Supplementary Materials for

ATR blocks telomerase from converting DNA breaks into telomeres

Charles G. Kinzig[1,2], George Zakusilo[1,2], Kaori K. Takai[1], Logan R. Myler[1], Titia de Lange[1]*

Correspondence to: delange@rockefeller.edu

This PDF file includes:

Materials and Methods
Figs. S1 to S6

Materials and Methods

Cell culture

HeLa-superT cells were cultured in Dulbecco's modified Eagle medium (DMEM, Corning) supplemented with 15% fetal bovine serum (FBS, Gibco), non-essential amino acids (Gibco), 2 mM L-glutamine (Gibco), and 100 U/mL penicillin plus 100 µg/mL streptomycin (Gibco). Phoenix A and HEK293FT cells were cultured in DMEM supplemented with 10% bovine calf serum (BCS) and non-essential amino acids, L-glutamine, penicillin, and streptomycin, as above. All RPE1 cell lines were cultured in DMEM/F-12 medium (Gibco) supplemented with 10% FBS (Gibco) and 100 U/mL penicillin plus 100 µg/mL streptomycin (Gibco).

Immunoblotting

Whole-cell lysates were prepared via direct lysis of 10^4 cells per µL 2X Laemmli lysis buffer (100 mM Tris-Cl pH 6.8, 20% glycerol, 2% SDS, 0.025% bromophenol blue, 300 mM β-mercaptoethanol). Lysed samples were heated at 95°C for 5 min and then sonicated. The equivalent of 10^5 cells was fractionated by SDS-PAGE on Tris-glycine gels and transferred to nitrocellulose membranes. Membranes were blocked in 5% nonfat dry milk in PBST (0.1% Tween-20 in PBS) for 1 h at room temperature and then incubated with primary antibody using the specified antibody dilution and conditions (see antibody table below) overnight at 8°C. Membranes were washed 3 times in PBST, incubated for 1 h at room temperature in secondary antibodies in 5% milk/PBST, and washed 3 times more in PBST. Membranes were developed using chemiluminescence with SuperSignal West Pico PLUS (ThermoFisher).

Lentiviral and retroviral production and infection

Lentivirus and retrovirus were produced in HEK293FT or Phoenix A cells, respectively, as previously described (*39*). Two to six infections were performed at 12-h intervals, as required. Cells were selected for drug resistance until uninfected control cells were dead.

Neotelomere formation assays

For neotelomere formation assays using the TS site, cells were infected with the pLenti-sgTS-TaqMan-TS lentivirus (or the sgLuc control) shown in Fig. 1A. HeLa-superT cells were plated at 0 h at 500,000 cells per 10-cm plate in the presence of 2.0×10^7 PFU Cas9 adenovirus (AdCas9; Vector Biolabs) (MOI = 40) and 4 µg/mL polybrene. RPE1 cells were plated at 0 h at 700,000 cells per 10-cm plate in the presence of 2.8×10^7 PFU AdCas9 (MOI = 40) and 8 µg/mL polybrene. 24 h after plating, the media was changed. Genomic DNA was harvested at the indicated intervals. Control cells were plated at the same density and with the same concentration of polybrene but without AdCas9. For cells treated with drugs during the assay, drug treatment was initiated on plating with AdCas9 and continued until harvest unless indicated otherwise.

For neotelomere formation assays using the I-SceI site, RPE1-superT cells were infected with the pLenti-TaqMan-I-SceI lentivirus. This lentivirus uses the same backbone as the pLenti-sgTS-TaqMan-TS lentivirus. However, the U6-sgRNA cassette has been removed, and the TS cassette has been replaced by the I-SceI cassette shown in Fig. 4M. These cells were subsequently infected with a lentivirus (pCW-DD-I-SceI-GR-Puro) encoding a copy of I-SceI whose expression and nuclear translocation could be induced upon the simultaneous addition of

doxycycline, Shield1, and triamcinolone (TA) (*25*). Cells were plated at 0 h at 500,000 cells per 10-cm plate in the presence of 2 μg/mL doxycycline hyclate (Sigma-Aldrich D5208), 1 μM Shield1 (Takara 632189), and 0.1 μM triamcinolone acetonide (MedChemExpress HY-B0636). Control cells were plated at the same density but with the appropriate vehicle controls (ddH_2O, ethanol, and DMSO, respectively) instead of active drugs. Genomic DNA was harvested at 72 h. For cells treated with additional drugs during the assay, drug treatment was initiated on plating with AdCas9 and continued until harvesting at 72 h.

Genomic DNA extraction

Genomic DNA was isolated from cells using the Zymo Research Quick-DNA Miniprep kit (Zymo Research). Adherent cells were washed once with 1X PBS and then lysed by adding ~1 mL Genomic Lysis Buffer with 0.5% β-mercaptoethanol. The lysate was scraped into a microcentrifuge tube, vortexed for 30 s, and incubated at room temperature for at least 30 min. Column-based purification of genomic DNA was performed according to the manufacturer's protocol and eluted with ddH_2O. DNA concentrations were estimated using via UV spectrophotometry with a NanoDrop ND-1000 and samples were diluted in ddH_2O to a final concentration of 25 ng/μL before storage at –20°C.

Endpoint neotelomere PCR

Endpoint neotelomere PCR reactions for assays using the sgTS-TaqMan-TS construct were performed with HotStarTaq DNA polymerase (Qiagen). The forward and reverse neotelomere primer sequences were 5'-AAGTACCCCTATCGCGTGTG-3' and 5'-ACCCTAACCCTAACCCTAACTCTG-3', respectively. In general, PCR reactions were performed in a 20-μL volume containing 100 ng genomic DNA template (estimated using a NanoDrop ND-1000), 1X PCR buffer (containing a final $MgCl_2$ concentration of 1.5 mM), 500 nM forward primer, 500 nM reverse primer, 200 μM each dNTP, and 0.5 U HotStarTaq DNA polymerase. When reaction volumes other than 20 μL were used, the quantities of input genomic DNA and DNA polymerase were scaled accordingly. PCR was carried out on C1000 Touch Thermal Cycler (Bio-Rad) device with the following thermocycling conditions: 15 min initial denaturation at 95°C, followed by 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min, with a final extension for 10 min at 72°C. PCR products were resolved by agarose gel electrophoresis in 1X TBE and detected with EthBr staining.

Neotelomere TaqMan qPCR

The TaqMan qPCR assay used a standard TaqMan probe originally used for the detection of cytomegalovirus (CMV) DNA. Apart from the sequence of the reverse neotelomere primer, the reaction composition and protocol were identical for the Cas9 and I-SceI assays. The forward primer for both assays was 5'-AAGTACCCCTATCGCGTGTG-3', as above. The reverse neotelomere primer for the Cas9 assay was 5'-ACCCTAACCCTAACCCTAACTCTG-3'. The reverse neotelomere primer for the I-SceI assay was 5'-CTAACCCTAACCCTAACCCTTATCC-3'. The custom neotelomere TaqMan probe had a sequence of 5'-TGGCCCAGGGTACGGATCTTATTCG-3' and was labeled with 6-carboxyfluorescein (FAM) at the 5' end as the reporter fluorophore and with Black Hole Quencher 1 (BHQ1) at the 3' end as the quencher (Biosearch Technologies). qPCR reactions were performed in a 20-μL volume containing 100 ng genomic DNA template (estimated by UV spectrophotometry, NanoDrop ND-1000), 1X AmpliTaq Gold 360 Buffer (ThermoFisher Scientific), 3 mM $MgCl_2$, 400 nM forward primer, 400 nM reverse primer, 200 nM TaqMan probe, 200 μM each dNTP, and 50 nM ROX reference dye (ThermoFisher Scientific). qPCR was

carried out on a QuantStudio 12K Flex Real-Time PCR System or QuantStudio 3 device (Rockefeller University Genomics Resource Center, ThermoFisher Scientific) with the following thermocycling conditions: 10 min initial denaturation at 95°C, followed by 45 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Fluorescence data were collected during the 72°C step. C_T values were calculated by the QuantStudio software using a C_T fluorescence threshold value of 0.192813. At least three technical replicates were performed for each reaction, alongside a ddH_2O and/or genomic DNA negative control and a 10^{-2} or 10^{-3} neotelomeres per haploid genome positive/standardization control. For the Cas9 assay, the absolute number of neotelomeres per haploid genome (N) was calculated using the equation $C_T = -3.290\log_{10}(N) + b$, where the y-intercept b was calculated for each reaction plate based on the C_T value calculated for the standardization control. For the I-SceI assay, the equation was $C_T = -3.428\log_{10}(N) + b$. These values were then normalized to the quantity of input genomic DNA calculated based on qPCR with primers targeting genomic *ACTB* (see below).

A standard curve for the Cas9 assay was generated by spiking a TaqMan Neotelomere Control plasmid into 100 ng/µL human genomic DNA at a known copy number. Serial 10-fold dilutions of this mixture were prepared from 10^1 to 10^{-6} copies of the TaqMan Neotelomere Control plasmid per haploid genome. These standards were used as templates for the Cas9 qPCR reaction described above. The average C_T values were plotted as a function of the copy number of the TaqMan Neotelomere Control plasmid per haploid genome (N) and fitted to the equation $C_T = A\log_{10}(N) + b$ by linear regression in GraphPad Prism 9.0, which yielded $A = -3.290$ and $b = 25.13$ with $R^2 = 0.9999$.

A standard curve for the I-SceI assay was generated in a similar fashion. A chemically synthesized double-stranded template was spiked into 100 ng/µL human genomic DNA at a known copy number. Serial 10-fold dilutions of this mixture were prepared from 10^1 to 10^{-4} copies of the template per haploid genome. These standards were used as a templates for the I-SceI TaqMan qPCR reaction described above. The average C_T values were plotted as a function of the copy number of the control template per haploid genome (N) and fitted to the equation $C_T = A\log_{10}(N) + b$ by linear regression in GraphPad Prism 9.0, which yielded $A = -3.428$ and $b = 24.37$ with $R^2 = 0.9977$.

To normalize to the quantity of input genomic DNA, a parallel qPCR reaction with SYBR Green quantification was performed with primers targeting *ACTB* genomic DNA (NCBI Gene ID 60). The forward and reverse *ACTB* primers were 5'-GTGCTGTGGAAGCTAAGTCCTGC-3' and 5'-GTCTTTGCGGATGTCCACGTCAC-3', respectively. qPCR reactions were performed in a 20-µL volume containing 25 ng genomic DNA template (estimated by UV spectrophotometry, NanoDrop ND-1000), 1X SYBR Green PCR Master Mix (ThermoFisher), 500 nM forward primer, and 500 nM reverse primer. qPCR was carried out on a QuantStudio 12K Flex Real-Time PCR System or QuantStudio 3 device (Rockefeller University Genomics Resource Center, ThermoFisher Scientific) with the following thermocycling conditions: 10 min initial denaturation at 95°C, followed by 35 cycles of 95°C for 15 s and 60°C for 1 min, followed by melt-curve analysis. Fluorescence data were collected during the 60°C step. C_T values were calculated by the QuantStudio software using an automatically calculated C_T fluorescence threshold value. The inferred quantity of input DNA (Q) in ng/µL was calculated using the equation $C_T = -3.477\log_{10}(Q) + b$, where the y-intercept b was calculated for each reaction plate based on the C_T value determined for the standardization control.

A standard curve for the *ACTB* qPCR described above was generated using 2-fold serial dilutions of human genomic DNA. The average C_T values were plotted as a function of the input quantity of DNA (Q) in ng/µL and fitted to the equation $C_T = A\log_{10}(Q) + b$ by linear regression in GraphPad Prism 9.0, which yielded $A = -3.477$ and $b = 29.23$ with $R^2 = 0.9997$.

Copy number measurement

To determine the average number of integration sites of the pLenti-sgTS-TaqMan-TS lentivirus in the cell lines generated here, the Cas9 neotelomere qPCR assay was performed as described above, except 5'-GGGGAGACCGCTTAACTCTG-3' was used as the reverse primer to detect intact cassettes. Representative genomic DNA samples from uninduced cells were used as templates. A standard curve for this reaction was generated by spiking the pLenti-sgTS-TaqMan-TS plasmid into 100 ng/µL human genomic DNA at a known copy number. These standards were used as templates for the modified TaqMan qPCR reaction described above. The average C_T values were plotted as a function of the copy number of the pLenti-sgTS-TaqMan-TS plasmid per haploid genome (N) and fitted to the equation $C_T = A\log_{10}(N) + b$ by linear regression in GraphPad Prism 9.0, which yielded $A = -3.407$ and $b = 27.22$ with $R^2 = 0.9963$. Normalization to the input quantity of genomic DNA was performed using qPCR for *ACTB*, as described above.

TRAP assays

TRAP assays were performed with the TRAPeze Telomerase Detection Kit (Millipore Sigma) largely according to the manufacturer's protocol. Briefly, cells were harvested by washing once with 1X PBS, dislodging with trypsin-EDTA, and quenching with serum-containing growth medium. 4×10^5 cells were lysed by resuspending in 160 µL 1X CHAPS Lysis Buffer and incubating on ice for 30 min. The lysate was cleared by centrifuging at 12,000 *g* for 20 min at 4°C, and the supernatant was transferred to a fresh microcentrifuge tube, giving a lysate containing 2500 cells/µL. Serial fivefold dilutions in 1X CHAPS Lysis Buffer were prepared as necessary. Heat-treated negative controls were prepared by heating the 2500 cells/µL lysate at 95°C for 15 min. An additional negative control was performed with 1X CHAPS Lysis Buffer in each trial.

Telomerase-mediated extension and subsequent amplification of TRAP products were conducted in 25-µL reactions containing 1 µL of cell lysate and 2 U of Titanium Taq DNA polymerase (Takara). The other kit components—TRAP reaction buffer, dNTP mix, TS primer, TRAP primer mix, and ddH_2O—were added in the proportions specified by the manufacturer. Reactions were incubated at 30°C for 30 min and 95°C for 2 min and then underwent 32 cycles of PCR at 94°C for 15 s, 59°C for 30 s, and 72°C for 1 min. TRAP assay products were resolved on a 1X TBE 12.5% native polyacrylamide gel at 100 V for 3 hours. Bands were visualized by staining in 1 µg/mL EthBr in ddH_2O for 30 min and then de-stained in ddH_2O for 30 min.

E. coli *exonuclease I digestion*

For *Exo*I assays, genomic DNA harvested with the Quick-DNA Miniprep kit (Zymo Research) was digested with 100 µg/mL RNase A in 1X TNE for 3 h and then with 100 µg/mL proteinase K in 1X TNES for 1 h more. Genomic DNA was extracted with phenol-chloroform-isoamyl alcohol and precipitated with isopropyl alcohol overnight. 15 µg genomic DNA was digested with 60 U *E. coli Exo*I (New England BioLabs) in 1X *Exo*I buffer (67 mM glycine-NaOH pH 9.5, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol) overnight at 37°C. Another 60 U *Exo*I was added the following morning, and the incubation was performed for an additional 2 h at 37°C. As a negative control, 15 µg genomic DNA was incubated in the same way in 1X *Exo*I buffer

without the enzyme. DNA was extracted, precipitated, and used as input for the Cas9 qPCR assay and telomeric overhang gels.

In-gel analysis of single-stranded telomeric DNA

After *Exo*I treatment, genomic DNA samples were digested overnight with *Mbo*I (New England BioLabs) at 37°C according to the manufacturer's specifications. Following digestion, DNA concentrations were measured by Hoechst fluorimetry, and 1.5 µg DNA was resolved by agarose gel electrophoresis in TAE. In-gel hybridization with a γ-^{32}P-ATP end-labelled $(AACCCT)_4$ probe was performed, as previously described (*39*)

Bal31 digestion

Genomic DNA was treated with 0.5 U of Bal31 (Takara) per 2 µg DNA for 20 min at 30°C in the manufacturer's buffer. Bal31-digested DNA was isolated by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation and digested with *Mbo*I for analysis of telomeric restriction fragments as above or with *Pvu*II for analysis of the neotelomeres at 16p as described below. Gels were processed by Southern blotting with the appropriate probes as described below.

AsCpf1 knockouts

Bulk knockout by AsCpf1 was achieved by infecting cells four times with pLenti-AsCpf1 lentiviruses containing the oligonucleotide crRNA cassettes listed below. In scenarios where a satisfactory bulk knockout could not be achieved with a single crRNA cassette, cells were co-infected with two pLenti-AsCpf1 lentiviruses encoding distinct crRNAs as indicated in the table below.

Pif1 RT-qPCR

Total RNA was isolated from a sub-confluent 10-cm plate of RPE1 cells using TRIzol (ThermoFisher) according to the manufacturer's protocol. RNA was digested with Rnase-free Dnase I (Roche) to remove contaminating genomic DNA according to the manufacturer's protocol, reextracted with TRIzol, and precipitated once again with isopropyl alcohol overnight. cDNA was synthesized with SuperScript IV reverse transcriptase (ThermoFisher) using 1 µg of total RNA and the $(dT)_{20}$ primer. The RNA-primer mixture was pre-annealed by heating to 65°C for 5 min and then snap-cooling on ice for at least 1 min. Reverse transcription was carried out with the following thermocycling program: 10 min at 50°C, 5 min at 55°C, and 10 min at 80°C. cDNA was diluted 25-fold for qPCR.

qPCR reactions were performed with SYBR Green PCR Master Mix (ThermoFisher) to detect Pif1 cDNA, using GAPDH cDNA as a normalization control. The forward and reverse Pif1 primers were 5'-AGGAGCTGCCAGGTAAGGTA-3' and 5'-GCTAACAGGACACTGGGCAT-3', respectively. The forward and reverse GAPDH primers were 5'-AGCCACATCGCTCAGACAC-3' and 5'-GCCCAATACGACCAAATCC-3', respectively. qPCR reactions were performed in a 20-µL volume containing 1 µL 1-to-25 diluted cDNA, 1X SYBR Green PCR Master Mix (ThermoFisher), 500 nM forward primer, and 500 nM reverse primer. qPCR was carried out on a QuantStudio 12K Flex Real-Time PCR System or QuantStudio 3 device (Rockefeller University Genomics Resource Center, ThermoFisher Scientific) with the following thermocycling conditions: 10 min initial denaturation at 95°C, followed by 40 cycles of 95°C for 15 s and 61°C for 1 min, followed by melt-curve analysis. Fluorescence data were collected during the 61°C step. C_T values were calculated by the QuantStudio software using an automatically calculated C_T fluorescence threshold value. Pif1

qPCR reactions that had more than one melting-curve peak were discarded. The amplification factor for the Pif1 qPCR reaction was determined to be 1.8845, and that for the GAPDH qPCR reaction was assumed to be 2. The quantity of Pif1 mRNA was normalized to the quantity of GAPDH mRNA.

sgRNA expression

For sgRNA expression, cells were infected with lentiviruses containing the oligonucleotide cassettes listed in the table below inserted downstream of a human U6 promoter.

shRNA knockdown

For shRNA-mediated knockdown, cells were infected with pLKO.1 lentiviruses (Open Biosystems) containing the oligonucleotide cassettes listed in the table below. In scenarios where a satisfactory knockdown could not be achieved with a single shRNA, cells were co-infected with pLKO.1 lentiviruses encoding distinct shRNAs.

siRNA knockdown of RPA70

48 hours prior to infection with AdCas9, 500,000 RPE1-superT cells were reverse-transfected with Silencer Select siRNAs (ThermoFisher) using Lipofectamine RNAiMAX (ThermoFisher) according to the manufacturer's protocol. 48 h later, one of three replicate transfected plates was trypsinized, counted, and harvested for immunoblotting. On one of the two remaining plates, the media was changed to RPE1 media plus 8 µg/mL polybrene. On the other remaining plate, AdCas9 was added to an MOI of 40 in RPE1 media containing 8 µg/mL polybrene. Genomic DNA was harvested 96 h after siRNA transfection.

Isolation of HeLa-superT clones containing stable neotelomeres

A clonal HeLa-superT cell line was derived by integrating a construct containing the elements shown in Fig. 3A and S3A into the region between the *LUC7L* and *FAM234A* genes on chromosome 16p13.3 (hg38 chr16:229,557–229,641). HeLa-superT cells were nucleofected with a linearized template containing diphtheria toxin A (DT-A) cassettes on both ends plus two AsCpf1 expression plasmids, each encoding a crRNA targeting the region just proximal to the *LUC7L* transcriptional start site: 5'-CCCTCCGTAGAGACTCGTTTGAG-3' and 5'-GTTCCGCCGTTGGACAACTTGCG-3'. Following nucleofection, cells were selected in 10 µg/mL blasticidin for 10 days, and resistant cells were subcloned by flow sorting. 96 clones were screened in 16-clone pools, and then positive pools were screened individually using the 5'/centromeric and 3'/telomeric PCR primers below. The HeLa-superT 16p-targ clone was identified as having an intact cassette at the appropriate locus. This clone was assumed to contain a single insertion.

The HeLa-superT 16p-targ clone was infected with pLenti-sgTS-Puro and selected with 0.6 µg/mL puromycin to instate constitutive expression of an sgRNA targeting the integrated TS site. 500,000 cells were infected with 2.83×10^7 PFU AdCas9 (MOI = 56.7) in 8 µg/mL polybrene. 24 h later, the media was changed, and the cells were treated with another 5.67×10^7 PFU AdCas9 in media containing 16 µg/mL polybrene. 10 days after the first AdCas9 infection, the cells were split into 15-cm plates at 5,000 cells per plate in 50 µM ganciclovir (Sigma). Surviving colonies were isolated with cloning cylinders and expanded in 50 µM ganciclovir. Cells were screened for potential neotelomere formation by PCR with primer pairs to detect telomeric repeat addition at the TS site, retention of the 5'/centromeric junction of the TS cassette, and loss of the 3'/telomeric junction of the TS cassette. The primers used to detect

telomeric repeat addition were the same as those used in the Cas9 neotelomere qPCR assay. The forward and reverse primers were 5'-AAGTATATGCCAGACTATGCACACA-3' and 5'-CGCAGGCGCATAACATCAAA-3', respectively, for the 5'/centromeric junction and 5'-CGGCTCCATACCGACGATAT-3' and 5'-GACTTCTCTCAGGCAGGCG-3' for the 3'/telomeric junction. PCR was carried out with Q5 High-Fidelity 2X Master Mix (New England BioLabs) using the conditions recommended by the manufacturer for 35 cycles, except as specified below. In all reactions, each primer was present at a concentration of 500 nM, and extension was conducted at 72°C for 35 s. Annealing was conducted for 30 s at 68°C for the neotelomere primers, 66°C for the 5'/centromeric junction primers, and 67°C for the 3'/telomeric junction primers. PCR products were resolved on 1X TBE agarose gels and stained with EthBr.

Metaphase FISH

BAC probes were obtained from BACPAC Genomics to identify the q arm (Chr16q13, RP11-109J21) and distal p arm of chromosome 16 (Chr16p13.3, RP11-344L06). Preparation of metaphases and probe labeling by nick-translation were performed as previously described (*33*), except that probes were denatured at 76°C for 10 min rather than at 80°C for 8 min. Slides were treated with 50 µg/mL RNase A in 2X SSC at 37°C for 10 min and then rinsed with 1X PBS. Thereafter, slides were crosslinked in 4% formaldehyde in 1X PBS for 5 min, rinsed thrice with 1X PBS, and dehydrated in an ethanol series. After preheating to 65°C for 1 h, slides were denatured at 76°C for 110 sec in 70% formamide in 2X SSC and then dehydrated in a chilled ethanol series. Where applicable, as in Fig. 3D, slides were hybridized at room temperature for 1 hour with 50 nM Cy3-TelG PNA probes (PANAGENE F1006) in hybridization solution (10 mM Tris-HCl pH 7.2, 70% formamide, 0.5% Roche 11096176001 blocking reagent). Slides were washed twice with 2X SSC for 5 min each and then dehydrated in an ethanol series, as before. Thereafter, all slides were hybridized with labeled BAC probes overnight in a humidified chamber at 37°C and washed thrice with 1X SSC at 65°C for 5 min each and then twice with 0.1X SSC at 50°C for 5 min each. All subsequent steps were performed as previously described (*33*), except the following antibodies were used at a 1:1000 dilution to detect the FISH probes: AlexaFluor 488 mouse monoclonal anti-digoxin (JacksonImmunoResearch 200-542-156) and AlexaFluor 594 mouse monoclonal anti-biotin (JacksonImmunoResearch 200-582-211). The FISH signals observed with the chromosome 16q probe are sometimes split into two signals per chromatid. This behavior is usually due to a fragile site and becomes more prominent in response to aphidicolin treatment. Although there is no common fragile site at the position of the 16q FISH probe, it is possible that HeLa cells carry a weakly expressed fragile site at this locus.

Southern blotting for the TS cassette

Genomic DNA was harvested from cells lysed in TNES containing 50 µg/mL proteinase K overnight at 37°C, then extracted with phenol-chloroform-isoamyl alcohol, and precipitated with isopropyl alcohol. Genomic DNA was subsequently digested with 100 µg/mL Rnase A in 1X TNE for 4 hours at 37°C, and an equal volume of TNES containing 100 µg/mL proteinase K was added and incubated for 1 h more. DNA was re-extracted with phenol-chloroform-isoamyl alcohol and precipitated with isopropyl alcohol. Samples were digested overnight with *Pvu*II-HF (New England BioLabs) at 37°C according to the manufacturer's specifications. DNA concentrations were measured by Hoechst fluorimetry, and 8 µg DNA was resolved by agarose gel electrophoresis in 0.5X TAE. The DNA was depurinated in situ with 0.25 M HCl for 30 min, denatured with 1.5 M NaCl and 0.5 M NaOH twice for 30 min each, and then neutralized twice with 3 M NaCl and 0.5 M Tris-Cl pH 7.0 for 30 min. DNA was blotted onto a Hybond membrane (GE Healthcare) in 20X SSC. The membrane was UV-crosslinked in a Stratalinker

and prehybridized for 1 hour at 65°C with Church mix (0.5 M sodium phosphate buffer at pH 7.2, 1 mM EDTA, 0.7% SDS, 0.1% BSA). A Klenow α-^{32}P-dCTP-labeled probe was prepared from a gel-purified 904-bp PCR product spanning ψ, *gag*, and part of the TS sequence, as indicated in Fig. 3A. Before hybridization with the membrane, 2 µg *Mbo*I-digested human gDNA was added to the probe, and the mixture was heated to 95°C for 5 min and then incubated at room temperature for 10 min to reduce background. The membrane was hybridized with this mixture in Church mix overnight at 65°C, washed thrice for 15 min each with Church wash (40 mM sodium phosphate buffer at pH 7.2, 1 mM EDTA, 1% SDS) and exposed to a PhosphorImager screen overnight.

T7 endonuclease I assay

PCR products were amplified from the specified genomic DNA samples with Q5 High-Fidelity 2X Master Mix (New England BioLabs) for 35 cycles according to the manufacturer's protocol. The forward and reverse primers were 5'-GACGTTGGGTTACCTTCTGC-3' and 5'-TCCGATCGCGACGATACAAG-3', respectively, and annealing was conducted at 67°C for 30 s. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and eluted in 10 mM Tris-HCl pH 8.0. 200 ng of the purified PCR product was digested with T7 endonuclease I (New England BioLabs), as specified by the manufacturer. Reactions were quenched with EDTA, resolved on 1X TBE agarose gels, and stained with EthBr.

Thymidine cell cycle block

To assess the cell cycle dependence of neotelomere healing events, 1 million RPE1-superT cells bearing the pLenti-sgTS-TaqMan-TS construct were incubated with or without 2.5 mM thymidine for 30 h, then infected with 2.0×10^7 PFU AdCas9, and then maintained with or without 2.5 mM thymidine another 48 h (see Fig. S6). Two plates were prepared for each condition: one for genomic DNA and one for fluorescence-activated cell sorting (FACS). 47 h after AdCas9 infection, BrdU was added to 10 µM to plates destined for FACS. 48 h after infection, all plates were harvested.

Cell cycle analysis via fluorescence-activated cell sorting

For fluorescence-activated cell sorting (FACS), cells were trypsinized and fixed overnight in 70% ethanol at –20°C. Cells were denatured in 2 N HCl for 25 min at room temperature, neutralized with 0.1 M sodium tetraborate at pH 8.0, and then permeabilized with 0.1% Triton X-100 for 5 min in 3% BCS/PBS. Cells were incubated with a FITC-conjugated anti-BrdU antibody (BD Biosciences 347583) for 30 min at room temperature and then washed with 1% BCS/PBS. Cells were resuspended in 1% BCS/PBS containing 0.5 µg/mL Rnase A and incubated at room temperature for 20 min, after which propidium iodide was added to a final concentration of 75 µg/mL Cells were incubated for another 20 min at room temperature and then transferred to ice. FACS analysis was conducted on an Accuri C6 (BD Biosciences).

In vitro neotelomere formation assay

In vitro neotelomere assays were performed using 2.5 nM sgTS-TaqMan-TS plasmid in 20 µL of TRAP assay buffer (20 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween-20, 1 mM EGTA, 50 µM dNTPs) preincubated for 5 min with Cas9 (abm) + sgRNA (final concentration of 100 nM for each) followed by addition of 1-5 µL of telomerase (see figure legend) purified from transfected HEK293T cells (a kind gift of Joachim Lingner). Reactions (25 µL final volume) were incubated for 2.5 hours at 37°C and 1 µL of a 1:5 dilution of the telomerase reaction was added to a 20 µL PCR reaction with HotStarTaq DNA polymerase

(Qiagen). The forward and reverse primer sequences were 5′-AAGTACCCCTATCGCGTGTG-3′ and 5′-ACCCTAACCCTAACCCTAACTCTG-3′, respectively. PCR reactions were performed in a 20-µL volume containing 1 µM forward primer, 1 µM reverse primer, 200 µM each dNTP, and 0.5 U HotStarTaq DNA polymerase. PCR was carried out on GeneAmp PCR System 9700 (Applied Biosystems) device with the following thermocycling conditions: 5 min initial denaturation at 95°C, followed by 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension for 5 min at 72°C. PCR products were resolved by PAGE on 10% TBE pre-cast gels (ThermoFisher) and visualized by ethidium bromide staining for 15 min. The dsDNA control was made by annealing two oligos (ThermoFisher): 5′-AAGTACCCCTATCGCGTGTGTTCTATGGCCCAGGGTACGGATCTTATTCGCTTTGAACGTAATAGGAGGTGGGTGGGGCGAGCAGAGTT-3′ and 5′-AACTCTGCTCGCCCCACCCACCTCCTATTACGTTCAAAGCGAATAAGATCCGTACCCTGGGCCATAGAACACACGCGATAGGGGTACTT-3′ and purifying the annealed product by gel extraction from a 2% agarose gel. The ssDNA control sequence was: 5′-AAGTACCCCTATCGCGTGTGTTCTATGGCCCAGGGTACGGATCTTATTCGCTTTGAACGTAATAGGAGGTGGGTGGGGCGAGCAGAGTT-3′. Both ssDNA and dsDNA controls were added to the *in vitro* neotelomere assay at a concentration of 0.25 nM.

Molecular cloning

pBABE-WT-TERT-U3-hTR-500-Neo was produced by Gibson assembly. The puromycin *N*-acetyltransferase (Puro) gene was excised from pBABE-WT-TERT-U3-hTR-500-Puro via double digestion with *Avr*II and *Cla*I (New England BioLabs) and replaced with a PCR-amplified Tn5 aminoglycoside phosphotransferase (Neo) gene. The Neo gene was amplified from pBABE-Neo (Addgene 1767) using the following primers: 5'-CTATTCCAGAAGTAGTGAGGAGGC-3' and 5'-ACAGGTGGGGTCTTTCATTCC-3'.

pCW-DD-I-SceI-GR-Puro was produced by Gibson assembly. pCW-Cas9-Puro (Addgene 50661) was digested with *Nhe*I-HF and *Bam*HI-HF to remove Cas9, which was replaced with the PCR-amplified gene encoding the fusion protein consisting of the destabilizing domain (DD) of mutant FKBP12, I-SceI, and the ligand-binding domain of the glucocorticoid receptor (GR). The DD-I-SceI-GR fusion was amplified from a vector kindly provided by Simon Powell (*25*) with the following primers: 5'-CGTTTAGTGAACCGTCAGATCGCCTGGAGAATTGGGCTAGCGCTACCGGTATGG-3' and 5'-AGGGCTGCCTTGGAAAAGGCGCAACCCCAACCCCGGATCCAATTGCATTCATTTTATGTTTCAGGTTCAGG-3'.

The pET-TaqMan Neotelomere Control plasmid was cloned via sequential ligation of phosphorylated and annealed oligonucleotide pairs after *Eco*RI-HF and *Hind*III-HF (New England BioLabs) double digestion of pET-Duet (EMD Biosciences 71146) to produce an intermediate and then again after *Kpn*I-HF and *Mfe*I-HF (New England BioLabs) double digestion to yield the final product. The oligonucleotide pair for the *EcoR*I-*Hind*III insertion was 5′-AATTCAAGTACCCCTATCGCGTGTGTTCTATGGCCCAGGGTACGGATCTTATTCGCTTTGAACGTAATATC-3′ and 5′-AGCTGATATTACGTTCAAAGCGAATAAGATCCGTACCCTGGGCCATAGAACACACGCGATAGGGGTACTTG-3′. The oligonucleotide pair for the *Kpn*I-*Mfe*I insertion was 5′-AATTGGATCCGGAGGTGGGTGGGGCGAGCAGAGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGGTAC-3′ and

5′-CCCTAACCCTAACCCTAACCCTAACCCTAACTCTGCTCGCCCCACCCACCT CCGGATCC-3′.

pLenti-sgRNA-Puro plasmids targeting firefly luciferase, TS, and Down (see Fig. S2H) were cloned by ligating the phosphorylated and annealed oligonucleotide cassettes listed below into the *Bsm*BI (New England BioLabs) sites of pLenti-sgRNA-Puro.

pLenti-AsCpf1-Puro plasmids expressing the crRNA cassettes listed below were cloned by ligating phosphorylated and annealed oligonucleotide cassettes into the *Bsm*BI (New England BioLabs) sites of pLenti-AsCpf1-Puro.

pLKO.1 plasmids for shRNA knockdown experiments were produced via ligation of the phosphorylated and annealed oligonucleotides listed below into pLKO.1 double digested with *Eco*RI-HF and *Nhe*I-HF (New England BioLabs).

pLenti-sgLuc-TaqMan TS-Blast and pLenti-sgTS-TaqMan TS-Blast were cloned via a series of Gibson assemblies. The TaqMan TS cassette shown in Fig. 1A was inserted into the *Bgl*II site of a pQCXIB vector (Clontech) via Gibson assembly with the following PCR product derived from overlapping oligonucleotides: 5'-CCAGCCCTCACTCCTTCTCTAGGCGCCGGAATTGAAGATCAAGTACCCCTATCGCGT GTGTTCTATGGCCCAGGGTACGGATCTTATTCGCTTTGAACGTAATAGGAGGTGGGT GGGGCGAGCAGAGTTAAGCGGTCTCCCCTCCAGGATGTCTTTTGGGGGATCGATCCT CTAGAGTCCGTTACATAACTTACGG-3'. The *gag*-TaqMan TS-CMV cassette from this pQCXIB plasmid was amplified by sequential PCR reactions using the following overlapping primers: 5'- TTACAGGGACAGCAGAGATCCACTTTGGCGC-3', 5'-GAGATCCACTTTGGCGCCGGCGGCCAGACTGTTACCACTC-3', 5'-GTACTCGGTCATGGTGGCGGATCCGTTAATTAAGCGTACG-3', and 5'-GCGCACCGTGGGCTTGTACTCGGTCATGGTGGC-3' (underlining denotes regions of primer overlap). This *gag*-TaqMan TS-CMV cassette was inserted via Gibson assembly into pLenti-sgLuc-Puro and pLenti-sgTS-Puro double digested with *Bam*HI-HF and *Xho*I (New England BioLabs), yielding pLenti-sgLuc-TaqMan TS-Puro and pLenti-sgTS-TaqMan TS-Puro, respectively. The Puro gene was excised via double digestion with *Mlu*I and *Bam*HI (New England BioLabs) and replaced via Gibson assembly with the blasticidin S deaminase (Blast) gene amplified from the aforementioned pQCXIB vector by sequential PCR reactions with the following overlapping oligonucleotides: 5'-GCCGCACCGGTAGGCCTCGTACGCTTAATTAACGGATCCG-3', 5'-ACGCTTAATTAACGGATCCGCCACCATGGCCAAGCCTTTG-3', 5'-TGATTGTCGACTTAACGCGTTTAGCCCTCCCACACATAAC-3', and 5'-CAAATTTTGTAATCCAGAGGTTGATTGTCGACTTAACGCG-3' (underlining denotes regions of primer overlap).

pLenti-TaqMan-I-SceI-Blast was derived from pLenti-sgTS-TaqMan TS-Blast via Gibson assembly. An 837-bp gBlocks Gene Fragment (IDT) was constructed that contained the central polypurine tract and central termination sequence of HIV-1 (cPPT/CTS), *gag*, and the I-SceI cassette shown in Fig. 4M. This fragment was inserted by Gibson assembly into pLenti-sgTS-TaqMan TS-Blast double digested with *Ava*I and *Xba*I (New England BioLabs).

Quantification and statistical analysis

The number of biological replicates performed is indicated in each figure and corresponds to the number of data points plotted. All metaphase FISH images were blinded before scoring. Statistical analysis was performed in GraphPad Prism. Data plotted throughout are mean ± standard deviation (SD). The level of statistical significance is indicated by asterisks: ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Two-way comparisons were performed via two-tailed unpaired or two-tailed ratio-paired *t*-tests, as indicated in the figure legends. Two-tailed ratio-paired *t*-tests were performed to determine whether the fold-change resulting from a given intervention was significantly different from 1.

Cell Lines

Line	Species	Source/Reference
Phoenix A	*Homo sapiens*	ATCC CRL-3213
HEK293FT	*Homo sapiens*	ThermoFisher R70007
HeLa-superT	*Homo sapiens*	(*10*)
TP53$^{-/-}$ *RB1*$^{-/-}$ RPE1	*Homo sapiens*	(*12*)
TP53$^{-/-}$ *RB1*$^{-/-}$ RPE1-superT	*Homo sapiens*	This publication
HeLa-superT 16p targeted clone	*Homo sapiens*	This publication

shRNA Sequences

Gene	Sequence
shDNA2-1	CCGGCCAGCTTTGAAGATGGATTAACTCGAGTTAATCCATCTTCAA AGCTGGTTTTTTG AATTCAAAAAACCAGCTTTGAAGATGGATTAACTCGAGTTAATCC ATCTTCAAAGCTGG
shDNA2-2	CCGGCCCTCTGATATTGGTATTATTCTCGAGAATAATACCAATATC AGAGGGTTTTTTG AATTCAAAAAACCCTCTGATATTGGTATTATTCTCGAGAATAATAC CAATATCAGAGGG
shScramble	CCGGCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAA CCTTAGGTTTTTG AATTCAAAAACCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCG ACTTAACCTTAGG

siRNAs

siRNA	Source
siControl	ThemoFisher 4390843, Silencer Select Negative Control #1 siRNA
siRPA70-1	ThermoFisher 4392420-s12127, Silencer Select RPA70 siRNA
siRPA70-2	ThermoFisher 4392420-s12128, Silencer Select RPA70 siRNA

Cas9 sgRNA Sequences

Target	Sequence
sgDown	CACCGAGAGTCCGTTACATAACTTA AAACTAAGTTATGTAACGGACTCTC
sgLuc	CACCGACAACTTTACCGACCGCGCC AAACGGCGCGGTCGGTAAAGTTGTC

Target	Sequence
sgTS	CACCGTGGGGCGAGCAGAGTTAAG AAACCTTAACTCTGCTCGCCCCAC

AsCpf1 crRNA Sequences

Gene/Locus	Sequence
cr53BP1	AGATGCTGAGGTAACTGCTCAATGAATTTCTACTCTTGTAGATAGT CAGAGAAAGGTGCAACG AAAACGTTGCACCTTTCTCTGACTATCTACAAGAGTAGAAATTCAT TGAGCAGTTACCTCAGC
crATRIP	AGATTATTGTGCCTGAAGTACCTCTAAAATTTCTACTCTTGTAGAT GTGCTAACATGTCCCTTCCCCAC AAAAGTGGGGAAGGGACATGTTAGCACATCTACAAGAGTAGAAA TTTTAGAGGTACTTCAGGCACAATA AGATCAGGTGATCATAAGGTCCACAGAAATTTCTACTCTTGTAGAT AACTGGAGTTCAGACTGCAATGA AAAATCATTGCAGTCTGAACTCCAGTTATCTACAAGAGTAGAAAT TTCTGTGGACCTTATGATCACCTG
crBLM	AGATGGTAGTGCATACAACTTCCTTCGAATTTCTACTCTTGTAGAT GCAGCCAGCAAATCTTCCACAGC AAAAGCTGTGGAAGATTTGCTGGCTGCATCTACAAGAGTAGAAAT TCGAAGGAAGTTGTATGCACTACC
crBRCA2	AGATTTCAGAAGCTCCACCCTATAAATTTCTACTCTTGTAGATGTC CTGTTGTTCTACAATGT AAAAACATTGTAGAACAACAGGACATCTACAAGAGTAGAAATTTA TAGGGTGGAGCTTCTGAA
crClaspin	AGATATAGCTGCCCTGTCCACTATCTGAATTTCTACTCTTGTAGAT AGCTGATTGGCTCCACGATTCCA AAAATGGAATCGTGGAGCCAATCAGCTATCTACAAGAGTAGAAAT TCAGATAGTGGACAGGGCAGCTAT
crControl	AGATCGTTAATCGCGTATAATACGAATTTCTACTCTTGTAGATCAT ATTGCGCGTATAGTCGC AAAAGCGACTATACGCGCAATATGATCTACAAGAGTAGAAATTCG TATTATACGCGATTAACG
crCtIP	AGATCACACACAGAGTGCTCCAATTTAAATTTCTACTCTTGTAGAT ACATTCCTTACACGTGTGCCCAA AAAATTGGGCACACGTGTAAGGAATGTATCTACAAGAGTAGAAAT TTAAATTGGAGCACTCTGTGTGTG AGATATCGGACAACACATGAAGAGTATAATTTCTACTCTTGTAGAT ATACTAGAGGTAGCTCCAAATAC AAAAGTATTTGGAGCTACCTCTAGTATATCTACAAGAGTAGAAAT TATACTCTTCATGTGTTGTCCGAT

Gene/Locus	Sequence
crExo1	AGATCCTTAAGAAGATTGGCTTGTCGTAATTTCTACTCTTGTAGAT CAGCCAAAAGCTAGGAGATCCGA AAAATCGGATCTCCTAGCTTTTGGCTGATCTACAAGAGTAGAAATT ACGACAAGCCAATCTTCTTAAGG
crFANCJ	AGATAACTATCCAAGCACACCACCTTCAATTTCTACTCTTGTAGAT CTCGGGATGAACTAGATAGTATG AAAACATACTATCTAGTTCATCCCGAGATCTACAAGAGTAGAAAT TGAAGGTGGTGTGCTTGGATAGTT AGATTGTTGAAGTTACCGACTACCTCAAATTTCTACTCTTGTAGAT AGTATTCTGGAAGGTAGCACAGA AAAATCTGTGCTACCTTCCAGAATACTATCTACAAGAGTAGAAAT TTGAGGTAGTCGGTAACTTCAACA
crhSSB1	AGATCCTCCCATAGGTACGCTTCAGTTAATTTCTACTCTTGTAGAT TCTGTTCCGTTCAGGCCGAGTGA AAAATCACTCGGCCTGAACGGAACAGAATCTACAAGAGTAGAAAT TAACTGAAGCGTACCTATGGGAGG AGATGTGAGCCGGATAATGTCCCCAGGAATTTCTACTCTTGTAGAT TTCTGAGGTTCCTAACTTCAGTG AAAACACTGAAGTTAGGAACCTCAGAAATCTACAAGAGTAGAAAT TCCTGGGGACATTATCCGGCTCAC
crKu70/80	AGATCCCTTGCAACAGGCATCTTCCTTAATTTCTACTCTTGTAGAT GCTTAGGTGGCCATGGGCCTTCC AAAAGGAAGGCCCATGGCCACCTAAGCATCTACAAGAGTAGAAA TTAAGGAAGATGCCTGTTGCAAGGG
crLig3	AGATACTGGAGAAGTCACCTGGCCAGTAATTTCTACTCTTGTAGAT CACGGTGATGTGTACCTAACAGT AAAAACTGTTAGGTACACATCACCGTGATCTACAAGAGTAGAAAT TACTGGCCAGGTGACTTCTCCAGT
crLig4	AGATACCATACAGCAAGTAAACGACCTAATTTCTACTCTTGTAGAT ATACTCTCTCTCGTGTTGGGTCT AAAAAGACCCAACACGAGAGAGAGTATATCTACAAGAGTAGAAA TTAGGTCGTTTACTTGCTGTATGGT
crMdc1	AGATGTGGTGCCCATGGACCAGAAAAAAATTTCTACTCTTGTAGA TGCTTCATCGACAGCGACACTGAT AAAAATCAGTGTCGCTGTCGATGAAGCATCTACAAGAGTAGAAAT TTTTTTCTGGTCCATGGGCACCAC
crMLH1	AGATCTTACCCTGATCCCGGTGCCAATTTCTACTCTTGTAGATCAA CATAGCCACGAGGAGAA AAAATTCTCCTCGTGGCTATGTTGATCTACAAGAGTAGAAATTGGC ACCGGGATCAGGGTAAG

Gene/Locus	Sequence
crMRE11	AGATATCGAGTGTTACAAACGTATCATAATTTCTACTCTTGTAGATTTGACAAACATTCGATAGAGCCTAAAAAGGCTCTATCGAATGTTTGTCAAATCTACAAGAGTAGAAATTATGATACGTTTGTAACACTCGAT AGATTGGAGAAAGATGCAGTCAGAGGAAATTTCTACTCTTGTAGATTATCTCACAACCTGGAAGCTCAGAAAACTGAGCTTCCAGGTTGTGAGATAATCTACAAGAGTAGAAATTTCCTCTGACTGCATCTTTCTCCA
crPARP1	AGATTATCTTCTCCATACACCCCTTGCAATTTCTACTCTTGTAGATGCTCGTCCTTGATGTTCCAGATCAAAAGATCTGGAACATCAAGGACGAGCATCTACAAGAGTAGAAATTGCAAGGGGTGTATGGAGAAGATA
crPif1	AGATGCAGATATGACTTCCCTGTTAATTTCTACTCTTGTAGATGTTGAAGGAGCTGGCTAACAAAAATGTTAGCCAGCTCCTTCAACATCTACAAGAGTAGAAATTAACAGGGAAGTCATATCTGC
crPINX1	AGATATATGATCTGTGGCTCCTTGCTCAATTTCTACTCTTGTAGATGCCTTGAGGAAAAGTCCAAAATCAAAAGATTTTGGACTTTTCCTCAAGGCATCTACAAGAGTAGAAATTGAGCAAGGAGCCACAGATCATAT
crRad51	AGATGCGCATAGGCAACAGCCTCCACAAATTTCTACTCTTGTAGATAGCCAGGCAGATGCACTTGGCCAAAAATGGCCAAGTGCATCTGCCTGGCTATCTACAAGAGTAGAAATTTGTGGAGGCTGTTGCCTATGCGC AGATGTGTAGCAGTGGTAATCACTAATAATTTCTACTCTTGTAGATTCAGCTTTGGCTTCACTAATTCCAAAAGGAATTAGTGAAGCCAAAGCTGAATCTACAAGAGTAGAAATTATTAGTGATTACCACTGCTACAC
crRad52	AGATGTTACAATGGCTGGGCACACTCCAATTTCTACTCTTGTAGATGTGATCTCAGGTAGTCTTTGTCCAAAAGGACAAAGACTACCTGAGATCACATCTACAAGAGTAGAAATTGGAGTGTGCCCAGCCATTGTAAC
crRadX	AGATGCAGTCTCCATTCTGGTTTCACCAATTTCTACTCTTGTAGATCCTTGGCATCATACGTATACGGCAAAAGCCGTATACGTATGATGCCAAGGATCTACAAGAGTAGAAATTGGTGAAACCAGAATGGAGACTGC AGATGTCATATCGCTGGATTCACATTGAATTTCTACTCTTGTAGATAGGATAAACACGGCTGTAGTGTAAAAATACACTACAGCCGTGTTTATCCTATCTACAAGAGTAGAAATTCAATGTGAATCCAGCGATATGAC

Gene/Locus	Sequence
crRev7	AGATGATAAAGAGCACCGCCCAGTAATTTCTACTCTTGTAGATAG ATCACCCAGCCTCCACTG AAAACAGTGGAGGCTGGGTGATCTATCTACAAGAGTAGAAATTAC TGGGCGGTGCTCTTTATC
crSLX4	AGATACAACAGCAGTGCCAAGTCCCTCAATTTCTACTCTTGTAGAT CGCCCACAAGTTCGTGCTTTATG AAAACATAAAGCACGAACTTGTGGGCGATCTACAAGAGTAGAAAT TGAGGGACTTGGCACTGCTGTTGT AGATCACGGGTAGGAGCATCGGCACATAATTTCTACTCTTGTAGA TGCGTGAGTGAGCTCGTTCACCTG AAAACAGGTGAACGAGCTCACTCACGCATCTACAAGAGTAGAAAT TATGTGCCGATGCTCCTACCCGTG AGATCTCCAGGCTATCATCATGTGCCGAATTTCTACTCTTGTAGAT TGAGATCTGGAGCTCGAATGGTC AAAAGACCATTCGAGCTCCAGATCTCAATCTACAAGAGTAGAAAT TCGGCACATGATGATAGCCTGGAG
crWRN	AGATGCATGAGTCTATCAGATGGGGATAATTTCTACTCTTGTAGAT GAATGAGTTGGTTCTACCGTGCC AAAAGGCACGGTAGAACCAACTCATTCATCTACAAGAGTAGAAAT TATCCCCATCTGATAGACTCATGC AGATACCAGACTGTTAAGGCTCCAGGTAATTTCTACTCTTGTAGAT TCTGATGGAAGACCAAGTGCTAC AAAAGTAGCACTTGGTCTTCCATCAGAATCTACAAGAGTAGAAAT TACCTGGAGCCTTAACAGTCTGGT
crXPF	AGATCCAGGCCTACTCGCCCTGTAAATAATTTCTACTCTTGTAGAT CGCAAAGCAGTGAGATAGCGTTG AAAACAACGCTATCTCACTGCTTTGCGATCTACAAGAGTAGAAAT TATTTACAGGGCGAGTAGGCCTGG
crChr16p	AGATCCCTCCGTAGAGACTCGTTTGAG AAAACTCAAACGAGTCTCTACGGAGGG AGATGTTCCGCCGTTGGACAACTTGCG AAAACGCAAGTTGTCCAACGGCGGAAC

Plasmids

Plasmid	Source/Reference
pBABE-CD hTERT-Puro	(*13*)
pBABE-WT hTERT-U3-hTR-500-Puro	(*14*)
pBABE-WT hTERT-U3-hTR-500-Neo	Cloned here
pCW-DD-I-SceI-GR-Puro	Cloned here
pET-TaqMan Neotelomere Control	Cloned here
pLenti-AsCpf1-Puro	(*40*)

Plasmid	Source/Reference
pLenti-sgRNA	Addgene 71409
pLenti-TaqMan-I-SceI-Blast	Cloned here
pLenti-TaqMan TS-sgLuc-Blast	Cloned here
pLenti-TaqMan TS-sgTS-Blast	Cloned here
pLKO.1	Open Biosystems

Antibodies

Protein	RRID	Antibody
53BP1	AB_399824	BD Biosciences 612523, mouse mAb, 1:1,000, 5% milk/PBST
ATR	AB_2227860	CST 2790, rabbit pAb, 1:500, 5% milk/PBST
ATRIP	AB_823659	CST 2737, rabbit pAb, 1:1,000, 5% milk/PBST
BLM	AB_2290411	Abcam ab2179, rabbit pAb 1:2,000, 5% milk/PBST
BRCA2	AB_2797730	CST 10741, rabbit mAb, 1:500, 5% milk/PBST
Claspin	AB_2082886	CST 2800, rabbit pAb, 1:250, 5% milk/PBST
CtIP	AB_10828593	CST 9201, rabbit mAb, 1:1000, 5% milk/PBST,
DNA2	AB_3075877	Abcam ab197283, rabbit pAb, 1:500, 5% milk/PBST
Exo1	AB_10675762	Abcam ab95068, rabbit pAb, 1:500, 5% milk/PBST
FANCJ	AB_2061832	CST 4578, rabbit pAb, 1:1,000, 5% milk/PBST
hSSB1	AB_1860975	Abcam ab85752, rabbit pAb, 1:2,000, 5% milk/PBST
I-SceI	AB_3075878	Abcam ab216263, rabbit pAb, 1:1,000, 5% milk/PBST
Ku70	AB_2809922	Invitrogen MA5-32645, rabbit mAb, 1:40,000, 5% milk/PBST
Ku80	AB_10983840	Invitrogen MA5-12933, mouse mAb, 1:10,000, 5% milk/PBST
Lig3	AB_3075879	Abcam ab185815, rabbit pAb, 1:1,000, 5% milk/PBST
Lig4	AB_2750871	CST 14649, rabbit mAb, 1:1,000, 5% milk/PBST
Mdc1	AB_2266361	Calbiochem DR1018, rabbit pAb, 1:10,000, 5% milk/PBST
MLH1	AB_2145615	CST 3515, mouse mAb, 1:5,000, 5% milk/PBST
MRE11	AB_2145100	CST 4895, rabbit pAb, 1:1,000, 5% milk/PBST
PARP1	AB_11001350	Enzo BML-SA249, mouse mAb, 1:1,000, 5% milk/PBST
PINX1	AB_2164405	Proteintech 12368-1-AP, rabbit pAb, 1:2,000, 5% milk/PBST
Rad51	AB_3075881	Bioacademia 70-002, rabbit pAb, 1:25,000, 5% milk/PBST
Rad52	AB_10851346	Santa Cruz sc-365341, mouse mAb, 1:100, 5% milk/PBST
RadX	AB_2687552	Novus NBP2-13887, rabbit pAb, 1:500, 5% milk/PBST
Rev7	AB_2890174	Abcam ab180579, rabbit mAb, 1:500, 5% milk/PBST
SLX4	AB_3075880	Abcam ab169114, mouse pAb, 5% milk/PBST, 1:500
WRN	AB_10692114	CST 4666, mouse mAb, 1:500, 5% milk/PBST
XPF	AB_2798227	CST 13465, rabbit mAb, 1:500, 5% milk/PBST
γ-tubulin	AB_532292	Sigma T5326, mouse mAb, 1:30,000, 5% milk/PBST

Drugs/Chemicals/Recombinant Proteins

Drug/Chemical/Protein	Supplier/Solvent
BIBR1532	Tocris 2981, DMSO

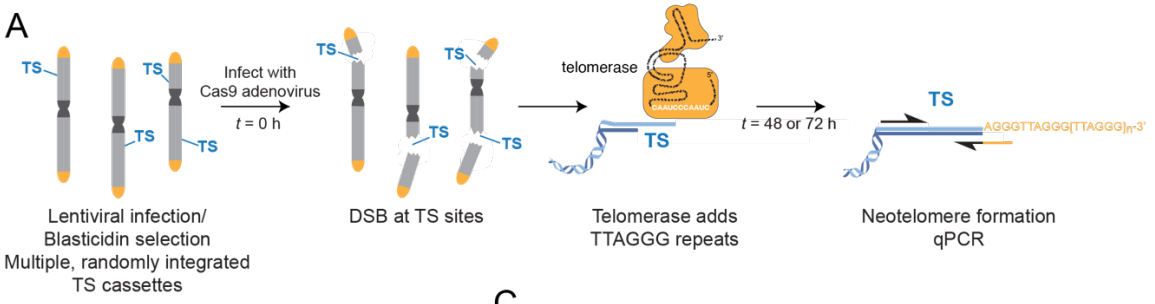
Drug/Chemical/Protein	Supplier/Solvent
KU55933	Selleckchem S1092, DMSO
AZD-6748	Selleckchem S8843, DMSO
VE-821	Selleckchem S8007, DMSO
M4344/Gartisertib	MedChemExpress HY-136270, DMSO
CHIR124	Selleckchem S2683, DMSO
MK-8776	Selleckchem S2735, DMSO
CCT245737	Selleckchem S8253, DMSO
Ganciclovir	Sigma-Aldrich G2536, DMSO
Shld1	Takara 632189, ethanol
Triamcinolone acetonide	MedChemExpress HY-B0636, DMSO
Doxycycline hyclate	Sigma-Aldrich D5208, ddH_2O
Cas9 nuclease	Abm K108

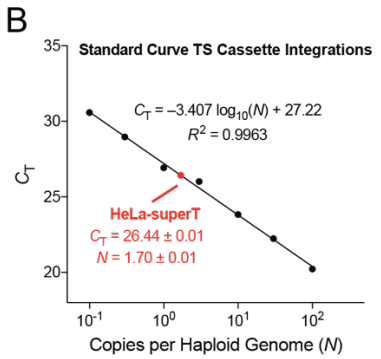
Kinzig et al., fig. S1

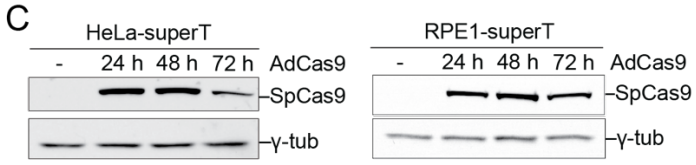
ID	Locus	Sequence	Ref.
TI (TS)	16p13.3	GGAGGTGGGTGGGGCGAGCAGAGTTAGGGTT	*(8)*
BO	16p13.3	CGTGTGTACAGAGAAGCAAGGGGGTTAGGGT	*(41)*
CMO	16p13.3	TAGCCGAGGTTCTGTGTTCAGAGGTTAGGGT	*(42)*
IdF	16p13.3	TAAGTGGGGCTGGGGAACCTCCAGGGTTAGG	*(42)*
TAT	16p13.3	CCCCAGGGTCCCCTTATATACAGGGTTAGGG	*(42)*
NT	22q13.33	GGGGGTGGAGAGGGGGGTGGTGGAGAGGGTT	*(43)*
FB336R	7q32.3	GAAGGGCAGAAATATGGCCACGAGAGGGTTA	*(44)*
BR	16p13.3	GTCTGCATAGAGTCCTATACTTAGGGTTAGG	*(45)*
HW	16p13.3	AAGTATCACGTAGTCACGATGGTAAGGGTTA	*(45)*
23	1p36.32	CCAGCTTGTGGCTGACTGTCACTGGGTTAGG	*(46)*
P3	9q34.3	TGTGGGGCTGAGCGGAGAAGACCGGTTAGGG	*(47)*
P40	9q34.3	TTTTAATTTACTCTTGAGACAGGGTTAGGGT	*(47)*
1	4p16.3	GGGGCGCCACAGGGGCAGAGGCTGGTTAGGG	*(48)*
2	4p16.3	TCTGTTCCATTGGTCTGCGTGTCGGTTAGGG	*(48)*
4	4p16.3	AACCTTGACCTTCCAGGCTCACGTGTTAGGG	*(48)*
5	4p16.3	TGCCACACTGCAGCCCGTGAAGTAGGGTTAG	*(48)*
6	4p16.3	TTTCCTGGATGTTCCAGAGAGTTTAGGGTTA	*(48)*
7	4p16.2	CAGCAGCAACAACGACAACAGAATAGGGTTA	*(48)*
8	4p16.2	CATACCTAAAACATCTCTAGTAGGGTTAGGG	*(48)*
P01	22q13.33	AGGCGGGCAGACTACCTGAGGTCGGTTAGGG	*(49)*
P04	22q13.33	GCTCAGGCCGGGACTCTGGGTCAGGGTTAGG	*(49)*
P05	22q13.31	AGCCAGGGGTTAGTTATAGCCAGGGTTAGGG	*(49)*
P06	22q13.32	CCCAGCTGAGGTCCACAGATCACGGTTAGGG	*(49)*
P12	22q13.31	AGAGAGAGACTTGAGTTGGAACTGTTAGGGT	*(49)*
P13	22q13.33	AGAGTGAAGGTAAAGGCAGTTTTAGGGTTAG	*(49)*
P14	22q13.31	TGGAAAGTCCTTAGGCATGTGCAGTTAGGGT	*(49)*
P20	22q13.2	CTGAAAAGAATATAAGGTTCTCTTGGTTAGG	*(49)*
P31	22q13.33	GGGTGGAGAGGGGGGTGGTGGAGGGTTAGGG	*(49)*
P32	22q13.33	GGAGAGGGGGGTGGTGGAGGGGGTTAGGGTT	*(49)*
P36	22q13.2	CTTTGAACAAATGAGAGATTGAAGTTAGGGT	*(49)*
P1	18q21.33	CAGTTGGGGCACTGGGAGAAACAGGTTAAGG	*(50)*
P2	18q21.33	ATCCCAGCTACTCAGGAGGCTGAGGTTAGGG	*(50)*
P3	18q21.32	CATAAGAGCAGACAACATAGGCATGTTAGGG	*(50)*
P4	18q21.32	TTAGAAATACATACGTAATAGACATTTAGGG	*(50)*
P5	18q21.31	CAAATTATAATATCTTCAGAAAAAGGGTTAG	*(50)*
P6	18q22.1	TAACGCCACTCCATTTCCTTGGCTTAGGGTT	*(50)*

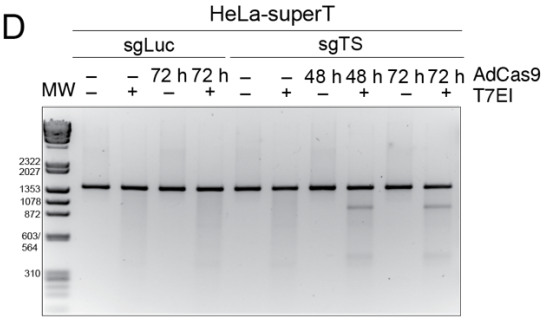
Fig. S1.
Breakpoint sequences from suspected neotelomere formation events in patients with terminal chromosomal deletions. Candidate neotelomere formation events were culled from the literature. The 25-nt sequence proximal to unambiguous telomeric sequence—that is, telomeric sequence not present in the reference genome—is provided followed by the first telomeric repeat in orange text. Breakpoint homology to the hTR template sequence is indicated by blue, bold text. No microhomology is present at the breakpoints in patients NT and P4. TS is derived from patient TI (underlined, top). In patient P1, telomerase appears to have mis-incorporated an A instead of a G. Distal to this A, P1's DNA shows TTAGGG repeats.

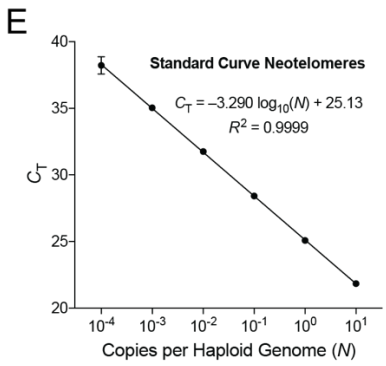
Kinzig et al., fig. S2

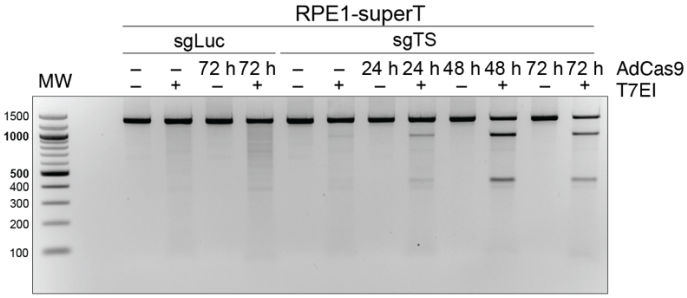


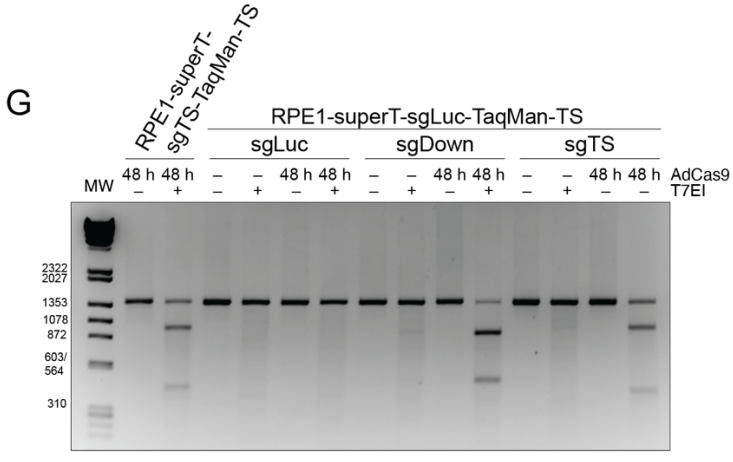












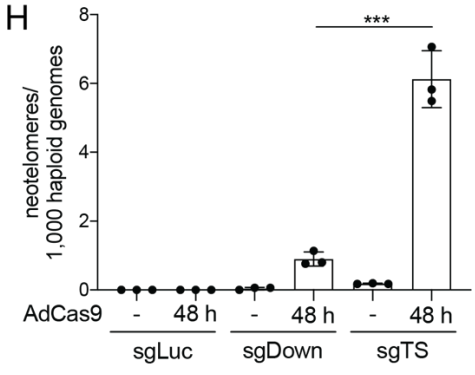


Fig. S2.

Cas9 neotelomere formation assay workflow and relevant information. (A) Schematic depicting the neotelomere formation assay strategy. (B) Standard curve for copy number measurement of the pLenti-sgTS-TaqMan-TS cassette using a plasmid template spiked into human genomic DNA at a known copy number. The data plotted in black are the mean C_T of three technical replicates versus the base-10 logarithm of the number of plasmid template copies per haploid genome. The data were fitted by linear regression in GraphPad Prism 9 to obtain the displayed equation. The red data point corresponds to the mean C_T value and the calculated number of integrated pLenti-sgTS-TaqMan-TS cassettes in HeLa-superT cells per haploid genome from three biological replicates. The copy number is 5.6 ± 0.3 in RPE1-superT cells and 15.4 ± 1.4 in RPE1 cells. (C) Immunoblots for SpCas9 expression at the indicated time points after infection of HeLa-superT and RPE1-superT cells. γ-tub, loading control. (D) EthBr-stained agarose gel showing the result of a T7 endonuclease I assays performed on PCR products corresponding to the TS site in RPE1-superT and HeLa-superT cells expressing sgLuc or sgTS at the indicated times after infection with AdCas9. T7EI cleavage products are expected at ~400 and ~1000 bp. (E) Standard curve for the Cas9 TaqMan neotelomere formation assay using a positive-control plasmid template spiked into human genomic DNA at a known copy number. Three independent qPCRs were performed, each in technical quadruplicate. The data plotted are mean C_T ± SD versus the base-10 logarithm of the number of template copies per haploid genome. Most error bars are too small to be displayed. The data were fitted by linear regression in GraphPad Prism 9 to obtain the displayed equation. (F) Schematic showing an additional Cas9 cut site downstream of the TS site (denoted as Down). The sequence of the TS PCR cassette, primers, TaqMan binding site, TS sequence, Cas9 cleavage sites, sgRNA binding sites, and PAMs are highlighted, as in Fig. 1A. Cells containing this cassette were transduced with lentiviruses to constitutively express either sgLuc (control), sgDown, or sgTS sgRNAs and then induced with AdCas9. Neotelomere formation at the TS site was assessed via TaqMan qPCR using the primer pair and TaqMan probe shown. (G) EthBr-stained agarose gel showing the result of T7EI assays performed on PCR products corresponding to the TS site and the downstream Cas9 site (Down, as indicated in panel (F)) at the indicated times after infection with AdCas9. T7EI cleavage products are expected at ~400 and ~1000 bp for the sgTS sgRNA and ~900 and ~500 bp for the sgDown sgRNA. (H) Quantification of neotelomere formation at the TS site by TaqMan qPCR in the RPE1-superT cells expressing sgLuc, sgDown, or sgTS. Mean ± SD of 3 biological replicates. *** $p < 0.001$, two-tailed unpaired *t*-test.

Kinzig et al., fig. S3

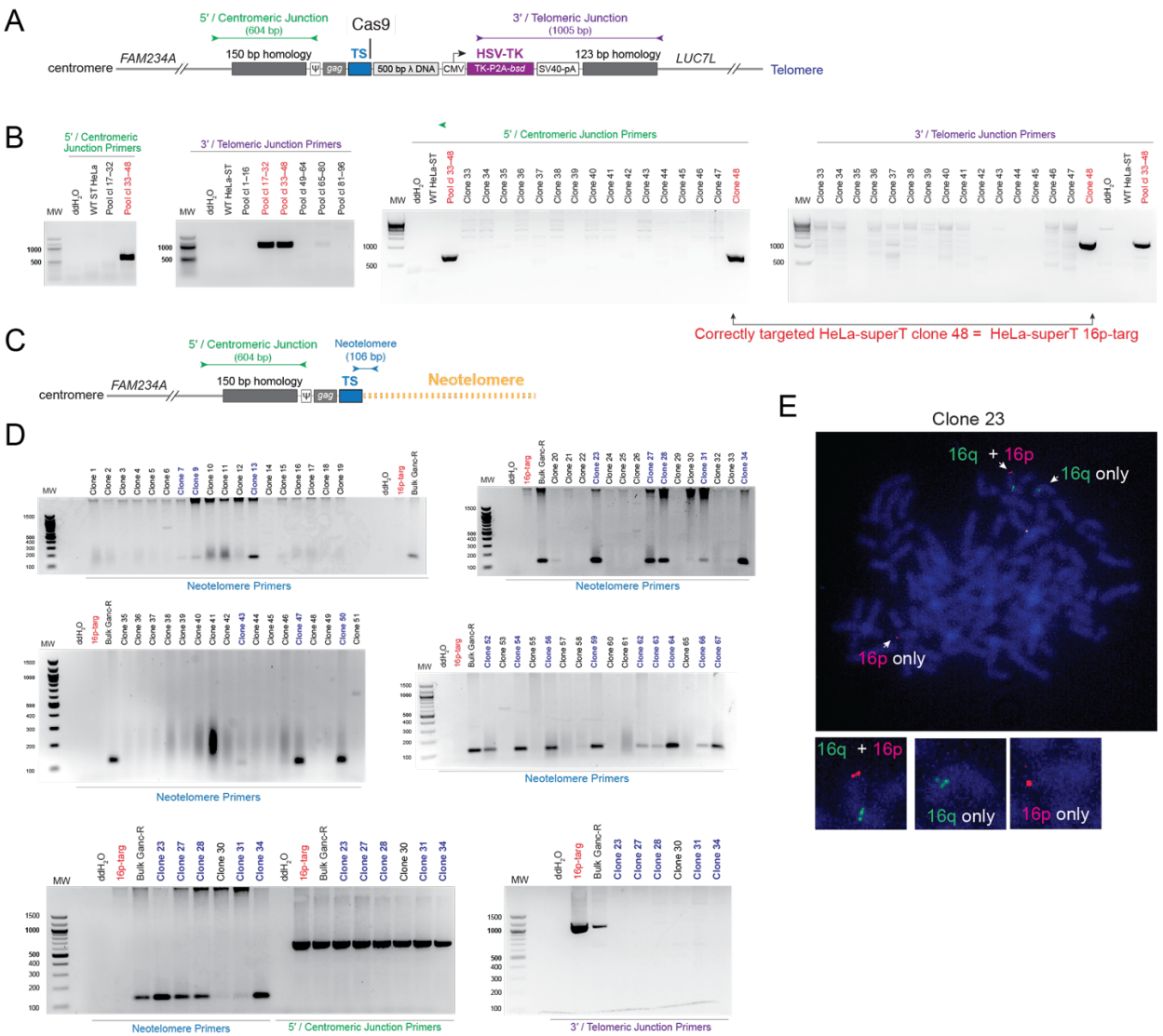


Fig. S3.

Identification of HeLa-superT clones bearing neotelomeres at a predetermined locus. (A) Schematic depicting the CRISPR/Cas12a-edited chromosome 16 in the HeLa-superT 16p targeted clone (16p-targ) and the PCR-based strategy to identify an HeLa-superT clone containing the knock-in cassette at the appropriate location. PCR primers were designed to target amplicons spanning the 5′/centromeric and 3′/telomeric junctions between the endogenous chromosome 16 and knock-in cassette. Abbreviations are as in Fig. 3A. (B) EthBr-stained agarose gels showing PCR products obtained from targeted HeLa-superT pools and clones to identify a clone that contains the TS insert at the 16p locus. Single-cell clones were screened first in pools and then individually with the two primer pairs in (A) to identify clones yielding the expected centromeric and telomeric junction PCR products. Clone 48 (red) was the only clone identified as containing the correct insertion at both ends of the knock-in cassette. This clone was renamed HeLa-superT 16p-targ. (C) Schematic depicting the predicted map of the edited copy of

chromosome 16 in HeLa-superT 16p-targ following successful neotelomere formation at the TS site. The binding sites of primer pairs designed to screen for 5′/centromeric junction retention and neotelomere formation are shown. (D) EthBr-stained agarose gels from PCR assays used to identify HeLa-superT 16p-targ derived clones with neotelomeres at TS. Genomic DNA was harvested from the indicated Ganc-R clones and PCR amplified with the indicated primer pairs shown in (A) and (C). ddH_2O and genomic DNA from the parental HeLa-superT 16p-targ (16p-targ) were used as negative controls. Genomic DNA derived from a mixed population of ganciclovir-resistant (Ganc-R) clones was used as a positive control. A subset of clones was further analyzed for retention and loss of the expected segments at the target locus (bottom gels). Neotelomere clones are highlighted in bold, blue type. (E) Representative metaphase from ganciclovir-resistant clone 23 hybridized with FISH probes for 16q and 16p (see Fig. 3) showing the presence of one intact chromosome 16, one chromosome with only the 16q signal (consistent with neotelomere formation), and one chromosome with only the 16p signal.

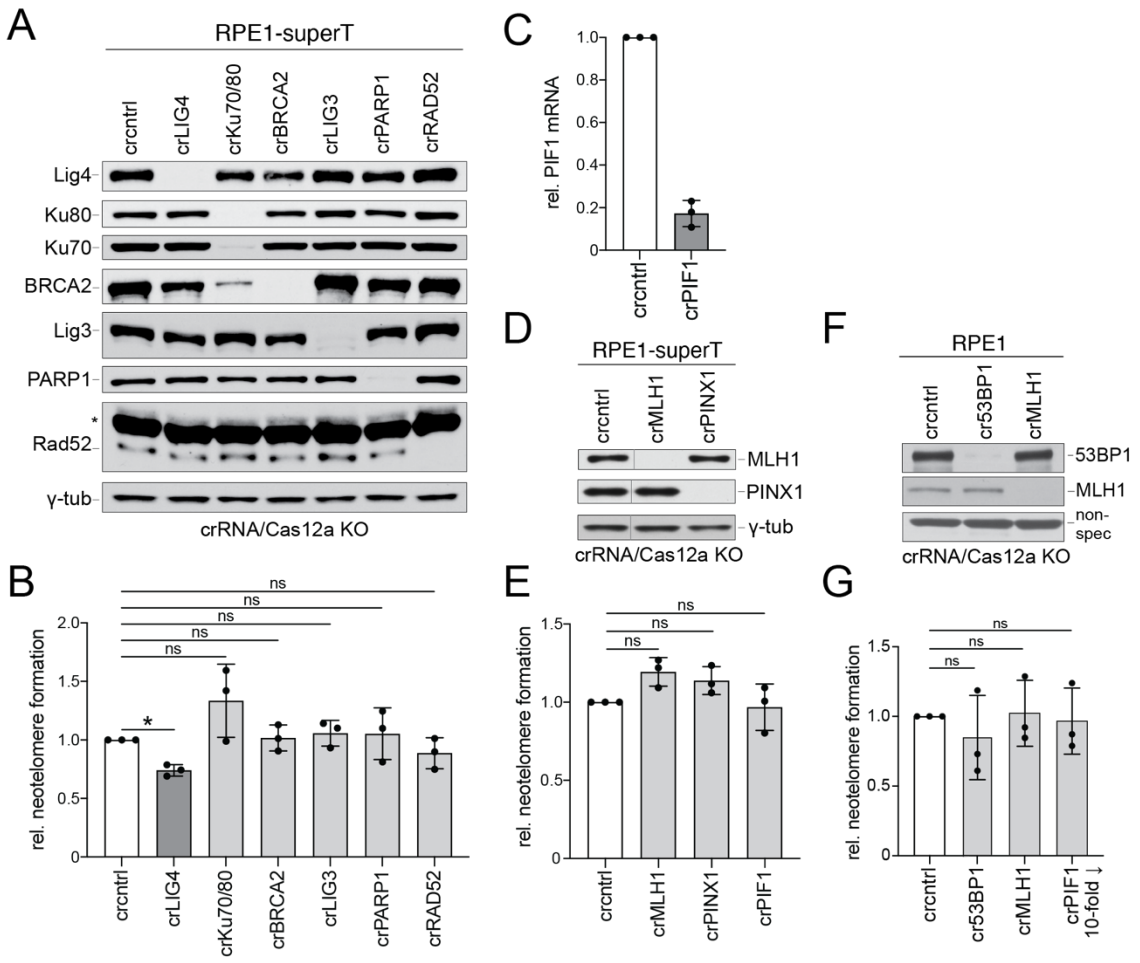


Fig. S4.

DSB repair genes that do not affect neotelomere formation. (A) Immunoblots for the indicated proteins from RPE1-superT cells treated with Cas12a and crRNAs targeting LIG4, Ku70/80 (combination of crKu70 and crKu80), BRCA2, LIG3, PARP1, or RAD52 or a non-targeting control (cntrl) crRNA. γ-tub, loading control; * non-specific band. (B) Relative neotelomere formation based on qPCR in the RPE1-superT cells shown in (A) normalized to cells treated with the control crRNA. (C) Abundance of PIF1 mRNA as assessed by RT-qPCR in RPE1-superT cells treated with Cas12a and a crRNA targeting PIF1 or the control crRNA. Relative PIF1 mRNA levels were normalized to GAPDH mRNA. (D) Immunoblots for the indicated proteins from RPE1-superT cells treated with Cas12a and crRNAs targeting MLH1 or PINX1 or a non-targeting control (cntrl) crRNA. γ-tub, loading control. The blot was cut between lanes 1 and 2 to remove irrelevant knockout samples. (E) Relative neotelomere formation based on qPCR on RPE1-superT cells treated with crRNAs targeting MLH1, PINX1, or PIF1 normalized to that of cells treated with a non-targeting control (cntrl) crRNA. (F) Immunoblots for the indicated proteins from RPE1 cells treated with Cas12a and crRNAs targeting 53BP1 or MLH1 or a non-targeting control (cntrl) crRNA. n.s., non-specific band used as a loading control. (G) Relative neotelomere formation based on qPCR on RPE1 cells treated with crRNAs targeting 53BP1, MLH1, or PIF1 normalized to that of cells treated with a non-

targeting control (cntrl) crRNA. PIF1 mRNA was found to be reduced 10-fold in RPE1 cells treated with crRNAs targeting PIF1 relative to cells treated with the control crRNA. Mean ± SD of 3 biological replicates. ns $p > 0.05$, * $p < 0.05$, two-tailed ratio-paired *t*-test in (B), (E), and (G).

Kinzig et al., fig. S5

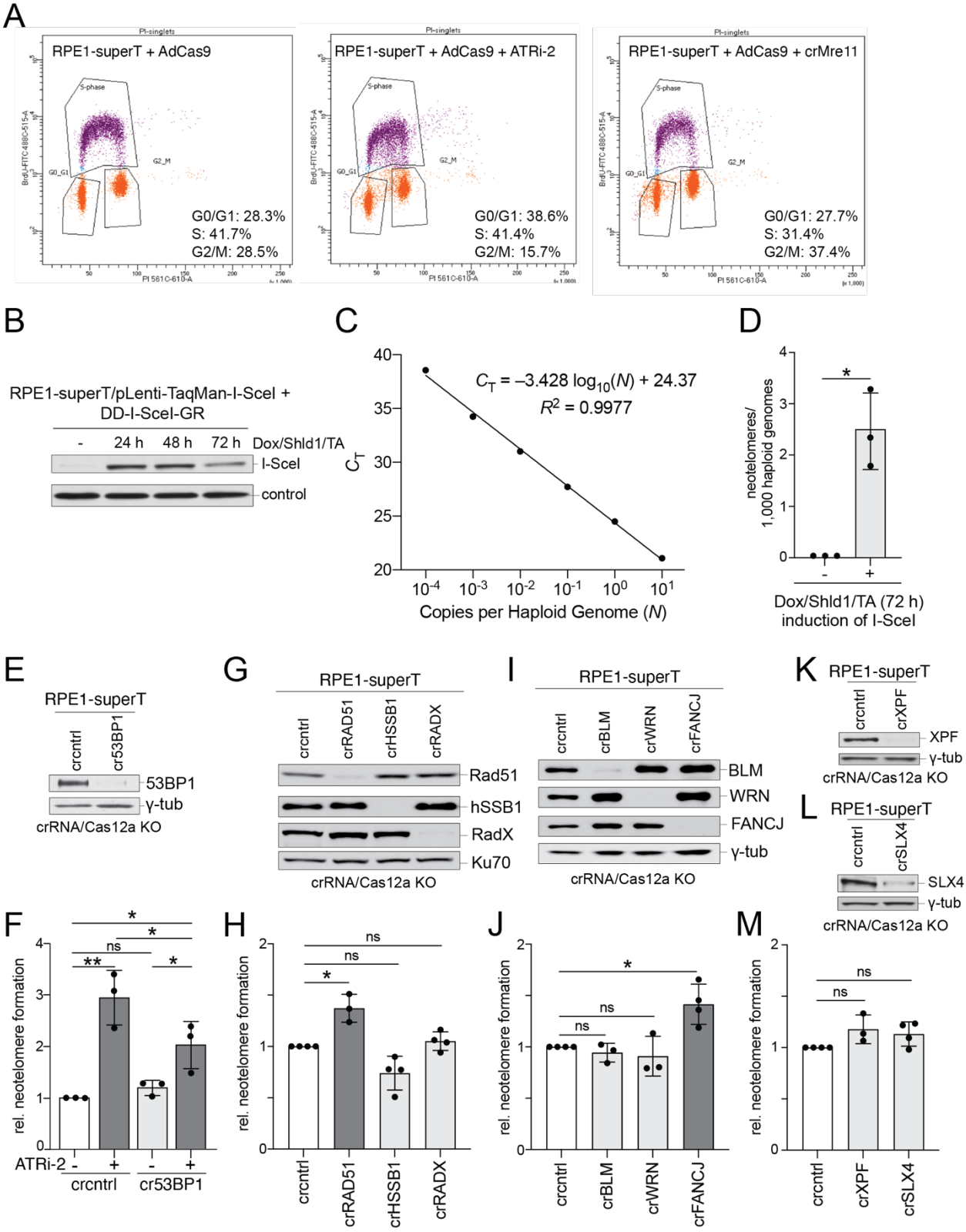


Fig. S5.

Potential downstream effectors of ATR-mediated telomerase inhibition at DSBs. (A) FACS profiles of the RPE1-superT cells treated with AdCas9 alone (left), AdCas9 plus ATRi-2 (center), or AdCas9 plus MRE11 crRNA knockout (right). Cells were harvested 48 h after AdCas9 infection. The intensities of the propidium iodide (PI) and BrdU-FITC signals for PI singlet cells are plotted on the *x*- and *y*-axes, respectively. Regions of the plot corresponding to G0/G1, S, and G2/M phases and the proportion of cells in each assigned phase are shown. (B) Immunoblot for I-SceI in RPE1-superT cells infected with pLenti-TaqMan-I-SceI and pCW-DD-I-SceI-GR-Puro and then induced with doxycycline (Dox), Shield1 (Shld1), and triamcinolone acetonide (TA) for the indicated amount of time. n.s., non-specific band used as a loading control. (C) Standard curve for the I-SceI TaqMan neotelomere formation assay using a positive-control template spiked into human genomic DNA at a known copy number. The data plotted are mean C_T versus the base-10 logarithm of the number of template copies per haploid genome. The data were fitted by linear regression in GraphPad Prism 9 to obtain the displayed equation. (D) TaqMan-qPCR quantification of neotelomeres formed after 72 h in the I-SceI neotelomere formation assay in RPE1-superT assay. (E) Immunoblot for 53BP1 from RPE1-superT cells infected with the pLenti-sgTS-TaqMan-TS lentivirus and treated with Cas12a and crRNAs targeting 53BP1 or a control crRNA. γ-tub, loading control. (F) Relative neotelomere formation based on qPCR in the RPE1-superT cells shown in (E) with or without ATRi. (G) Immunoblots for the indicated proteins from RPE1-superT cells treated with Cas12a and crRNAs targeting RAD51, hSSB1, or RADX or a control (cntrl) crRNA. Ku70, loading control. (H) Quantification of neotelomere formation by TaqMan qPCR in the RPE1-superT cells shown in (G) normalized to cells treated with the control crRNA. (I) Immunoblots for the indicated proteins from RPE1-superT cells treated with Cas12a and crRNAs targeting BLM, WRN, or FANCJ or a control (cntrl) crRNA. γ-tub, loading control. (J) Quantification of neotelomere formation by TaqMan qPCR in the RPE1-superT cells shown in (I) normalized to cells treated with the control crRNA. (K and L) Immunoblots for the indicated proteins from RPE1-superT cells treated with Cas12a and crRNAs targeting XPF (K), SLX4 (L), or a control (cntrl) crRNA. γ-tub, loading control. (M) Quantification of neotelomere formation by TaqMan qPCR in the RPE1-superT cells shown in (K) and (L) normalized to cells treated with the control crRNA. Mean ± SD of at least 3 biological replicates. ns $p > 0.05$, * $p < 0.05$, two-tailed unpaired *t*-test in (D) and two-tailed ratio-paired *t*-test in (F), (H), (J), and (M). ATRi-2, gartisertib/M4344, 1 μM.

Kinzig et al., fig. S6

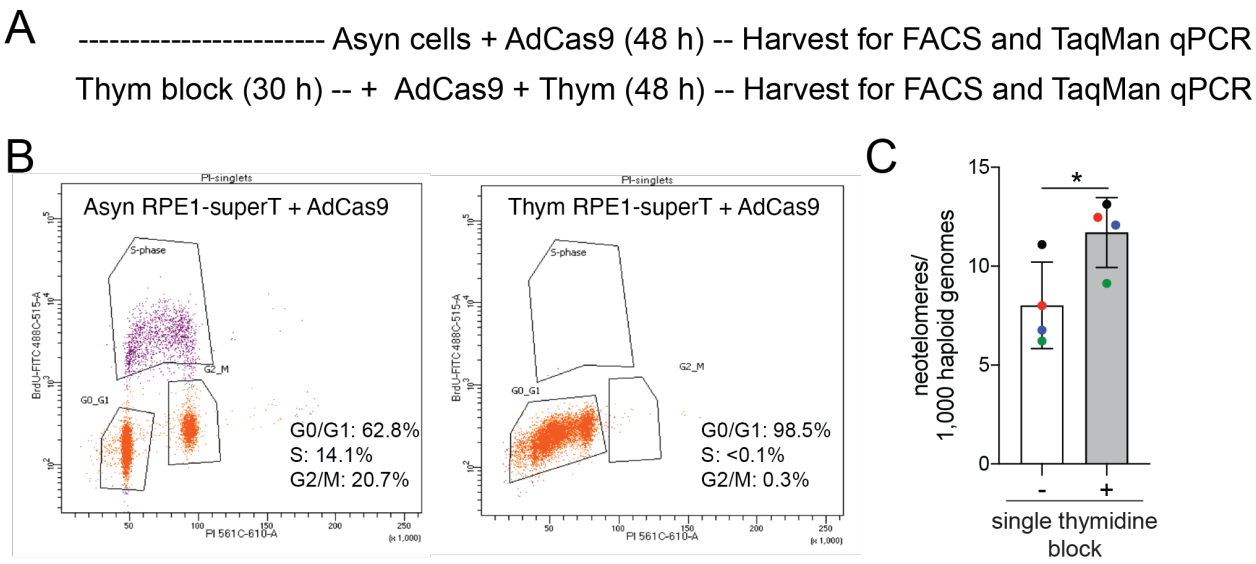


Fig. S6.

Neotelomere formation can take place in G1. (A) Timeline depicting the experimental strategy used to determine whether neotelomere events can occur during G1. RPE1-superT cells bearing the pLenti-sgTS-TaqMan-TS construct were either maintained in asynchronous culture or treated with thymidine for 30 h before induction with AdCas9. Thereafter, cells continued with or without thymidine treatment for another 48 h before harvest for FACS cell cycle profiling and qPCR for neotelomere quantification. (B) FACS profiles of the RPE1-superT cells described in (A) at harvest. The intensities of the propidium iodide (PI) and BrdU-FITC signals for PI singlet cells are plotted on the *x*- and *y*-axes, respectively. Regions of the plot corresponding to G0/G1, S, and G2/M phases and the proportion of cells in each assigned phase are shown. (C) Quantification of neotelomere formation by TaqMan qPCR in the RPE1-superT cells treated with or without thymidine blockade. Data points bearing the same color belong to the same biological replicate. * $p < 0.05$, two-tailed ratio-paired *t*-test.