SUPPLEMENTARY DATA

Supplementary Material and Methods

Q-FISH and CO-FISH

Q-FISH and CO-FISH were performed according to established protocols using strand specific telomeric PNA C-rich (TelC-Cy3, Panagene, F1002) and LNA G-rich (TelG-FAM, Exiqon, custom made) probes (1).

Briefly, metaphase spreads were fixed in 4% formaldehyde in PBS for 2 min, washed 3 x 5 min in PBS 1x, treated with pepsin (1 mg/ml in 0.05 M citric acid pH 2) for 10 min at 37°C, post-fixed for 2 min, washed, and dehydrated though an ethanol series. For CO-FISH, treatments with Hoechst, UV and ExoIII were performed as described (1) to degrade the neosynthesized DNA strand containing BrdU/C. For both Q- and CO-FISH, 40 µl of a hybridization mix containing 100 nM TelC-Cy3 probe in 70% formamide, 10 mM Tris pH 7.4, and 1% blocking reagent (Roche, 11096176001) was applied to each slide. Slides were co-denatured in the presence of the probe by heating to 80°C for 3 min. After 2 hour hybridization at room temperature, slides were washed 2 x 15 min in 70% formamide, 20 mM Tris pH 7.4, then 3 x 5 min in 50 mM Tris pH 7.4, 150 mM NaCl, 0.05% Tween-20, dehydrated in successive ethanol baths and air-dried. For Q-FISH, slides were mounted in Vectashield with DAPI (Vector, H-1200). For CO-FISH, slides were next hybridized for 2 hours with 100 nM TelG-FAM probe in 50% formamide, 2XSSC, 1% blocking reagent prior to 2 x 15 min washes in 50% formamide, 2XSSC, and 3 x 5 min washes in 50 mM Tris pH 7.4, 150 mM NaCl, 0.05% Tween-20. Slides were dehydrated and mounted as already described for Q-FISH.

Images of labeled metaphase spreads were acquired using Zeiss Axioplan 2 imaging system. Telomeric signals were quantified using the iVision software (BioVision Technologies).
Signals were first segmented using the Segmentation tool and then manually corrected to include individual telomeres at all metaphasic chromosome ends. Average pixel intensities were quantified and corrected for the average local background intensities of metaphase spreads. Telomere intensity values coming from the human species were normalized with the mean of the telomere intensities coming from Muntjac chromosomes.

**Chromatin preparation**

Cells were crosslinked directly in cell culture dishes using 1% formaldehyde (Thermo Scientific, 28908) for 10 min at 37°C, before adding 0.125 M of glycine (Sigma) for 5 min at room temperature. After two washes in cold PBS, nuclei were prepared by homogenization in cell lysis buffer (5 mM PIPES pH 8, 85 mM KCl, 0.5% NP-40) and centrifugation at 1800 × g for 10 min. Finally, nuclei from two 300 cm² flasks were lyzed in 1 ml nuclei lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 1% SDS) and followed by sonication for 30 min (30 sec on / 30 sec off) in a Diagenode water bath-sonicator at speed 5. After centrifugation at 14000 rpm for 10 min at 4°C, the cleared supernatants were snap frozen in liquid nitrogen and stored at -80°C.

**Meta-TIF assay**

HT1080 and ST metaphases were prepared by centrifugation after a 7-minute hypotonic shock in 0.8% sodium citrate solution. Slides were immediately fixed with 3.7% formaldehyde in PBS, rinsed twice in double-distilled water, permeabilized in Solution KCM (10 mM Tris pH7.5, 120 mM KCl, 20 mM NaCl, 0.1% triton-X-100) and kept over night in PBS at 4°C. For the immunofluorescence (IF), slides were treated with RNase A (0.1mg/ml) in ABDIL buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2% BSA, 0.1 % Triton X-100) for 15 min at 37°C, incubated
with mouse anti-γ-H2AX antibody (Millipore, 1:1000), followed by the goat anti-mouse Alexa 488 (Invitrogen). All antibody incubation steps were performed in a humid incubator at 37°C for 45 min with antibodies diluted in the ABDIL buffer, followed by 3 x 2 min washes in PBST (0.1 % Tween-20 in PBS). For subsequent FISH experiments, IF slides were fixed with 3.7% formaldehyde in PBS for 10 minutes, rinsed once in PBS and dehydrated in 70%, 80%, 90% and 100% ethanol series. Telomere FISH was performed as described above in the Q-FISH protocol. Slides were washed 2 x 5 min in Wash A (70% formamide, 10 mM Tris pH7.5) and 2 x 5 min in Wash B (50 mM Tris pH 7.5, 150 mM NaCl, 0.8% Tween-20) prior to mounting in Vectashield with DAPI (Vector, H-1200).

DNA extraction

For the detection of C-circles, cells were collected by trypsinization, resuspended in 300 µl PBS and lysed with an equal volume of either 2x Hirt buffer (20 mM Tris pH 7.5, 200 mM NaCl, 20 mM EDTA, 01% SDS) or 2x Buffer T (20 mM Tris pH 7.5, 20 mM NaCl, 20 mM EDTA, 1% sarcosyl). Lysates were treated with RNase A for 1h at 37°C, over night with proteinase K at 55°C and extracted with phenol:chloroform:isoamylalcohol (Invitrogen) followed by chloroform:isoamylalcohol (Sigma) using 5 PRIME PhaseLock gel (Fischer, FP2302820). DNA was precipitated using 2 volumes of ethanol containing 1/10th volume of sodium acetate (3M, pH 5.2) for 1 hour at –80°C and centrifugation at 16 000 g for 30 min at 4°C. Ethanol washed pellets were dried with SpeedVac (no heat) and resuspended in 10 mM Tris 7.5 to prevent the acid hydrolysis of DNA.

2D gel assay for detection of T-circles
MboI digested restriction fragments were first separated by size in 0.4% Ultrapure agarose (Invitrogen) in 1xTBE at ~1V/cm overnight, then by shape in the second dimension using 1% agarose in 1xTBE containing 0.1 µg/ml ethidium bromide. Gels were blotted onto Biodyne B positively charged nylon membrane (Thermo Scientific) and probed with digoxigenin-labeled telomeric LNA probe (Exiqon, custom made). The hybridization was carried out over night at 42°C in modified Church buffer. Hybridization washes were performed in 100 mM Na₂HPO₄ and 2% SDS. The digoxigenin-labeled probe was detected using anti-digoxigenin alkaline phosphatase antibodies and CDP-Star detection kit (both Roche) following manufacturer’s instructions.

**C-circle assay**

5 µg of genomic DNA was digested overnight with Hinfl and RsaI (40 U each). In the morning, an additional 10 U of each enzyme were added and the digestion was continued for another 2 hours before precipitation of DNA with ethanol/sodium acetate. For the C-circle amplification, 500 ng of digested genomic DNA was incubated with the Phi 29 polymerase (Fermentas) in the presence of the buffer provided by the manufacturer and 2 mM dATP, dGTP and dTTP at 30°C for 12 hours and the reaction was stopped by heating samples to 65°C for 20 min. Phi 29 polymerase was omitted from the reaction as negative controls (No Phi). Amplified products were denatured in 0.2N NaOH/1 mM EDTA by heating to 95°C for 10 min, sot-blotted onto Biodyne B positively charged nylon membrane (Thermo Scientific) and detected by hybridization to a 3’ digoxigenin-labeled C-rich telomeric oligo TAA(CCCTAA)₄–DIG. Signal was revealed using the digoxigenin-detection kit (Roche) and CDP-Star reagent (Roche) following the manufacturer’s instructions. Images were obtained using the Luminescent image analyzer LAS-4000 mini (GE Healthcare).
RNA FISH for TERC detection

Cells were seeded in 4-well slides (VWR) with a density of 15,000 cells. The day after, cells were fixed for 10 min in 4% formaldehyde (Sigma), 1x PBS, and 10% acetic acid. After two washes in PBS 1x, cells were permeabilized in 70% ethanol overnight at 4°C. The following day slides were air-dried and rehydrated in 50% formamide and SSC 2x for 5 min and prehybridized for 1 hour at 37°C in solution RHM (50% deionized formamide, 10% Dextran sulfate (Fluka), 2x SSC, 2 mg/ml BSA, 10 mM RVC (Ribonucleoside Vanadyl Complex), 40 µg/µl tRNA from Escherichia coli, 1 µg/µl N50 (random oligo of about 50 bp). Slides were then incubated O/N at 37°C in RHM solution containing three hTR probes: hTR128–183 (GCT*GACATTTTT*TGT TTGCTCTCT*AGAATGAACGGT*GGAAGGCAGCGAGGGCT*T), hTR 331–383 (CT*CCGTTCCTTCTCT*CAGGCTGAAAGGCCT*GAACCTCGCCCT*CGCCCCCGAGT*G) and hTR 393–449 (AT*GTGTGAGCCGAGCT*TCCCACAGCTCAGGGAAT*CGCGCCGCGCT*C), where T* indicates aminoallyl-modified thymidines. The following day, slides were washed twice for 30 min in the Washing solution (50% formamide (Sigma), 2x SSC). Subsequently, immunofluorescence for the detection of Cajal bodies was performed. Slides were blocked with 10% goat serum in PBS, incubated with mouse anti-Coilin antibody (Sigma, 1/1000), followed by fluorescently labeled secondary antibody (Invitrogen, goat anti-mouse Alexa 488, 1/400). All incubation steps were done in a humid incubator at 37°C for 30 min. Slides were mounted in Vectashield containing 0.2 µg/ml DAPI.

Quantitative (q)PCR-TRAP assay

Whole cell CHAPs extracts were analyzed for the telomerase activity using the PCR-based semi-quantitative Telomerase Repeat Amplification Protocol (TRAP) (2).
For relative TRAP activities across cell lines (Figures 1E and 3B), whole cell extracts corresponding to 1000 cells were analyzed. For relative TRAP activities following depletion of RTEL1 by siRNA (Figure S7C), 200 ng of protein extracts were used. The assay was carried out using GoTaq qPCR Master Mix (Promega) and primers TS (5’-AATCCGTCGAGCAGAGTT-3’) and ACX (5’-GCACGGCTTACCCTACCCCTACCCTAAC-3’) at 200 nM. The reactions were run on a real-time PCR system (Applied, 7500) with the following protocol: 25ºC for 20 min, 95ºC for 10 min and 32-40 cycles with 95ºC for 30 sec, 60ºC for 30 sec and 72ºC for 1 min. To validate the specificity of TRAP, heat-inactivated (10 min at 85ºC) protein extracts were included in the assay. The PCR products and 10 bp DNA Ladder (Invitrogen) were resolved on NuPAGE Novex 20% TBE gel (Invitrogen) using DNA Loading buffer (Invitrogen). The gel was stained in 40 ml of 1xTBE containing SYBRgreen Gold nucleic acid gel stain (Invitrogen S-11494, 1/10,000) for 10 min and the characteristic DNA ladder for TRAP was visualized with UV.

Supplementary references

1. Ourliac-Garnier, I. and Londono-Vallejo, A. (2011) Telomere length analysis by quantitative fluorescent in situ hybridization (Q-FISH). Methods in molecular biology, 735, 21-31.
2. Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L., Coviello, G.M., Wright, W.E., Weinrich, S.L. and Shay, J.W. (1994) Specific association of human telomerase activity with immortal cells and cancer. Science, 266, 2011-2015.
3. Pickett, H.A., Cesare, A.J., Johnston, R.L., Neumann, A.A. and Reddel, R.R. (2009) Control of telomere length by a trimming mechanism that involves generation of t-circles. The EMBO journal, 28, 799-809.
**Supplementary Tables**

**Table S1. List of antibodies used for ChIP**

| Name   | Species | Reference/ Lot          |
|--------|---------|-------------------------|
| IgG    | Rabbit  | Abcam, ab37415/ GR12046-1 |
| TRF1   | Rabbit  | Abcam, ab1423           |
| TRF2   | Rabbit  | Novus, NB110-57130/ B2  |
| POT1   | Rabbit  | Epitomics, 5334-1/ YJ082315C5 |
| hTERT  | Sheep   | In house, S.B. Cohen, SBC-XXI-38b |

**Table S2. Consequences of experimentally induced RTEL1 dysfunction**

| Model system (Refs.) | Mouse ES cells (9,10) | Mouse fibroblasts (12,19,39) | Human primary fibroblasts, immortalized fibroblasts, most tumor cells (this study) | Super-telomerase human cells (high telomerase activity, very long telomeres) (this study) |
|----------------------|-----------------------|-------------------------------|---------------------------------------------------------------------------------|----------------------------------------------------------------------------------|
| Type of intervention | KO                    | Inducible KO                  | siRNA                                                                           | siRNA                                                                            |
| Telomere length      | Shortening (30%)      | Shortening, high heterogeneity | No effect                                                                        | Shortening (10-15%)                                                             |
| Telomere instability | No instability before differentiation, high level of instability upon differentiation (telomere loss, telomere fusion and damage) | High level of instability (sudden loss, T-loop excision, fragility, T-SCE) | No effect                                                                        | Low level of instability (small increase in T-SCE, limited telomere damage) |
| Telomere structure   | ?                     | T-loop excision, reversion at stalled replication forks | No effect                                                                        | Decrease of overhang length, decrease in POT1 association with telomeres        |
| Telomerase association with telomeres | ?                     | Increased association (with abnormal substrates?) | ?                                                                                | Increased association (with normal substrates?)                                  |
| Consequences         | Lower efficiency for telomere elongation | Block normal mechanisms of reversed fork resolution leading to deletion | None                                                                            | Lower efficiency for telomere elongation                                         |
Figure S1. Depletion of RTEL1 does not impact telomere length in HCA2, U2OS, HT1080 and HeLa I

(A) Western blot showing depletion of RTEL1 normalized with actin and relative to siControl.

(B) Representative metaphases after telomeric Q-FISH using TelC-Cy3 PNA probe (red) and counterstaining with DAPI.

(C) Telomere intensities analyzed in 30 metaphases of experimental conditions siControl (siC) and siRTEL1#1 (siR). Mean telomere intensities for each condition are indicated.
Figure S2. Depletion of RTEL1 affects all telomeres in HT1080-ST

(A) Histogram of telomere intensities analyzed by Q-FISH with a characteristic shift to the left (telomere shortening) upon RTEL1 depletion. n, number of telomeres analyzed from 30 metaphases. Mean, mean telomere intensities. P value, Wilcoxon rank-sum test.

(B) Percentage of telomere intensities below and above the chosen threshold showing an accumulation of short telomeres at dispense of the longest telomeres. Data are represented as mean ± confidence interval, α = 0.05.
Figure S3. Repetitive depletion of RTEL1 induces cell-cycle arrest in HT1080-ST
(A) Experimental outline. Cells were repeatedly, every 72 hours, transfected with siRNA against RTEL1 or control.
(B) Growth curves. Mean values of three independent cultures with standard deviation are shown.
(C) Cell cycle analysis after 72 hour transfection. Transfected cells were exposed to a 10-min pulse of BrdU prior to fixation and staining with anti-BrdU antibody (green) and propidium iodide (red, FLA-3). Each flow cytometry analysis includes over 18000 cells.
(D) Light microscopy on Day 6. Cells were seeded at equal concentrations on Day 3. Arrowheads, senescent cells, red color suggests polynucleation. Scale bar, 100 µm.
Figure S4. Depletion of RTEL1, while increasing recombination activities, does not lead to overt telomere replication stress or T-loop excision in HT1080-ST

HT1080-ST transfected with siRTEL1#1 or siControl were analyzed 72-hours post-transfection. (A) Abrupt telomere loss (T-loss) and (B) telomere (T-) fragility as detected by Q-FISH. No differences are detected between control and RTEL1-depleted cells. The mean values (n = 30 metaphases) ± SEM are indicated. P values, two-tailed student t-test.
(C) On the left, a schematic representation of different telomeric DNA species separated by two-dimensional gel electrophoresis to detect the presence of T-circles. On the right, depletion of RTEL1 does not significantly change the accumulation of T-circles, which are naturally present in HT1080-ST cells due to telomere trimming (1).

(D) RTEL1 induces sister chromatid exchanges (SCEs). BrdU was incorporated during two cell cycles prior to treatment of metaphases with UV and staining with DAPI to reveal exchanges between chromatids. On the left, representative images. Scale bars, 10 µM. On the right, quantification of the data. For each metaphase the number of SCEs per chromosome were determined. The mean values (n=30 metaphases) ± SEM are indicated. P value, two-tailed student t-test.

(E) RTEL1 also induces homologous recombination at telomeres as detected using the chromosome oriented (CO)-FISH assay used to score for telomeric (T)-SCEs. On the left, representative images. Scale bars, 10 µM. Enlarged intersections show a chromosome with a T-SCE for siRTEL1 and without for siControl. On the right, quantification of the data. The mean values (n=30 metaphases) ± SEM are indicated. P value, two-tailed student t-test.
Figure S5. Depletion of RTEL1 does not lead to C-circle accumulation
(A) Schematic representation of the C-circle assay.
(B) Phi 29 amplification products were spotted on the membrane and revealed using TAA(CCCTAA)_4-DIG probe. No amplification of C-circles is detected in untransfected HT1080-ST cells or cells transfected with siControl, siRTEL1#1 and #2, while C-circles are readily detectable in an ALT positive cell line U2OS-ST.
Figure S6. Colocalization of RTEL1 with telomeres

(A) Presence of RTEL1 at telomeres was scored in interphase nuclei stained with anti-RTEL1 antibodies (Alexa 488, green), TelC-Cy3 PNA probe (red) and DAPI (blue) using 3D microscopy. Representative average projections with ten-fold enlarged areas of interest are shown. Scale bars, 20 µm and 0.2 µm, respectively. Arrowheads point to the colocalization events.

(B) Quantification of A. Histogram shows the percentage of nuclei with one, two or three RTEL1-telomere colocalization events. Number of nuclei analyzed: HT1080, 200; HT1080-ST, 160; HCA2, 150. Pearson Chi-squared test, p-value = 1.293e-10.
Figure S7. RTEL1 is not required for telomerase biogenesis or recruitment of TERT to telomeres in HT1080-ST

(A) RTEL1 depletion does not impact TERC accumulation at Cajal bodies. ImmunoFISH showing colocalization of Coilin (green) with TERC (red) in nuclei (DAPI, blue) treated with siControl and siRTEL1#1. Scale bars, 10 µm.
(B) Quantification of A. Mean (n=30 nuclei) ± SEM. P value, two-tailed student t-test.
(C) RTEL1 depletion does not impact telomerase activity. Visualization of telomerase activity by gel electrophoresis following qPCR-TRAP using protein extracts from untransfected HT1080-ST or cells treated with siControl and siRTEL1#1. 10 bp DNA ladder is shown.
(D) RTEL1 depletion does not prevent telomerase recruitment to telomeres. Chromatin immunoprecipitation (ChIP) of telomeric DNA in HT1080-ST following downregulation of RTEL1. Serial dilutions of input chromatin and ChIPed material using antibodies against hTERT and IgG are shown.
(E) Quantification of D. ChIP signals were normalized to the input and siControl. Mean (n=3) ± SEM. P value, two-tailed student t-test.
(F) Experimental outline. HT1080-ST cultured in the presence of 10 µM BIBR1532 or DMSO for 40 days were transfected with siControl or siRTEL1 and analyzed by telomere Q-FISH.
(G) Telomere Q-FISH analysis. Mean telomere intensities (n, 30 metaphases) from two independent experiments ± SD. No additive affects of RTEL1 depletion and BIBR1532 on telomere shortening are observed.
Figure S8. RTEL1 depletion does not influence the level of single stranded telomeric G-rich repeats in HT1080 and HCA2 cells

|          | HT1080 | HCA2 |
|----------|--------|------|
| siContr  |        |      |
| siRTEL1#1|        |      |
| siRTEL1#2|        |      |

**Native/Denatured relative to siContr #1:**
1.99 .83 .81 .67 .37

**Native/Denatured relative to siRTEL1#2:**
1 .44

Native/Denatured relative to siContr #1:
Native Denatured
(AACCCT)3

**Relative enrichment of single strand telomeric G-rich DNA**

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**Note:** The image shows a gel electrophoresis result for the detection of telomeric G-rich repeats in HT1080 and HCA2 cells. The data indicates that RTEL1 depletion does not influence the level of single stranded telomeric G-rich repeats in both cell lines.
(A) In-gel hybridization for detection of telomeric single strand G-rich repeats. 6.5 µg of genomic DNA from HT1080-ST cells transfected with siControl#1, siControl#2, siRTEL1#1 and siRTEL1#2 was resolved on 0.7% agarose and hybridized under native condition using radioactively labeled telomeric C-rich probe. Following denaturation, the gel was re-hybridized using radioactively labeled telomeric C-rich probe to detect total telomeric G-rich repeats. ExoI, digestion of DNA with ExoI removing the G-rich overhang.

(B) Western blot showing downregulation of RTEL1 in HT1080 and HCA2, using a specific antibody against RTEL1. Depletion was normalized with actin and relative to siControl.

(C) In-gel hybridization for detection of telomeric single strand G-rich repeats. See A.

(D) Quantification of B. The amount of single stranded telomeric G-rich repeats were normalized with total telomeric G-rich repeats and are represented relative to siControl. Mean (n = 2) ± SEM.
Figure S9. RTEL1 depletion does not alter the shelterin composition in HT1080 cells

(A) Western blot showing efficient depletion of RTEL1 in HT1080 using two different siRNAs (#1 and #2). Depletion was normalized with actin and relative to siControl.

(B) Telomeric enrichment of TRF1, POT1 and TRF2 and IgG as determined by ChIP. Input dilutions for each chromatin preparation used for normalization are also shown.

(C) Quantification of B. Telomeric enrichments were normalized with inputs and are represented relative to siControl.
Figure S10. POT1 or POT1-OB-fold expression does not rescue single stranded telomeric G-rich repeats in HT1080-ST cells following depletion of RTEL1
Representative images of HT1080-ST metaphases analyzed by Q-FISH after down-regulation of RTEL1 or use of siControl and expression of GFP, GFP-POT1 and GFP-OB. The quantification is shown in Figure 5D-E. Scale bars, 10 µM.