cre as described previously (Celli and de Lange, 2005). Treatments of cultured cells were as follows. Synchronization of cells in G_0 was performed with primary MEF lines by contact inhibition and serum starvation. Primary MEFs were plated at $1*10^6$ cells per 10 cm dish or $0.5*10^6$ cells per 6 cm dish in media supplemented with 15% FCS. When cells reached confluence, serum levels were dropped gradually to 0.5% over a period of 5 days and the cells were maintained for an additional 2 days in media with 0.5% serum. Subsequently, cells were infected twice with Ad5-CMV-Cre (m.o.i. of 1000) and harvested 4 days later. shRNAs for Blm and ATR were introduced using 4 infections at 6 hr intervals of the shRNA bearing pSuperior puromycin retrovirus-containing supernatants from Phoenix cells supplemented with 4 µg/ml polybrene. Parallel infection with shLuciferase was used as a negative control. shRNA for Rtel1 was introduced using 2 infections at 12 hr intervals of lentivirus-containing supernatant from 293T cells. Parallel infection with PLK0.1 was used as a negative control. Puromycin selection was applied for 3 days at 2 µg/ml. Full-length mouse TRF1 (aa 2-421), $TRF1^{\Delta Ac}$ (aa 55-421) and $TRF1^{\Delta Myb}$ (aa 2-363) were cloned into pLPC-puro retroviral expression vector and introduced into MEFs by 3 retroviral infections at 12 hour intervals using supernatant from transfected Phoenix cells. Puromycin selection was applied for 3 days at 2 µg/ml.

SV40 Large T immortalized $Wrn^{-/-}$, $Blm^{-/-}$, and $Wrn^{-/-}$ $Blm^{-/-}$ mouse ear fibroblasts (a gift from Brad Johnson) were cultured in DMEM media containing 10% fetal calf serum. The

BLM mutation is a hypomorphic allele BLM^{m3} that has been previously shown to induce high levels of homologous recombination and increased rates of loss of heterozygosity (Luo et al., 2000). HeLa 1.3, BJ-hTERT and HTC75 cells were cultured in DMEM media supplemented with 10% bovine calf serum (BCS).

TRF1 gene targeting

The mTRF1 locus was isolated from 129 SV BAC library (Genome Systems) using full-length cDNA as a probe. The targeting vector contained a TK-Neomycin cassette flanked by Lox P sites cloned into a HindIII site in the first intron. A third Lox P site was introduced by inserting an oligonucleotide into a PvuII site upstream of exon 1. ES clones with the correct integration were identified by genomic blotting of HindIII digested DNA using a probe flanking the left arm of the construct. Cre recombinase was transiently expressed in the targeted ES clones to generate ES subclones that had lost the TK-neomycin cassette but retained exon 1 flanked by LoxP sites (floxed allele, F) and subclones that had lost both exon 1 and the TK-neomycin cassette (null allele, -). Two ES cell subclones for each allele were used to generate chimeras, which delivered offspring with the TRF1F/+ or TRF1+/- genotypes. TRF1 mice were maintained in a mixed background (129/C57Bl6). Genotyping PCR for TRF1 was performed using the following primers: F2: TGCTGCTGCTGCCATAACGCTCAA; F1: TATACTTACAGCGCTGGGAAG; and R: GGCCAAAAGACGGAAATTTGA. The amplification reaction was performed in a volume of 25 μl containing 1 μl DNA, 25 pmol of each primer, 0.1 μM dNTPs, 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH 8.0), and 0.5 U of Taq polymerase (Takara Taq). PCR conditions were as follows: 95°C for 1 min, 35 rounds of 95°C for 30 s, 57°C for 45 sec, and 72°C for 1 min and 72°C for 5 min.

Analysis of telomeric DNA by pulse-field gel electrophoresis and in-gel hybridization

Telomeric overhang signals and telomeric restriction fragment patterns were determined by in-gel analysis as previously described (Celli and de Lange, 2005). Briefly, a [CCCTAA]4 oligonucleotide was hybridized to native MboI cut genomic DNA fractionated on CHEF gels to determine the overhang signal. The DNA was denatured in situ, neutralized, and then rehybridized with the same probe to determine the total telomeric DNA signals. The overhang signal in each lane is normalized to the duplex telomeric signal so that comparison of these ratios reveal changes in the overhang status.

Telomere FISH and CO-FISH on metaphase spreads

FISH for telomeric DNA was performed as previously described (Dimitrova et al., 2008). Briefly, cells at the indicated time points and treatments were incubated for 1.5 h with 0.2 µg/ml colcemid. The cells were harvested, swollen in KCl, fixed in methanol/acetic acid (3:1) and dropped onto glass slides in a Thermotron Cycler (200C, 50% humidity). After aging overnight, the slides were washed in 1X PBS for 5 minutes followed by consecutive incubation with 75%, 95% and 100% ethanol. The slides were allowed to air dry for 30 minutes before applying Hybridizing Solution (70% formamide, 1 mg/ml blocking reagent (Roche), 10 mM Tris-HCl pH 7.2) containing FITC-OO-(CCCTAA)3 PNA probe (Applied Biosystems). The spreads were denatured for 3 min at 80ºC on a heat block and hybridized at RT for 2 hours. The slides were washed twice with 70% formamide/10 mM Tris-HCl (15 minutes each wash), followed by three washes in 0.1 M Tris-HCl, pH 7.0/0.15 M NaCl/0.08% Tween-20 (5 minutes each). The chromosomal DNA was counterstained with 4,6-diamidino-2-phenylindole (DAPI) added to the second wash. Slides were mounted in antifade reagent (ProLong Gold, Invitrogen) and digital images were captured with a Zeiss Axioplan II microscope with a Hamamatsu C4742-95 camera using Improvision OpenLab software.

For CO-FISH the cells were treated with 10 µM BrdU:BrdC (3:1) for 16 h and colcemid was added for the last 1.5 hour at a concentration of 0.2 µg/ml. Prior to hybridization the slides were treated with RNase A (0.5 µg/ml in PBS) for 10 min at 37^0C, stained with Hoechst 33258 (0.5 µg/ml in 2XSSC) for 10 min at RT and exposed to 365-nm UV light (Stratalinker 1800 UV irradiator) for 30 min. The BrdU/dC substituted DNA strand was digested with Exonuclease III (10 U/ml) for 10 min at RT. The slides were dehydrated through an ethanol series (75%, 95% and 100%) as above and hybridized with TAMRA-OO-(TTAGGG)3 PNA probe in hybridization solution for 2 hours. The slides were washed for few seconds with 70% formamide/10 mM Tris-HCl pH 7.2 and incubated with FITC-OO-(CCCTAA)3 PNA probe in hybridization solution for 2 hours. The subsequent steps were as for the FISH protocol.

Immunoblotting

Cells were harvested by trypsinization, suspended in media with serum, washed with PBS and lysed in Laemmli buffer (100 mM Tris-HCl pH 6.8, 200 mM DTT, 3% SDS, 20% glycerol, 0.05% bromophenol blue) at 10^4 cell per µl. The lysate was denatured for 10 min at 95ºC, and sheared (10 times) by forcing it through an insulin needle. Per lane,

lysate from 10^5 cells was resolved on SDS/PAGE (5% for ATM and ATR and 10% for all other proteins), transferred to a membrane, and blocked in PBS with 5% milk/0.1%Tween-20. The following primary antibodies were incubated in PBS/5% milk/0.1% Tween-20: TRF1 (1449, rabbit polyclonal); TRF2 (1254, rabbit polyclonal); Rap1 (1252, rabbit polyclonal); POT1a (1220, rabbit polyclonal); Chk2 (mouse monoclonal, BD Biosciences); Phopho-Chk1 (Ser 345) (mouse monoclonal, Cell Signaling); Chk1 (mouse monoclonal, Santa Cruz); ATM (mouse monoclonal, MAT3, Sigma); ATR (N-19) (goat polyclonal, Santa Cruz); BLM (rabbit polyclonal, Bethyl Laboratories); γ-tubulin (clone GTU 88, Sigma). After incubation with the appropriate secondary antibody, immunoblots were developed with enhanced chemiluminescence (ECL, Amersham).

IF-FISH and TIF assay

All steps were performed at room temperature unless indicated otherwise. Cells grown on coverslips were fixed for 10 min in 2% paraformaldehyde followed by three 5-min washes with PBS. For indirect immunofluorescence, coverslips were incubated in Blocking Solution (1 mg/ml BSA, 3% goat serum, 0.1% Triton X-100, 1 mM EDTA in PBS) for 30 min, followed by incubation with primary antibodies in Blocking Solution for 2 hours: 53BP1, 100-304A (rabbit polyclonal, Novus Biologicals); TRF1, 1449 (affinity purified rabbit polyclonal, raised against a GST-fusion of mouse TRF1 without the Myb domain); γ-H2AX, mouse monoclonal (Upstate Biotechnology); Rap1, 1252 (affinity purified rabbit polyclonal). After three 5-min washes with PBS, the coverslips were incubated with Rhodamine Red-X labeled secondary antibody raised against rabbit (RRX, Jackson) for 30 min and washed 3 times in PBS. Coverslips were dehydrated 70%, 95% and 100% ethanol, 5 min each, and allowed to air dry. FITC-OO-(CCCTAA)3 (Applied Biosystems) PNA probe in Blocking Solution (70% formamide, 1 mg/ml blocking reagent (Roche), 10 mM Tris-HCl pH 7.2) was added and the coverslips were denatured on a heat block (10 min at 80ºC). Hybridization was for 4 hours in the dark. The coverslips were washed twice with 70% formamide, 10 mM Tris-HCl pH 7.2 for 15 min and three times in PBS. DNA was counterstained with DAPI and slides were mounted in antifade reagent (ProLong Gold, Invitrogen). Digital images were captured with a Zeiss Axioplan II microscope with a Hamamatsu C4742-95 camera using Improvision OpenLab software. For the TIF assay, cells with at least five telomeric 53BP1 foci were

scored as TIF positive; n>100 for each experiment. Data reported are averages of three independent experiments and error bars indicate the standard deviations.

FACS

One day before harvesting, 10*6 cells were plated on 10 cm dishes. 10 μM BrdU was added one hour prior harvesting. Cells were collected by trypsinization, washed in PBS, and resuspended in PBS with 1 mM EDTA. Cells were fixed with ice cold 70% ethanol at 40^0C for at least 30 min. Cells were washed twice with 0.5% BSA in PBS and re-suspended in 0.4 ml 0.5% BSA in PBS containing 5 μg propidium-iodide and RNAseA (100 μg/ml). Samples were analyzed with a FACS calibur flow cytometer (Becton Dickinson) using FlowJo software.

Senescence-associated β-galactosidase staining

SA-β-GAL staining was preformed as previously described (Dimri et al., 1995). TRF1F/F cells were infected with vector control or pWZL Cre and selected with hygromycin for 5 days. At day 6 after infection 10*5 cells were plated in a 6-well cell culture plate. The following day, the cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS for 3 minutes and washed twice in PBS. The cells were then incubated with 3 ml of SA-β-GAL stain (1 mg/ml 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-Gal), 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$) at 37°C for 8 to 16 hrs in the dark. Cells were washed twice with PBS and photographed.

ChIP analysis

ChIP was performed as previously described (Loayza and de Lange, 2003). The TTAGGG signal was normalized to BamHI repeats. The following antibodies were used as crude sera: TRF1, 1449 (rabbit polyclonal); TRF2, 1254 (rabbit polyclonal); Rap1, 1252 (rabbit polyclonal); POT1a, 1220 (rabbit polyclonal); TPP1, 1150 (rabbit polyclonal).

Northern for TERRA

Total cellular RNA was prepared using RNeasy Mini Kit (Qiagen), according to the manufacturer instructions and Northern blot analysis was performed as previously described (Azzalin et al., 2007). Briefly, 10μg RNA was loaded onto 1.3% formaldehyde

agarose gels and separated by gel electrophoresis. RNA was transferred to a Hybond membrane. The blot was prehybridized at 60°C for 1 h in Church mix (0.5 M Na_2HPO_4 (pH 7.2), 1 mM EDTA, 7% SDS, and 1% BSA), followed by hybridization at 60°C overnight with 800-bp telomeric DNA probe from pSP73Sty11 labeled by Klenow fragment and [32P]dCTP. The blot was exposed to a PhosphorImager screen and scanned using Image-Quant software.

Telomere Length analysis

Cells were harvested by trypsinization, washed with cold Phosphate Buffered Saline (PBS), and lysed in Tris/NaCl/EDTA/SDS (TNE,10 mM Tris-HCl pH 7.4, 100 mM NaCl, 10 mM EDTA, 0.1%SDS) containing 0.1 mg/ml proteinase K at 37°C o/n. Two Phenol extraction steps with phenol/chloroform/isoamyl-alcohol (50:49:1) were performed in phase-lock tubes (Eppendorf). DNA was precipitated with iso-propanol and NaOAc, dissolved in TE (10 mM Tris-HCl pH 7.4, 1 mM EDTA) and digested with MboI/AluI. DNA concentrations were measured by Hoechst fluorimetry and 2 µg of DNA were fractionated on a 0.7% agarose gel. Hybridizations, washes and signal detection were performed as described by (Smogorzewska et al., 2000).

Semi quantitative RT-PCR analysis

Total cellular RNA was prepared from MEFs using RNeasy Mini Kit (Qiagen). 1 µg of RNA was subjected to reverse transcription using random oligo primer and ThermoScript RT-PCR System (Invitrogen) according to the manufacturer's protocol. Mouse RTEL1 cDNA was amplified by PCR with sense: 5'- CCT GAA TGG TGT GAC AGT GG-3' and antisense: 5'- CAG GAT GAC AAG GTC CGA CT- 3'; GAPDH cDNA was amplified by PCR with sense: 5'- GGG TGA GGC CGG TGC TGA GTA T -3' and antisense 5'- TTG GGG GTA GGA ACA CGG AAG G -3'. PCR reaction consisted of denaturing for 30 sec at 94^0C, annealing for 40 sec at 58^0C and elongation for 30 sec at 72^0C. The PCR products were examined at the indicated number of the cycles.

Supplemental References

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Supplemental Figures

Supplemental Figure 1

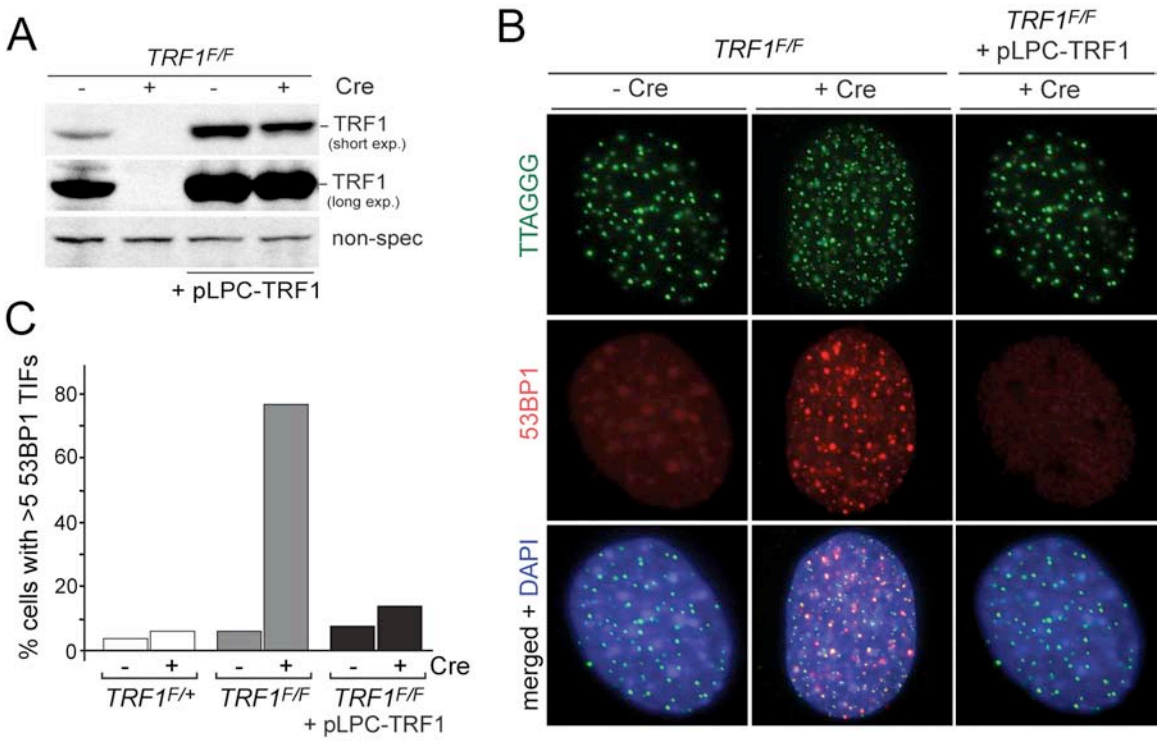


Figure S1. Exogenous TRF1 represses the phenotype of TRF1 deletion.
(A) Immunoblots for TRF1 in *TRF1*$^{F/F}$ MEFs and *TRF1*$^{F/F}$ MEFs expressing exogenous TRF1 (+pLPC Myc -TRF1) after H&R Cre infection or mock infection (-Cre).

(B) Cells of the indicated genotype and treatment were analyzed for TIF formation using FISH-IF with an antibody for 53BP1 (red) and a PNA probe (green) for telomeric DNA. DNA was counterstained with DAPI (blue).

(C) Quantification of the percentage of cells with 5 or more 53BP1 TIFs for the indicated treatment. Method as in (B).

Supplemental Figure 2

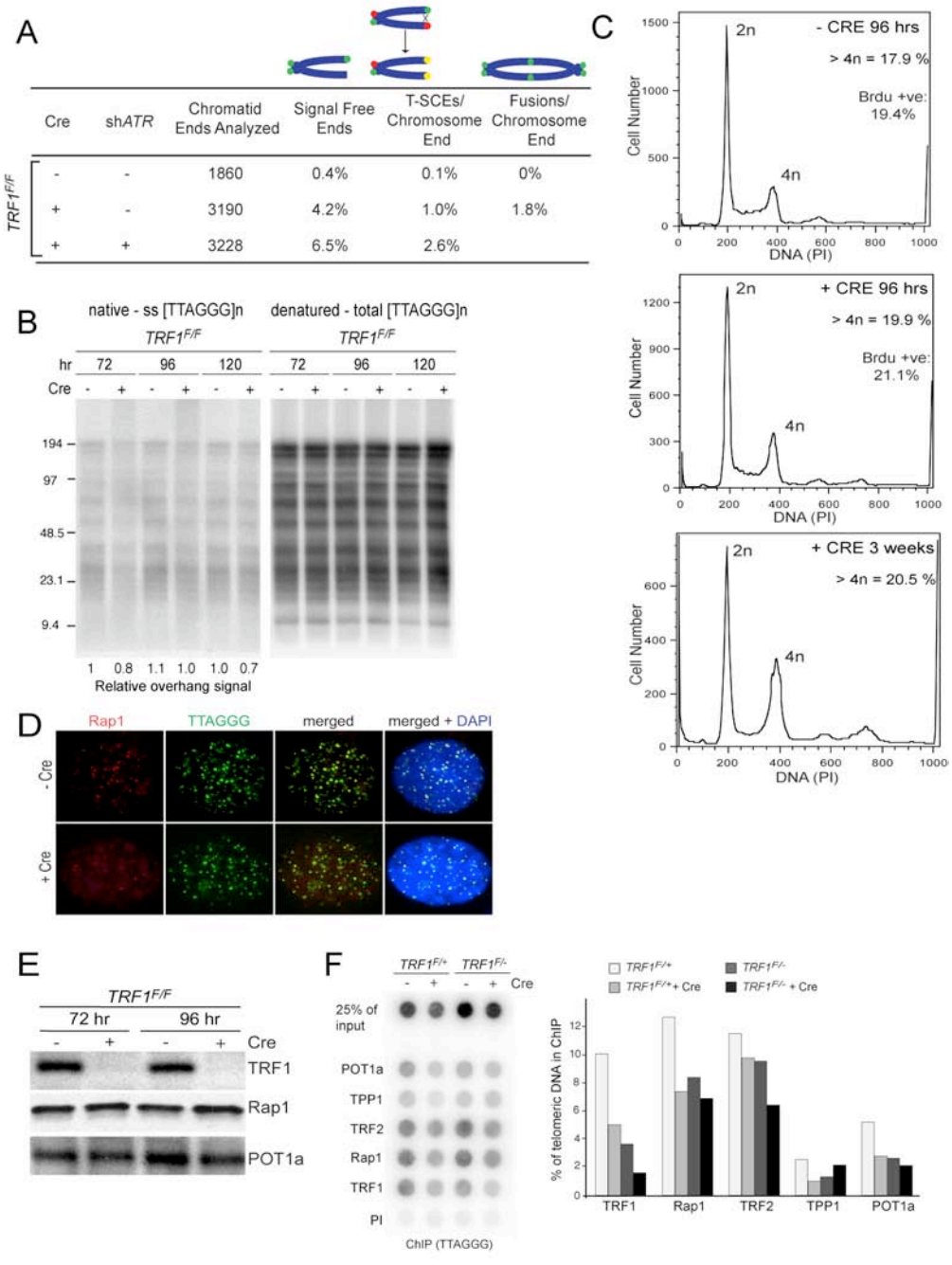


Figure S2. Effect of deletion of TRF1 from immortalized MEFs on telomere function and structure, cell cycle profiles, and other shelterin components.

(A) Frequencies of signal-free end, telomeric sister chromatid exchanges (T-SCEs) and telomere fusions in *TRF1*$^{F/F}$ MEFs with the indicated treatment and analyzed at day 4 after infection with Cre.

(B) In-gel detection of the structure of telomeric DNA from *TRF1*$^{F/F}$ MEFs at the indicated time points post Cre infection. The image on the left represents hybridization using a (CCCAAT)4 probe to detect the telomere overhang under native conditions. The image on the right represents total telomere hybridization signal obtained with the same probe after in situ denaturation of the DNA. The numbers on the bottom left represent the relative overhang signal.

(C) FACS profiles of *TRF1*$^{F/F}$ cells infected with pWZL-Cre or vector control analyzed at day 4 after infection. MEF lines infected with pWZL-Cre were also analyzed 3 weeks after infection. The percentage of cells >4n DNA content is noted within the FACS profile. Cells harvested at day 4 were pulsed with BrdU for 1 hr prior to harvesting to determine the S phase index. The percentage of Brdu positive cells is noted within the FACS profile.

(D) Immunofluorescence analysis for Rap1 at telomeres at day 4 post pWZL-Cre or vector control (-Cre) in *TRF1*$^{F/F}$ cells.

(E) Immunoblots to detect TRF1, Rap1 and POT1a at day 3 and day 4 after pWZL-Cre or vector control (-Cre) treatment of *TRF1*$^{F/F}$ cells.

(F) Telomeric DNA ChIP for shelterin. ChIPs with the indicated antibodies on *TRF1*$^{F/+}$ and *TRF1*$^{F/-}$ cells infected with pWZL-Cre or vector control and analyzed at day 4. Left: Dot blot of the precipitated telomeric DNA detected with a TTAGGG repeat probe. PI, pre-immune serum. Right: Bar graph of quantification of the % of TTAGGG repeats recovered in the IPs. The same results were obtained in a second, independent ChIP experiment.

Supplemental Figure 3

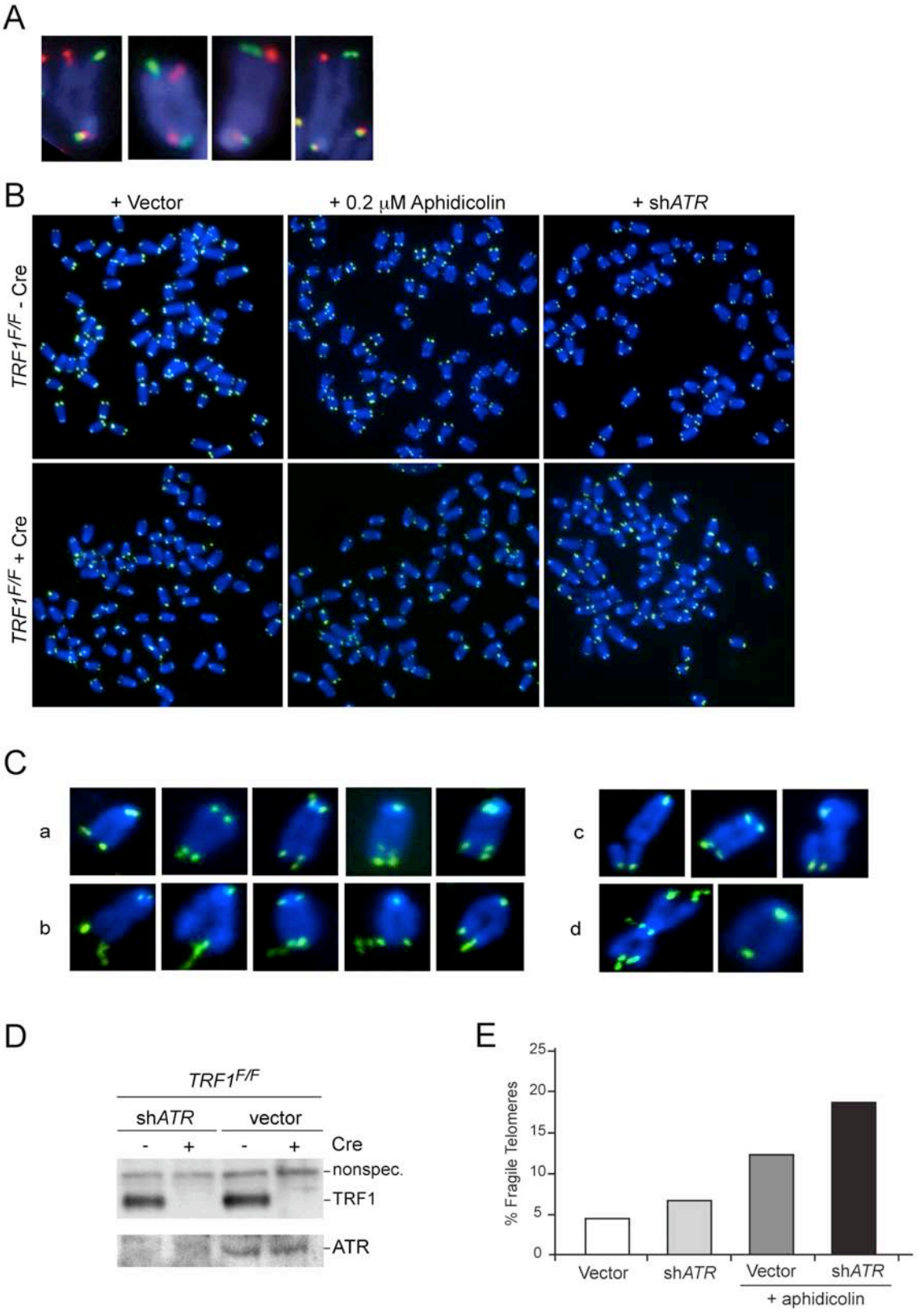


Figure S3. Fragile telomere phenotypes upon TRF1 deletion.

(A) Examples of fragile telomere phenotypes at both sister telomeres. TRF1$^{F/F}$ MEFs treated with H&R Cre and analyzed 4 days later using CO-FISH to visualize both sister telomeres. Among 471 fragile telomeres analyzed , 45% contained the parental G-strand

(B) Metaphase spreads of *TRF1$^{F/F}$* MEFs with the indicated treatment and stained for telomeric DNA (FITC PNA probe in green) and DAPI (blue).

(C) Examples of fragile telomeres (a) and (b), chromosome breaks (c), and telomere fusion (d).

(D) Immunoblot showing TRF1 deletion and ATR knockdown in the cells used for the data in Fig. 2. The non-specific band serves as a loading control.

(E) Quantification of the percentage of fragile telomeres in TRF1-proficient cells following treatment with 0.2 μM aphidicolin and/ or ATR shRNA.

Supplemental Figure 4

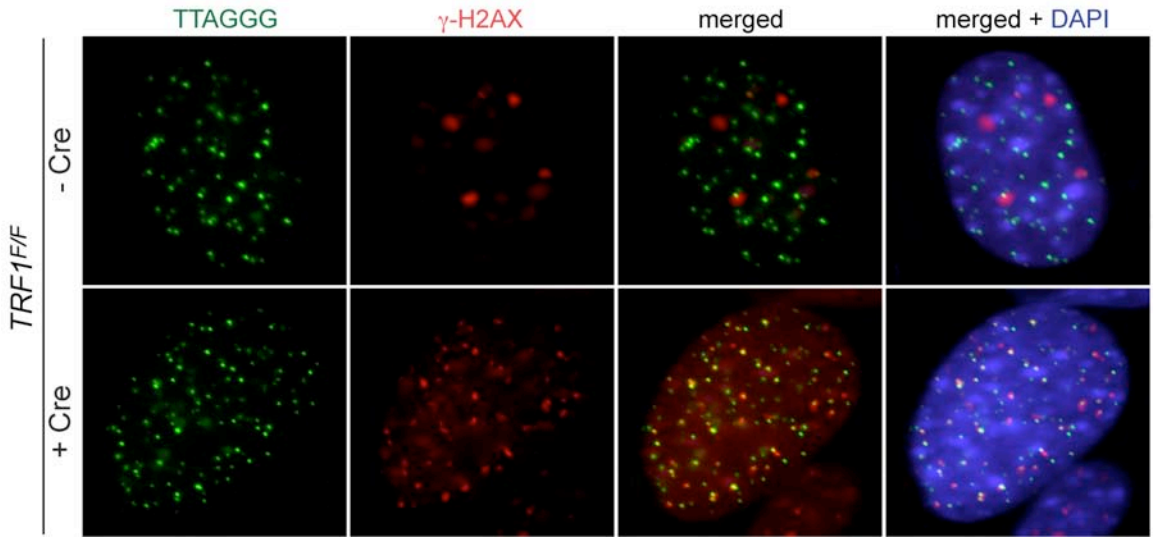


Figure S4. γ-H2AX at telomeres after deletion of TRF1.

TRF1$^{F/F}$ MEFs treated with H&R Cre or the empty vector were analyzed for FISH-IF by staining telomeres with a PNA probe (green) and γ-H2AX antibody (red). DNA was counterstained with DAPI (blue).

Supplemental Figure 5

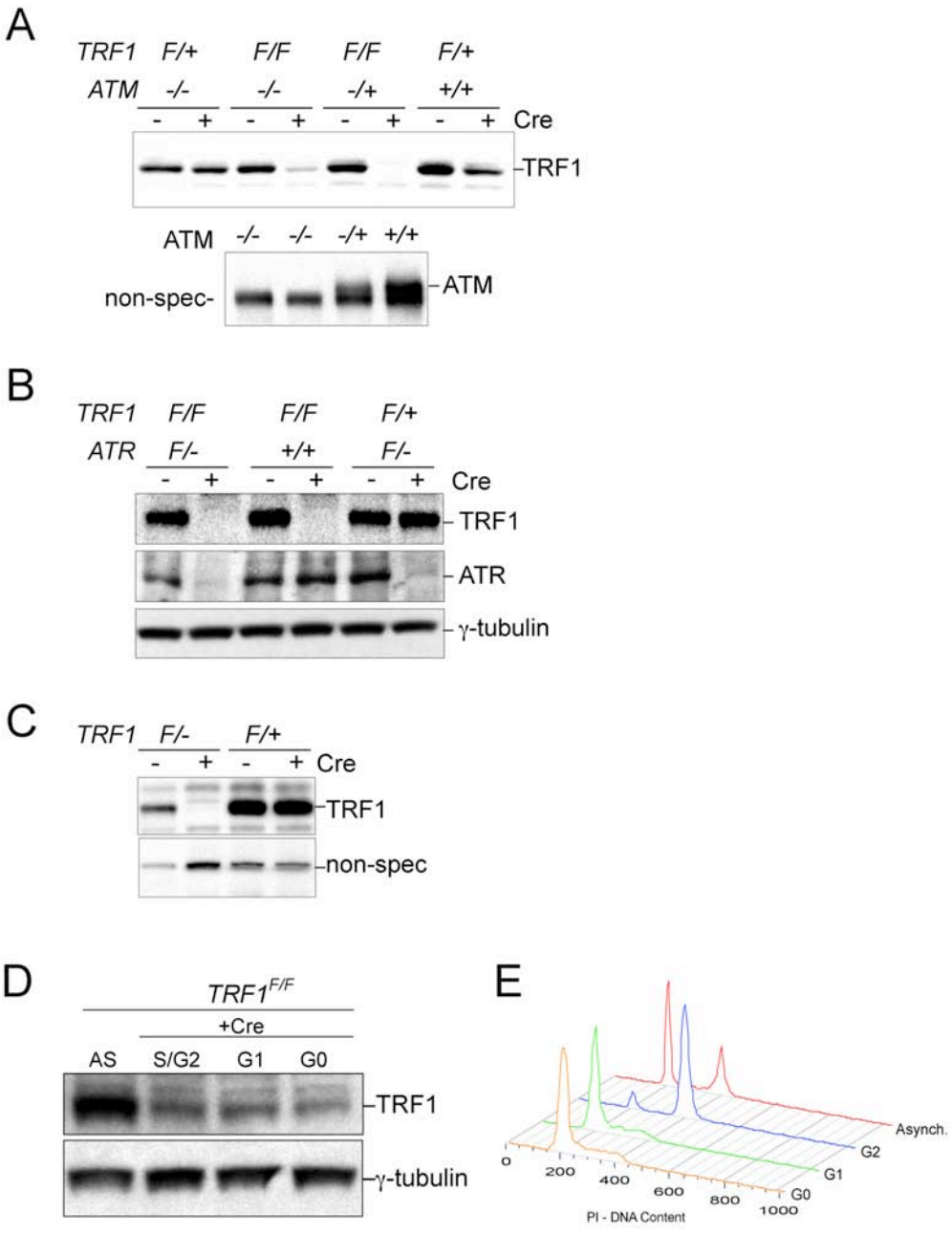


Figure S5. Immunoblots and FACS analysis relating to Figure 3.

(A) Immunoblots verifying ATM status and TRF1 deletion in the experiments shown in Fig. 3A and B.

(B) Immunoblots verifying ATR and TRF1 deletion in the experiments shown in Fig. 3A and B.

(C) Immunoblot showing TRF1 deletion from quiescent primary $TRF1^{F/-}$ cells used in Fig. 3A and B.

(D) Immunoblots showing partial deletion of TRF1 in the experiments in Fig. 3E.

(E) FACS profile (PI DNA content) of the samples used in Fig. 3E.

Supplemental Figure 6

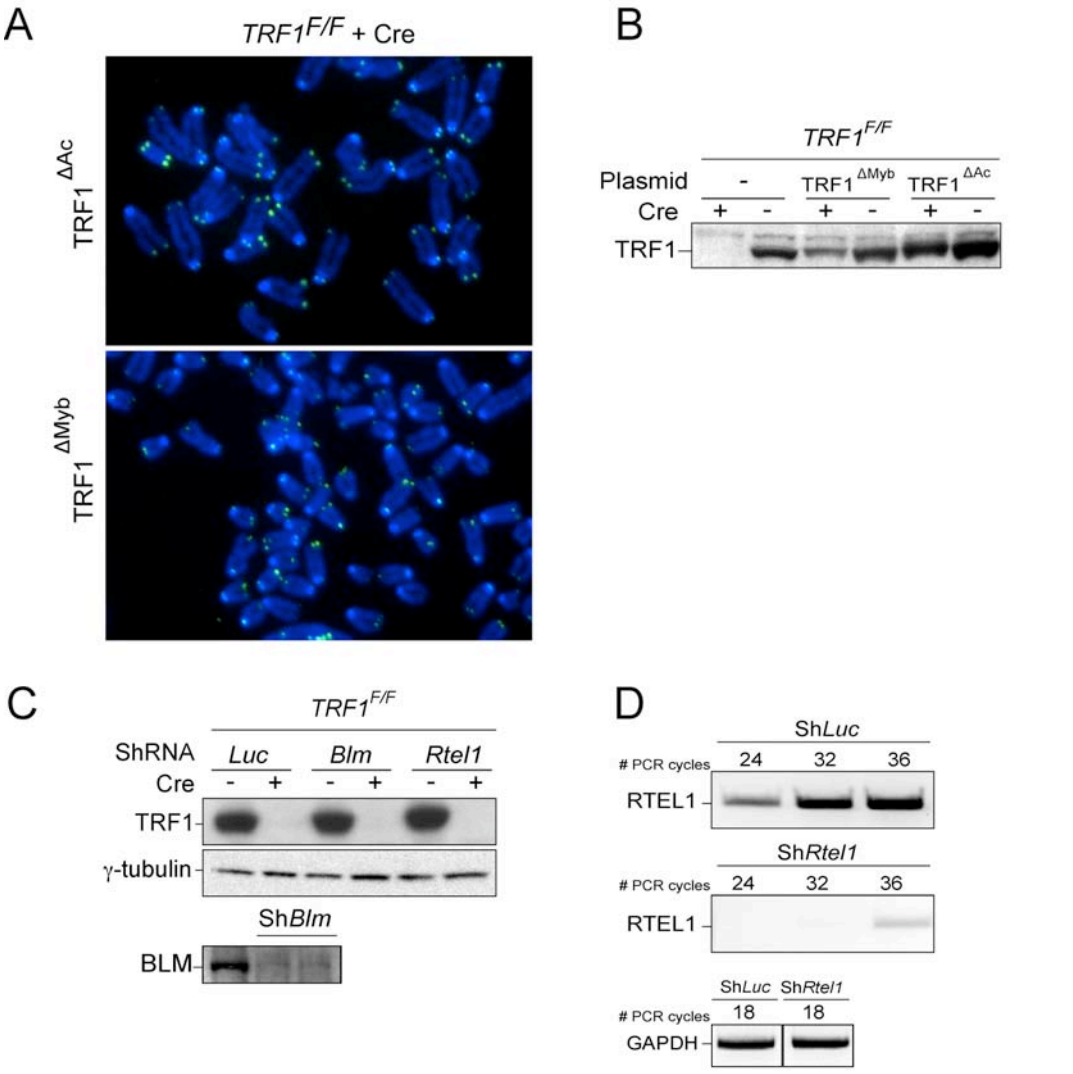


Figure S6. Metaphas spreads and Immunoblots relating to Figure 3.

(A) Metaphase spreads of *TRF1*$^{F/F}$ MEFs cells with the indicated treatment stained for telomeric DNA with a FITC PNA probe (green) and with DAPI (blue).

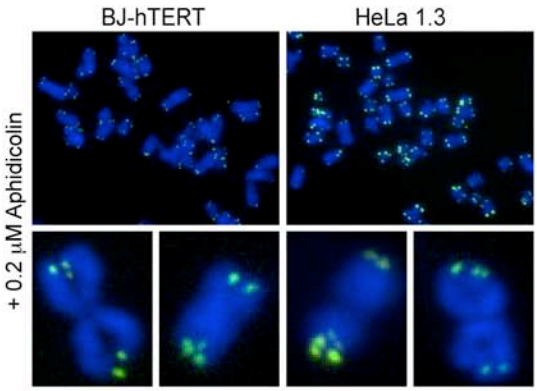
(B) Immunoblots of *TRF1*$^{F/F}$ MEFs expressing TRF1^{DAc} or TRF1^{DMyb} and treated with H&R Cre. The cells were analyzed at day 4 after Cre treatment.

(C) Immunoblot for TRF1 and *Blm* in *TRF1*$^{F/F}$ MEFs with indicated treatment at day 4 after Cre treatments.

(D) RT-PCR for *Rtel1*. RNA derived from TRF1 null cells infected with shRNA-encoding Lentivirus (*Rtel*1 and *Luc*) was processed to detect *Rtel* mRNA and *Gapdh* mRNA with RT-PCR.

Supplemental Figure 7

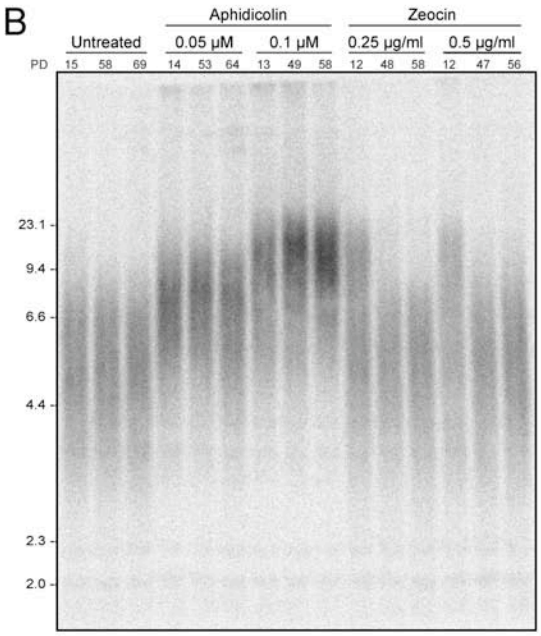
A



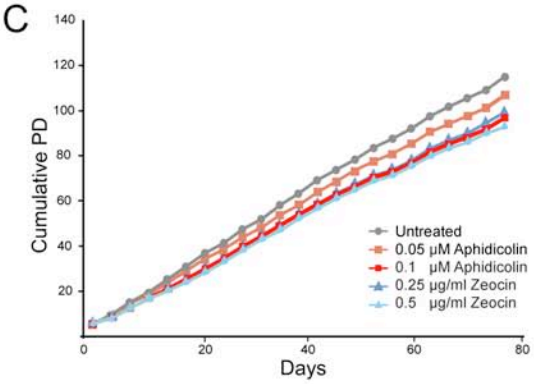
HeLa 1.3	Chromatid ends	Fragile Telomeres
Control	4650, 3496, 1793	5.3% ± 0.9
0.2 μM Aphidicolin	5112, 3189, 1448	9.9% ± 1.7

BJ-hTERT	Chromatid ends	Fragile Telomeres
Control	n= 1263, 1290	3.7%, 3.1%
0.2 μM Aphidicolin	n= 1539, 1715	7.5%, 7.1%

B



C



D

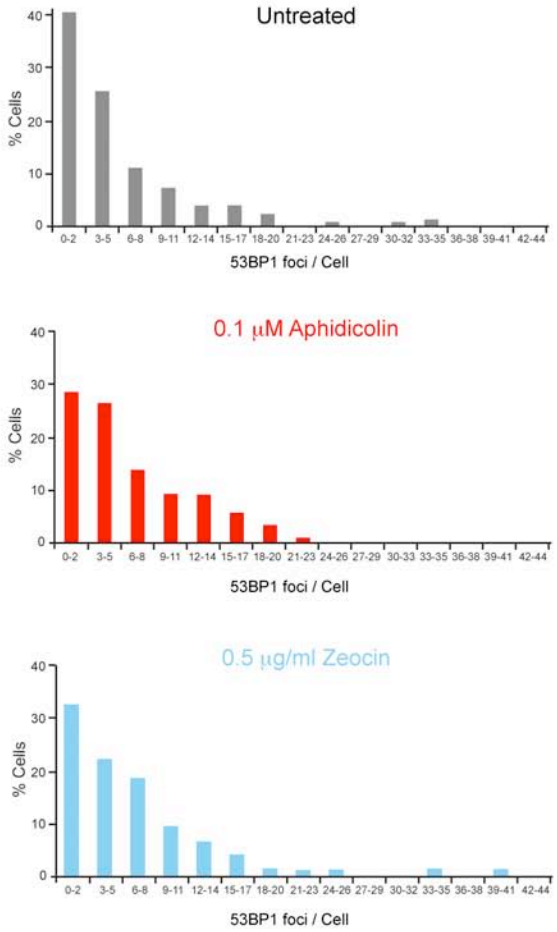


Figure S7. Fragile telomeres in human cells.

(A) Metaphase chromosomes of BJ-hTERT (human foreskin fibroblasts expressing ectopic telomerase) and Hela1.3 (a HeLa subclone with long telomeres) cells treated for 16hrs with 0.2 μM Aphidicolin and stained with a telomeric probe (FITC PNA probe in green) and DAPI (blue). The frequency of fragile telomeres in BJ-hTERT and Hela1.3 following treatment with 0.2 μM aphidicolin is represented in the tables.

(B) Genomic blot of telomeric restriction fragments for HTC75 cells with the indicated treatments and concentrations. DNA at the indicated PD was analyzed by Southern blotting using a double-stranded TTAGGG repeat probe.

(C) The number of 53BP1 damage foci/cell in untreated HTC75 cells (upper panel), and in cells treated with 0.1 μM aphidicolin (middle panel) or 0.5 μg/ml Zeocin (lower panel).

(D) Graph representing growth curves of untreated HTC75 cells as well as HTC75 treated with aphidicolin (0.1 and 0.05 μM) and Zeocin (0.25 and 0.5 μg/ml).