Supplemental methods:

hESC culture, editing, and differentiation

Culture of WIBR#3 hESCs[1] [NIH stem cell registry #0079] was carried out as previously described[2]. All targeting experiments in hESC were performed as previously described[3,4]. CAS9 and all sgRNAs were expressed using the pX330 plasmid or pX458 plasmids[5,6].

Fibroblast-like cells were derived from embryoid bodies (EBs) as previously described[7,8]. Cells were trypsinized, counted, and plated at a density of 500,000 cells per 10 cm dish. The medium was changed every 3-4 days and cells were passaged every 5-8 days. Growth curves were presented by plotting cumulative population doublings (PDs) against days after differentiation.

Deletion of exon 4-7 of *TINF2* in hESCs

hESCs were electroporated with 15 μg of two CAS9-P2A EGFP plasmids (pX458), each containing guide sequences TGTTCAAGTTCCTACAGCAG and CCTGACTCAGACTACCTACC respectively and were sorted for GFP expression 24 hours after electroporation. Targeting was confirmed by PCR (fw: GGCCACTAACCCACTTTTGA, rev: TGGCCATTTTCTTCCTCATC).

cDNA expression analysis

RNA was extracted with TRIzol [Ambion, 15-596-018] and treated with DNaseI [NEB, M0303]. 600 ng RNA were converted to cDNA with the qScript Reverse Transcriptase [Quantabio, 95047-100]. 2 μL cDNA from the qScript reaction mixture was used for the detection of the TIN2 transcripts. PCR was performed with Phusion Polymerase [NEB, M0530] and GC Buffer.

[Primers for detecting mutant/wt allele expression]

fw: CTAGCTGGCCGACACTTCAA rev1: CATGGCTAGGTCTGCTGTGT

[Primers for detecting all isoforms]

fw: CTAGCTGGCCGACACTTCAA rev2: CACAGAGACGGAGGACACAC

qRT-PCR

0.5 μL cDNA from the qScript reaction mixture was used for each reaction of KAPA SYBR Fast reaction mix [Kapa Biosystems, KK4601]. Relative expression levels were calculated based on Δ/ΔCt.

[GAPDH primers]

fw: TGCACCACCAACTGCTTAGC, rev: GGCATGGACTGTGGTCATGAG

[TIN2 primers]

fw: GATTTTGGAGGCACAGGAAA, rev: CTGCATCCAACTCAGCACAT

Immunofluorescence staining/TIF analysis

hESCs on Matrigel (Corning, 354234)-covered coverslips were fixed using 2% paraformaldehyde in phosphate-buffered saline (PBS). The fixed cells were permeabilized by 0.1% Triton X-100 in PBS and blocked in blocking solution (3% v/v horse serum, 1 mg/ml BSA, and 1 mM EDTA in PBS). Fixed cells were incubated in primary antibodies diluted in the blocking solution overnight at 4°C followed by PBS washes and incubated in diluted secondary antibodies at room temperature for 1 hour. Coverslips were washed with PBS, stained with DAPI, and

mounted using ProLong Gold Antifade reagent [ThermoFisher, L3000001]. Antibodies used: TIN2 [Novus Biologicals, NB600-1522SS], TRF1 [custom-made antibody raised against the peptide sequence DGRDADPTEEQMAETERNDEEQFEC-NH2 (aa17-41), Chinapeptide], TPP1 [Abcam, ab220740], γH2A.X [Millipore 05-636]. TIF(+) used in Figure 2: TPP1S KO line[9], TIF(+) used in Figure 3: WT/WT + TPP1ΔPBD line[10].

Z-stack images of the samples were taken using Nikon Eclipse TE2000-E Widefield EpiFluorescence microscope with ANDOR Zyla sCMOS camera. TIFs were scored blindly from the maximum projection images by detecting colocalization of TRF1 and γH2A.X using an ImageJ plugin ComDet v0.3.7 (Cell Biology Group of Utrecht University https://github.com/ekatrukha/ComDet). # TIFs per cell indicates the number of colocalizations of TRF1 and γH2A.X puncta in a cell. # Telomeres detected per cell indicates the number of TRF1 puncta detected in a cell.

Telomere restriction fragment assay

Genomic DNA was extracted using phenol chloroform following overnight Proteinase K digestion (0.25 mg/mL) and resuspended in TE buffer. Genomic DNA was digested with MboI, AluI and RNaseA overnight at 37°C. 2 µg of digested DNA was run on 0.75% agarose [Lonza, 12001-876]. The gel was dried for 2 hours at 50°C, denatured in 0.5 M NaOH, 1.5 M NaCl solution, and neutralized with 1 M Tris pH 6.0, 2.5 M NaCl. The gel was pre-hybridized in Church's buffer (1% BSA, 1 mM EDTA, 0.5 M NaPO$_4$ pH 7.2, 7% SDS) at 55°C before adding a $_{32}$P-end-labeled $(T_2AG_3)_4$ telomeric probe. The gel was washed three times, 5 minutes each, in 4 X SSC at 50°C, 15 minutes in 4 X SSC + 0.1% SDS at room temperature, and was exposed on a phosphorimager screen.

Immunoblotting

Protein samples were extracted in the lysis buffer of the following composition: 20 mM Hepes-KOH pH 7.9, 0.42 M KCl, 25% glycerol, 0.1 mM EDTA, 5 mM MgCl2, 0.2% NP-40, complete protease inhibitor cocktail [Sigma-Aldrich, 05892970001], and 1 mM PMSF. The lysate was quantified by Bradford reagent [Biorad, 500-0006] and diluted in the lysis buffer, sample buffer, and 2-Mercaptoethanol (5% final) to have 15 µg protein per well. After heating to 95°C for 5 min, they were resolved by SDS-PAGE. The protein was then transferred to PVDF or nitrocellulose membranes and subsequently incubated with the indicated antibodies diluted in 5% milk in Tris buffered saline with 0.1% Tween 20 (TBST). Antibodies used: Actin [Abcam, 8226], TIN2 [#864[11], gift from Titia de Lange], TRF1 [Chinapeptide, custom-made antibody raised against peptide sequence DGRDADPTEEQMAETERNDEEQFEC-NH2 (aa17-41)], TRF2 [Novus Biologicals, NB110-57130], TPP1 [Bethyl, A303-069A]. After washing with TBST, the membrane was visualized using ECL [Bio-Rad, 1705061].

Metaphase spreads/fluorescence in situ hybridization (FISH)

Cells were treated with colcemide at 100 ng/mL for 1 hour. The cells were collected using trypsin and incubated at 37°C in prewarmed 75 mM KCl for 5 minutes. The cells were pelleted and slowly resuspended in 3:1 methanol:acetic acid fixative while slowly vortexed. The resuspension was stored overnight at 4°C. The next day, cells were spread dropwise onto microscope slides and washed twice with 1 mL 3:1 methanol:acetic acid fixative. Slides were placed in a 50°C humidified hot plate chamber for 1 minute and dried at room temperature overnight. The spread was rehydrated in PBS for 5 minutes, fixed with 4% formadehyde in PBS for 2 minutes, washed in PBS three times for 5 minutes each, treated with 0.2 mg/mL pepsin in 10 mM glycine pH 2.0 at 37°C for 5 minutes, washed in PBS twice for 2 minutes each, fixed again with 4% formaldehyde in PBS for 2 minutes, and washed in PBS three times for 5 minutes each. The samples were dehydrated in 70%, 95%, and 100% ethanol for 5 minutes each. Hybridization mix was prepared by diluting TelC-Cy3 probe [PNA Bio, F1002] and CENPB-Cy5 probe [PNA Bio, F3005] 1:1000 in 10 mM Tris-HCl pH 7.2, 70% formamide, 0.5% blocking reagent (from 10% stock) [Roche, 11096176001]. The sample slide with the hybridization mix on top was placed on an 65°C humidified hot plate chamber for 5 minutes and kept at room temperature overnight in dark. The slides were washed in 10 mM Tris-HCl pH 7.2, 70% formamide in water twice for 15 minutes each and then in PBS three times for 5 minutes each. 0.5 mg/mL stock of DAPI was diluted 1:1000 in the second PBS wash. The slides were mounted using ProLong Gold Antifade reagent [ThermoFisher, L3000001].

Images of the samples were taken using Nikon Eclipse TE2000-E Widefield EpiFluorescence microscope with ANDOR Zyla sCMOS camera. Chromosomal abnormalities were blindly scored.

Telomere chromatin immunoprecipitation (ChIP)

hESCs were crosslinked in 1% formaldehyde in PBS for 15 min at room temperature on a rocker. Glycine was added to the final concentration of 0.2 M to quench the reaction for 5 minutes at room temperature on a rocker. The cells were washed twice with cold PBS and scraped in 10 mL cold PBS. Cells were spun at 1000 rpm for 5 minutes and resuspended in cold PBS with 1 mM PMSF. Supernatants were removed and the cell pellets were flash frozen and stored at -80°C until lysis. The pellets were lysed in ChIP lysis buffer (1% SDS, 10 mM EDTA pH 8.0, 50 mM Tris-Hcl pH 8.0 supplemented before use with complete protease inhibitor cocktail [Sigma-Aldrich, 04693159001] and 1 mM PMSF) for 15 minutes on ice. The lysate was sonicated 15 seconds on, 15 seconds off, for 15 iterations using Covaris S220 system at the peak power of 175.0, cycle/burst = 200, duty factor = 20, in 12x24 mm vials [Covaris, 520056]. After sonication, the lysates were spun down at max speed for 10 minutes at room temperature. 50 µL lysates were kept as the input fraction and processed later. 100 – 200 µL (~ 1 – 2 x10^6 cells) lysates were diluted with 1 mL of IP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.0, 150 mM NaCl supplemented with protease inhibitors and PMSF), and incubated on ice for 10 minutes. The lysates were incubated with 2 µg antibodies overnight at 4°C. On the next day, 30 µL magnetic beads (Cell signaling, 9006) were added to each IP and incubated for 30 minutes at 4°C on a rotator. The beads were washed with buffers A, B, C, and TE (Buffer A: 0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8.0, 20 mM Tris-HCl pH 8.0, 150 mM NaCl, supplemented with protease inhibitors and PMSF before use, Buffer B: 0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8.0, 20 mM Tris-HCl pH 8.0, 500 mM NaCl, Buffer C: 0.25 M LiCl, 1% NP-40, 1% Na-Deoxycholate, 1 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.0, TE: 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). The immunoprecipitated materials were eluted by incubating the beads in 250 µL 1% SDS/0.1 M NaHCO$_3$ by vortexing and incubating for 10 minutes. The process was repeated twice to pool 500 µL eluate. 450 µL 1% SDS/0.1 M NaHCO$_3$ were added to the 50 µL input fraction. 20 µL 5 M NaCl was added to all the samples of 500 µL volume and incubated at 65°C for 4-6 hours to reverse the crosslinks. 1 mL 100% ethanol was added to each sample and incubated at -20°C overnight. The samples were spun down at maximum speed for 10 minutes at 4°C and processed with cells and tissue DNA isolation micro kit (Norgen Biotek, 57300). The DNA was boiled and dot blotted on a membrane (Cytiva, RPN203S). The membrane was denatured in 1.5 M NaCl, 0.5 M NaOH, Neutralized in 3 M NaCl, 0.5 M Tris-HCl pH 7.0 and crosslinked in UV crosslinker. The membrane was prehybridized in Church buffer for 30 minutes at 65°C, and hybridized overnight with 800-bp Alu and telomere probes generated by random-priming labeling as described previously[12]. Membranes were washed four times in 2 X SSC at room temperature, 1 minute each, and exposed to PhosphorImager screen. The quantification of the signals was done with ImageJ. The percentage of each telomere immunoprecipitation sample was calculated based on the signal relative to the input DNA signal. The values were normalized to the wild-type cell level and the normalized values of three independent cell pellet replicates were plotted.

Antibodies used: rabbit IgG (Cell Signaling Technology, 2729S), TRF1 [Chinapeptide, custom-made antibody raised against peptide sequence DGRDADPTEEQMAETERNDEEQFEC-NH2 (aa17-41)], TPP1 [Bethyl, A303-069A].

Telomeric repeat amplification protocol

PCR-based telomeric repeat amplification protocol (TRAP) was performed as previously described[13] using primers TS (AATCCGTCGAGCAGAGTT) and ACX (GCGCGGCTTACCCTTACCCTTACCCTAACC) for amplification of telomeric repeats and TSNT (AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT) and NT (ATCGCTTCTCGGCCTTTT) as an internal control. Cell extract was generated using hypotonic lysis buffer (HLB) (20 mM HEPES, 2 mM MgCl$_2$, 0.2 mM EGTA, 10% Glycerol, 1 mM DTT, 0.1 mM PMSF) supplemented with 0.5% CHAPS (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) and samples were normalized using the Bradford assay. 200 ng of total protein were used for detection of fibroblasts telomerase activity. For hESCs, a series of diluted lysate, 200 ng were used. The TRAP products were resolved on 10% polyacrylamide /1 X TAE (Tris-acetate-EDTA) gel. Dried gels were visualized by phosphor imaging.

hTERC overexpression:

hTERC was overexpressed by targeting the AAVS1 locus of hESCs as previously described[7].

TeSLA (Telomere shortest length assay)

TeSLA was performed as described previously with slight modifications[14]. Briefly, genomic DNA was extracted using phenol chloroform following overnight proteinase K digestion (0.25 mg/mL). Purified DNA was resolved in TE buffer and quantified. 50 ng of isolated genomic DNA was used per sample for the ligation reaction. Then DNA was diluted to 40 pg per PCR reaction. 8 PCR reactions were run out per sample and quantified. PCRs were run on 0.75% agarose gels as described for telomere length analysis by telomere restriction fragment assay and detected by in-gel hybridization with a $_{32}$P-end-labeled $(T_2AG_3)_4$ telomeric probe. Quantification of the blots was performed using a MatLab plugin described in Lai et al., 2017[14].

STELA (Single telomere length analysis)

STELA was performed as previously described[15]. In short, 2-5 ng of genomic DNA was digested with EcoRI for 4 hours at 37°C. Telorette linker (TGCTCCGTGCATCTGGCATCCCTAACC) was annealed to the digested DNA for 10 min at 60°C and ligated for 12 hours at 35°C followed by ligase inactivation at 70°C. The resulting ligation product was diluted to 40 pg/μl for STELA PCR. STELA PCR was performed with the following primers and condition: Teltail (TGCTCCGTGCATCTGGCATC) and XpYpE2 primer (GTTGTCTCAGGGTCCTAGTG), 94°C for 2 min, 25 cycles of 94°C for 15 sec, 65°C for 30 sec, 68°C for 10 min, and final extension at 68°C for 20 min. The PCR products were resolved on a 0.5% agarose gel and transferred to Amersham Hybond-XL membrane [GE Healthcare]. The PCR products were detected using probes amplified with the following primers for XpYp telomeres: XpYpE2 (GTTGTCTCAGGGTCCTAGTG) and XpYpB2 (TCTGAAAGTGGACCTATCAG). Quantification of the blots was performed using a MatLab plugin described in Lai et al., 2017[14].

Generation of humanized mice and CFU (colony-forming unit) assay

The CFU assay used here generated three different colony types; burst-forming unit-erythroid (BFU-E), CFUs containing granulocytes and macrophages (CFU-GM), and CFUs containing granulocytes, erythrocytes, macrophages, and megakaryocytes (CFU-GEMM). Colonies of all three types were recorded, collected, and genotyped. gDNA was extracted from the colonies and used for the NGS PCR. The colonies were classified based on the % frameshift alleles they had (~100% wild-type allele: WT/WT, ~50% frameshift/~50% wild-type allele: WT/Mut, ~100% frameshift allele: Mut/Mut). Colonies with ambiguous allele frequencies were not included in # genotyped. For STELA, only the CFU-GM type, which arises most frequently in this assay[16], were used to eliminate potential variability between colony types. Not all colonies genotyped yielded enough gDNA to perform STELA. The '# genotyped' and the '# used for STELA' are indicated in the supplemental figures.

Donor HSPC culture

Adult donor G-CSF mobilized peripheral blood hCD34$^+$ cells were purchased from Fred Hutchinson Cancer Research Center and were maintained in StemSpan SFEM II [StemCell Technologies, 09655] medium supplemented with CC110 [StemCell Technologies, 02697].

Genome editing in patient HSPCs

Patient samples used in this study were collected by Alison Bertuch at Baylor College of Medicine under IRB protocol #4-49646. CD34$^+$ cells were sorted from the mononucleated cell population using anti-human CD34 antibody [BD Biosciences, 348791] staining. Fluorescence-activated cell sorted CD34$^+$ cells were recovered overnight in SFEM II medium supplemented with CC110. 30 pmole of synthetic gRNA and 50 pmole of purified CAS9 [UC Berkeley MacroLab] in a final volume of 4 μL was incubated at room temperature for CAS9 RNP assembly for 20 minutes. ~285,000 CD34$^+$ cells were resuspended in 20 μL of Lonza P3 solution + supplement [Lonza, V4XP-3024] and nucleofected using ER100 setting of Lonza 4D-Nucleofector. Nucleofected cells were cultured in SFEM II medium supplemented with StemSpan^{TM} CD34$^+$ Expansion Supplement [StemCell Technologies, 02691] for 5 days and collected for NGS analysis of editing frequency.

Mutant allele frequency analysis by NGS (next generation sequencing)

200-260 bp region around the Cas9 target site was amplified from extracted gDNA with the following primers with the sequence for NGS adaptor ligation.

TERT ex4 fw: GCTCTTCCGATCTgtctgttgtctggctgagca

TERT ex4 rev: GCTCTTCCGATCTggctcaaacgcacttctgtt

TIN2 ex6 fw: GCTCTTCCGATCTtctaggccgacgaagagttc

TIN2 ex6 rev: GCTCTTCCGATCTgcaagtcaactgggttctcc

TIN2 ex2 fw: GCTCTTCCGATCTgtaggacctggaaggggaaa

TIN2 ex2 rev: GCTCTTCCGATCTgatcccgcactataggtcca

After the adaptor ligation reaction, the library was run on Illumina iSeq or MiSeq sequencer using a paired-end read cycle. Data were demultiplexed using a custom script and analyzed using CRISPResso2[17]. The % frameshift alleles was calculated from the allele frequency table generated from CRISPResso2 analysis by summing up all the frameshift indels.
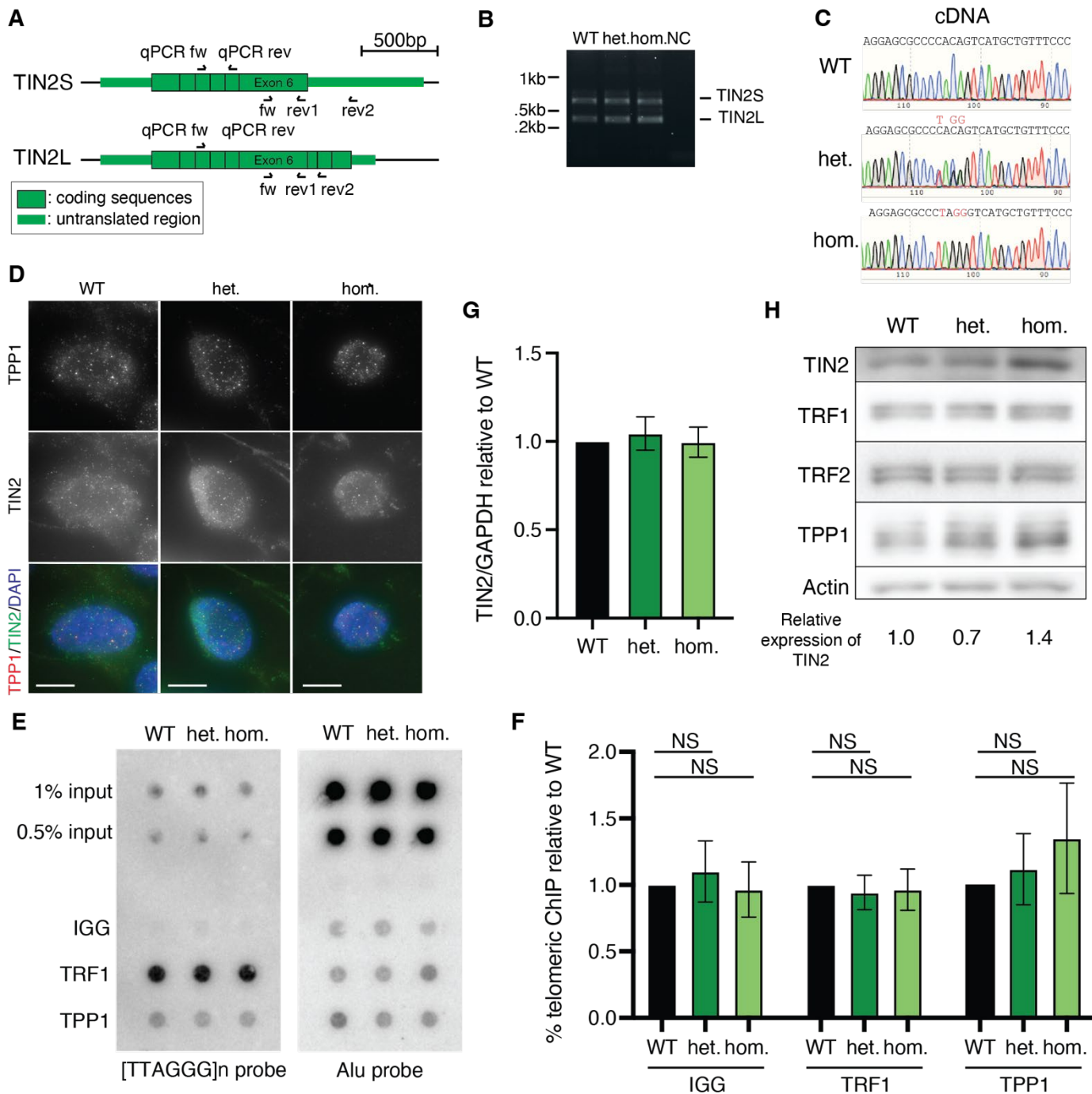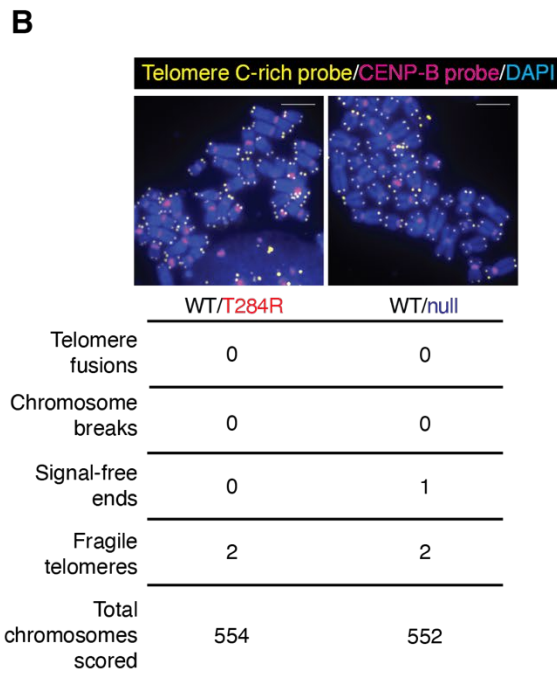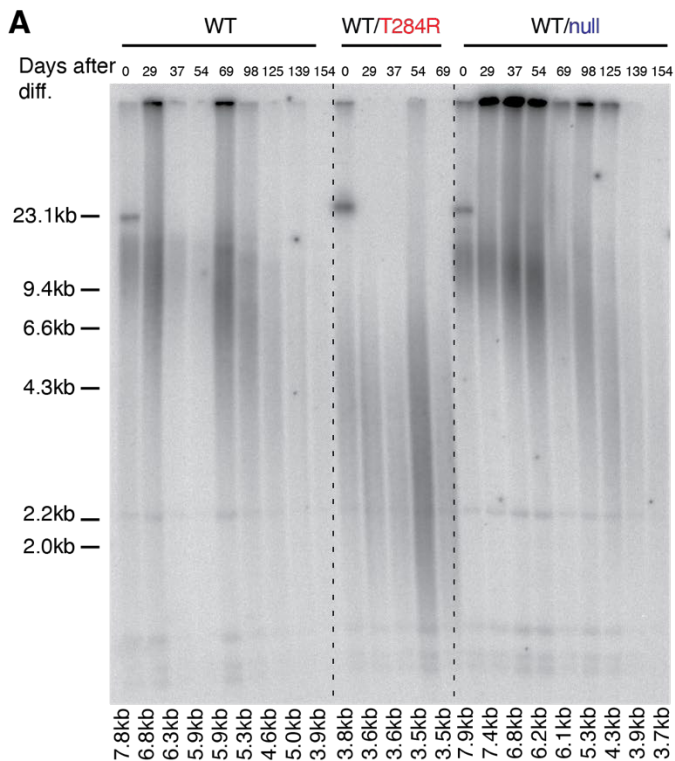
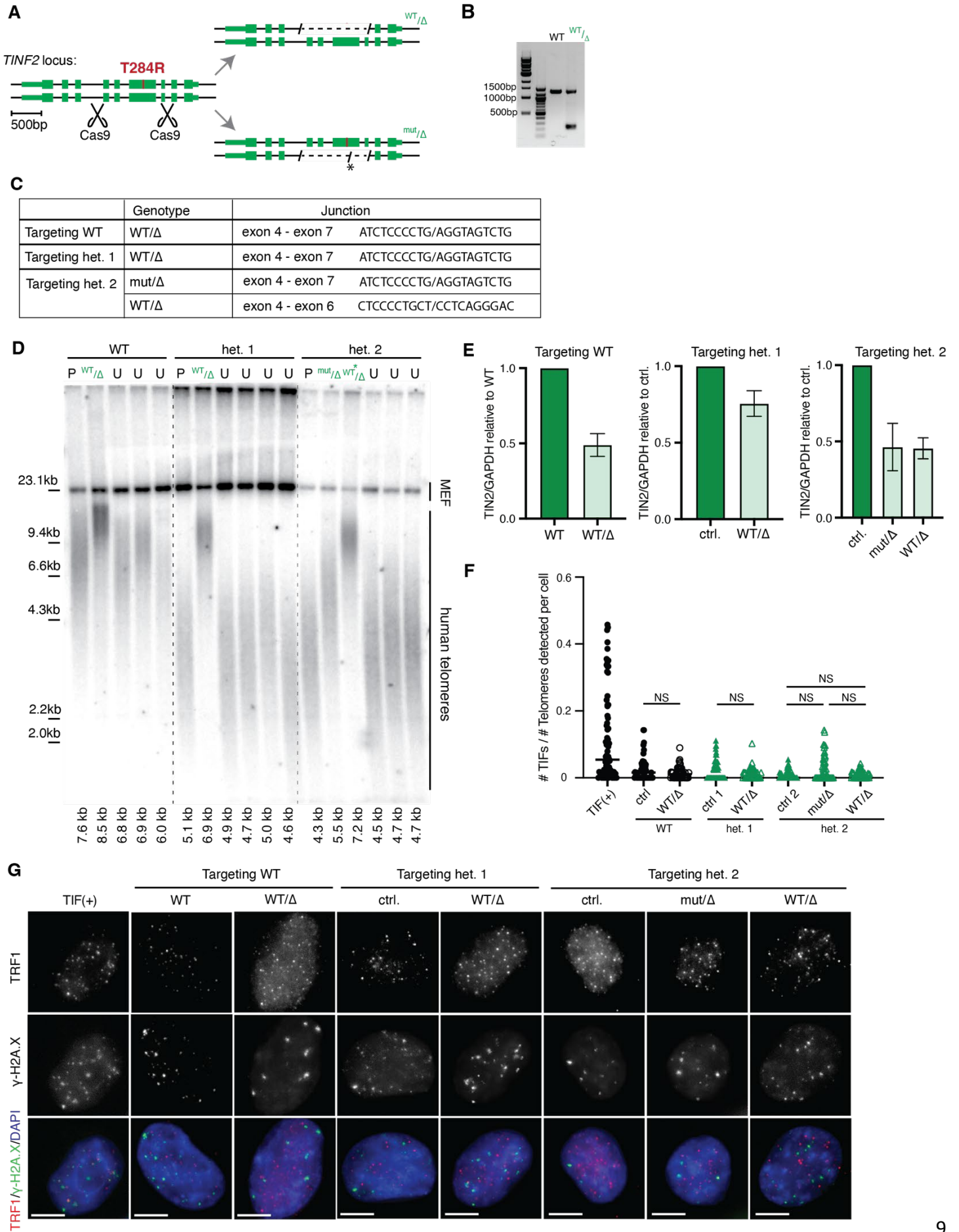**Supplemental Data Figure 1**: **Confirming TIN2 genotype and expression in the *TINF2*-DC hESC lines.** (A) Overview of short (TIN2S) and long (TIN2L) isoform transcripts (as described in Kaminker et al., 2009[18]) with primer pairs used for amplification of cDNA. (B) PCR products of the primer pair fw+rev2 amplified from cDNA of the wild-type (WT), heterozygous (het), and homozygous (hom) hESCs. (C) Sanger sequencing chromatograms of PCR product of cDNA of WT, het and hom hESCs using the primer pair fw+rev1. (D) IF staining of TPP1 (red) and TIN2 (green) of WT, het, and hom hESCs. DNA was stained with DAPI (blue). Scale bar: 10 μm. (E) Representative dot blot of ChIP of IGG, TRF1, and TPP1 hybridized with Alu and telomere probes. (F) Quantification of three independent replicates of telomere ChIP. (G) Relative expression level of TIN2 relative to the WT hESCs normalized to GAPDH. Error bars indicate the standard deviation, n = 2. (H) Western blot analysis of the WT, het, and hom hESCs.
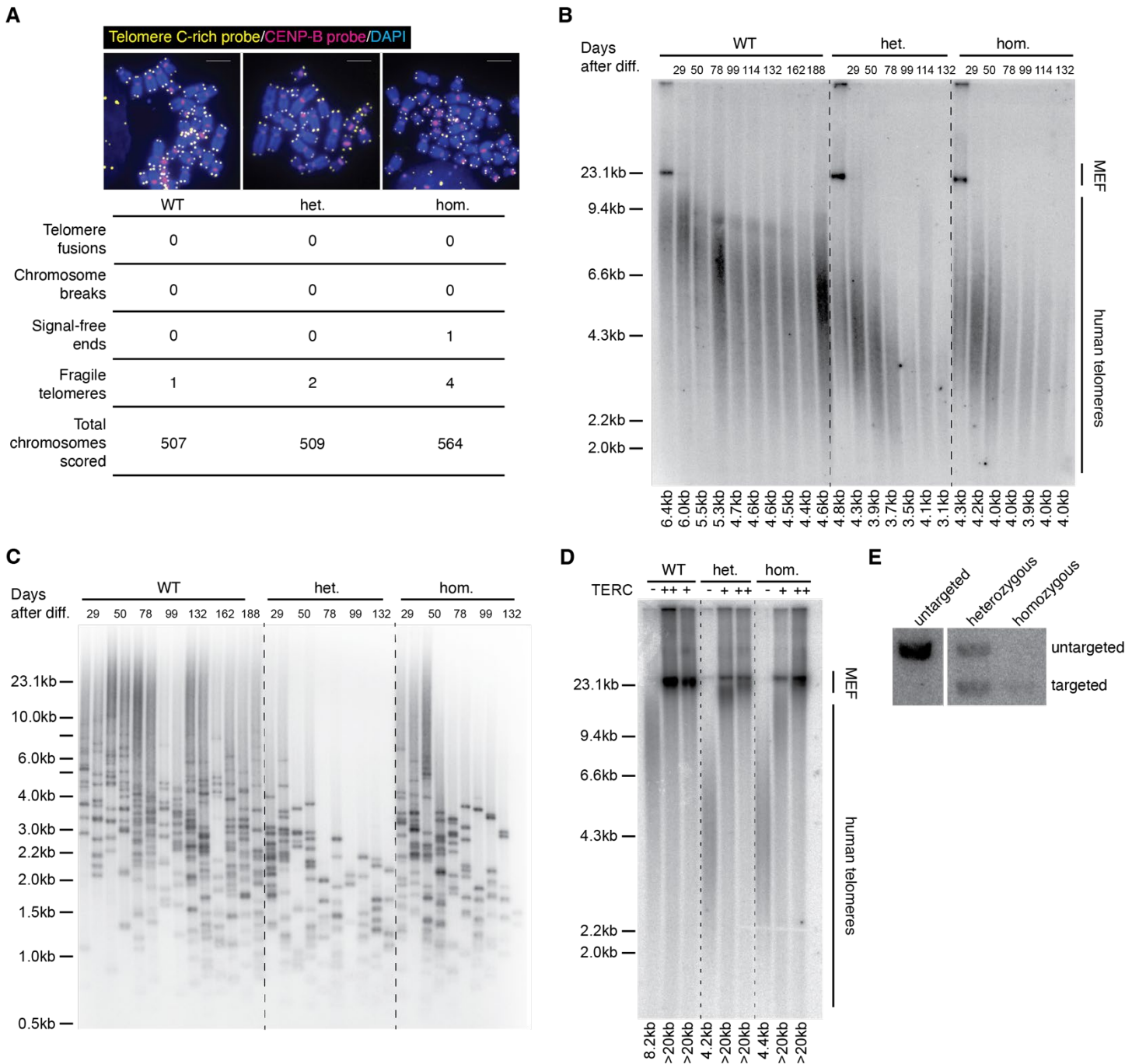
Supplemental Data Figure 2: **Chromosomal abnormality, telomere length analysis upon differentiation, and TERC overexpression.** (A) Images of telomere FISH of metaphase spreads of the wild-type (WT), heterozygous (het.), and homozygous (hom.) hESCs. DNA was stained with DAPI (top, scale bar = 5 µm). Quantification of chromosomal abnormalities (bottom). (B) Telomere length analysis by telomere restriction fragment blot of fibroblasts at indicated time points after differentiation. (C) Representative image of telomere length analysis by TeSLA of fibroblasts at indicated timepoint after differentiation, two lanes per timepoint. (D) Telomere length analysis after overexpression of TERC from the AAVS1 locus in WT, het, and hom hESCs. (- untargeted, + heterozygous insertion at the AAVS1 locus, ++ homozygous insertion at the AAVS1 locus) (E) Representative Southern Blot for the hESC clones untargeted, or with heterozygous and homozygous insertions at the AAVS1 locus. The probe used to confirm the targeted integration hybridizes at the AAVS1 locus outside of the homology regions.

A

WT | WT/T284R | WT/null

Days after diff.: 0 29 37 54 69 98 125 139 154 | 0 29 37 54 69 | 0 29 37 54 69 98 125 139 154

23.1kb —
9.4kb —
6.6kb —
4.3kb —
2.2kb —
2.0kb —

7.8kb 6.8kb 6.3kb 5.9kb 5.9kb 5.3kb 4.6kb 5.0kb 3.9kb | 3.8kb 3.6kb 3.6kb 3.5kb 3.5kb | 7.9kb 7.4kb 6.8kb 6.2kb 6.1kb 5.3kb 4.3kb 3.9kb 3.7kb

B

Telomere C-rich probe/CENP-B probe/DAPI

	WT/T284R	WT/null
Telomere fusions	0	0
Chromosome breaks	0	0
Signal-free ends	0	1
Fragile telomeres	2	2
Total chromosomes scored	554	552

Supplemental Data Figure 3: **Telomere length and proliferative capacity are restored upon hemizygous disruption of _TINF2_ in hESCs without inducing genomic instability.** (A) Telomere length analysis of wild-type (WT), het (WT/T284R), and het WT/c.568insT (WT/null) at indicated time points following differentiation into fibroblasts. Only the signal under 23kb were included for the quantification of mean telomere length. (B) Images of telomere FISH of metaphase spreads of the WT/T284R and WT/null hESCs. DNA was stained with DAPI (top, scale bar = 5 μm). Quantification of chromosomal abnormalities (bottom).

A

TINF2 locus:

T284R

Cas9   Cas9

500bp

WT/Δ

mut/Δ

*

B

WT   WT/Δ

1500bp
1000bp
500bp

C

	Genotype	Junction	
Targeting WT	WT/Δ	exon 4 - exon 7	ATCTCCCCTG/AGGTAGTCTG
Targeting het. 1	WT/Δ	exon 4 - exon 7	ATCTCCCCTG/AGGTAGTCTG
Targeting het. 2	mut/Δ	exon 4 - exon 7	ATCTCCCCTG/AGGTAGTCTG
	WT/Δ	exon 4 - exon 6	CTCCCCTGCT/CCTCAGGGAC

D

WT   het. 1   het. 2

P WT/Δ U U U   P WT/Δ U U U U   P mut/Δ WT/Δ* U U U

23.1kb

9.4kb

6.6kb

4.3kb

2.2kb
2.0kb

MEF

human telomeres

7.6 kb 8.5 kb 6.8 kb 6.9 kb 6.0 kb   5.1 kb 6.9 kb 4.9 kb 4.7 kb 5.0 kb 4.6 kb   4.3 kb 5.5 kb 7.2 kb 4.5 kb 4.7 kb 4.7 kb

E

Targeting WT

TIN2/GAPDH relative to WT

WT   WT/Δ

Targeting het. 1

TIN2/GAPDH relative to ctrl.

ctrl.   WT/Δ

Targeting het. 2

TIN2/GAPDH relative to ctrl.

ctrl.   mut/Δ   WT/Δ

F

TIFs / # Telomeres detected per cell

NS NS NS NS NS

TIF(+)   ctrl   WT/Δ   ctrl 1   WT/Δ   ctrl 2   mut/Δ   WT/Δ

WT   het. 1   het. 2

G

Targeting WT   Targeting het. 1   Targeting het. 2

TIF(+)   WT   WT/Δ   ctrl.   WT/Δ   ctrl.   mut/Δ   WT/Δ

TRF1

γ-H2A.X

TRF1/γ-H2A.X/DAPI

9

Supplemental Data Figure 4: ***TINF2* disruption by introducing two double strand breaks at exons 4 and 7 restores telomere length in the WT and het hESCs.** (A) Schematic overview of the *TINF2* locus with CAS9 cut sites in exon 4 and exon 7 for generation of a null allele. Possible outcomes of a heterozygous targeting event are WT/KO and mut/KO. The removal of exons 4-7 in heterozygous cells would lead to an allele with a premature termination codon (PTC) 53 nucleotides away from the last exon-exon junction, likely rendering the resulting mRNA sensitive to nonsense-mediated decay (NMD). (B) PCR products of the wild-type and the truncated *TINF2* alleles. (C) A table with the junctions after targeting in wild-type (WT) and heterozygous (het.) cell lines. (D) Telomere length analysis following targeting of WT and het hESCs. The genotype of the hemizygous cell lines is marked above each lane. (P = parental cell line, U = untargeted cell line, *incomplete removal of targeted region). (E) Relative expression level of TIN2 relative to the corresponding parental hESCs normalized to GAPDH. Error bars indicate the standard deviation, n = 3 for WT, het. 1, and het. 2 respectively. (F) Quantification of TIFs/detected telomeres per cell in the control and hemizygous cell lines. TIF (+): positive control. Mann-Whitney test. NS: p>0.05. (G) Representative IF images of indicated cell lines after targeting for exon 4-7 *TINF2* deletion. TRF1 (red), γ-H2A.X (green). DNA was stained with DAPI (blue). Scale bar: 10 μm.
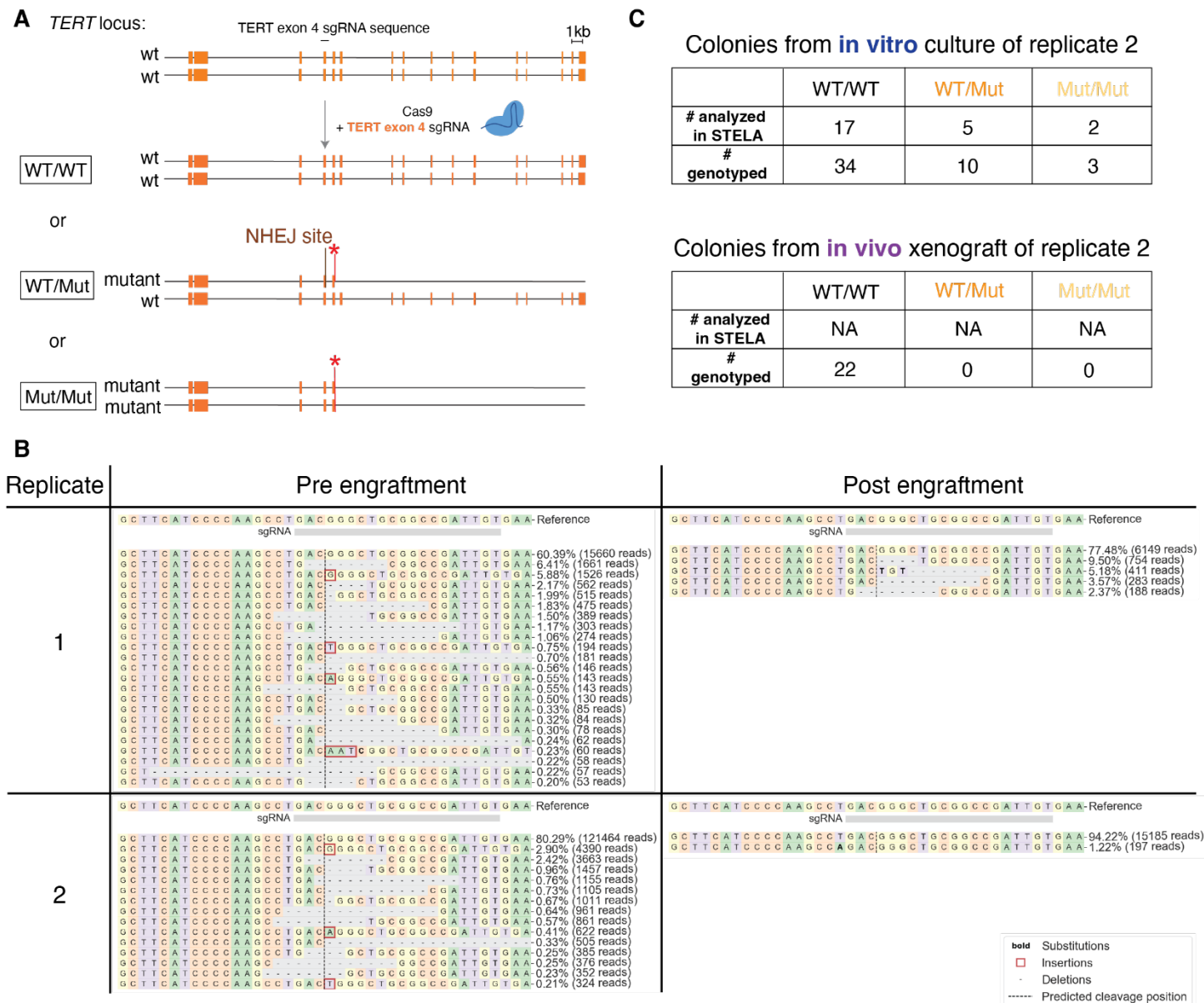
Supplemental Figure 5: *TERT* **hemizygous/homozygous knockout in donor HSPCs.** (A) Possible outcomes of CAS9-mediated editing at the *TERT* locus. The double-strand DNA cut introduced by CAS9 RNPs results in either a functional allele (WT) without insertion/deletions (indels), non-frameshift indels, or a mutant allele with frameshift indels. (B) Allele distributions of bulk hCD34^{+} cells before and after engraftment in NBSGW mice. The most frequently occurring alleles were +1 insertion and -8 deletion alleles, both of which lead to premature termination codons in exon 5. The resulting TERT mRNA was expected to trigger nonsense-mediated decay (NMD) or, if NMD is escaped, a truncated protein without the reverse transcriptase (RT) domain of TERT, which is required for telomerase activity[19]. (C) The number of colonies of each genotype derived in replicate 2.
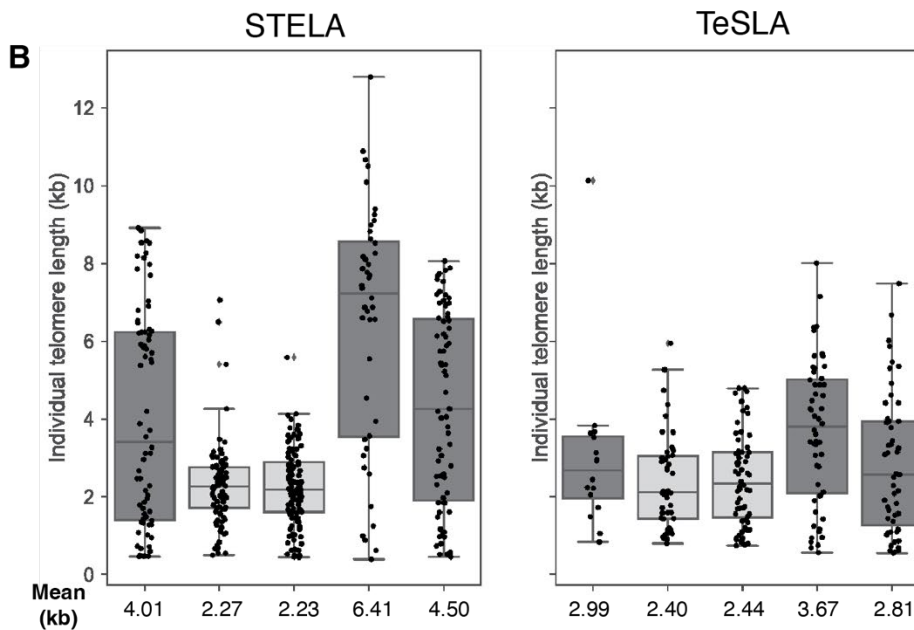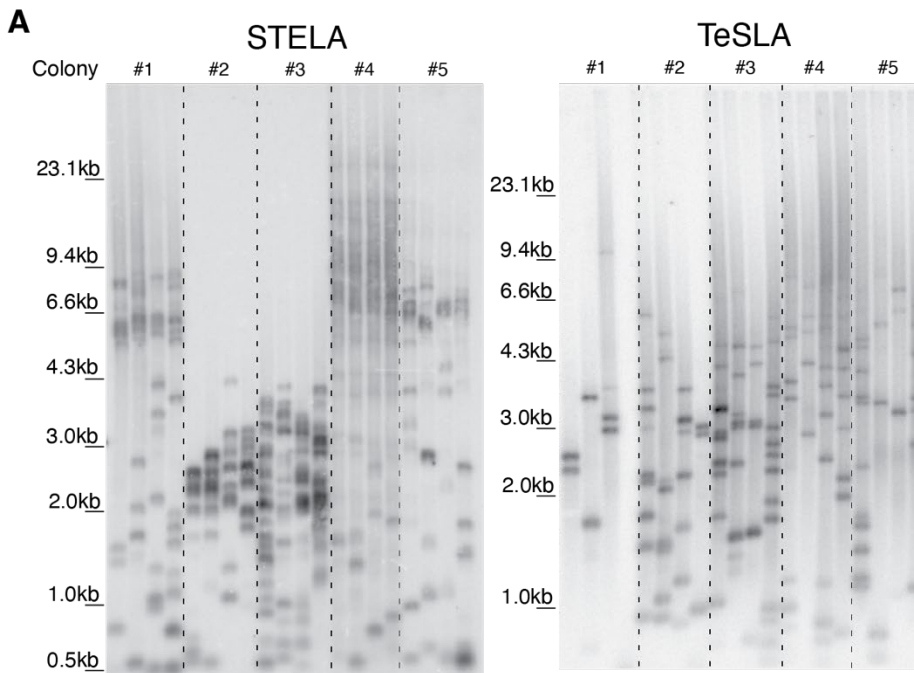
Supplemental Figure 6: Comparison of the detection ranges of STELA and TeSLA. (A) Representative images of telomere length analysis by STELA and TeSLA of 5 CFU colonies with different telomere length distributions. Four lanes per colony. (B) Quantification of the images in (A). The mean telomere length (kb) of each is shown at the bottom.
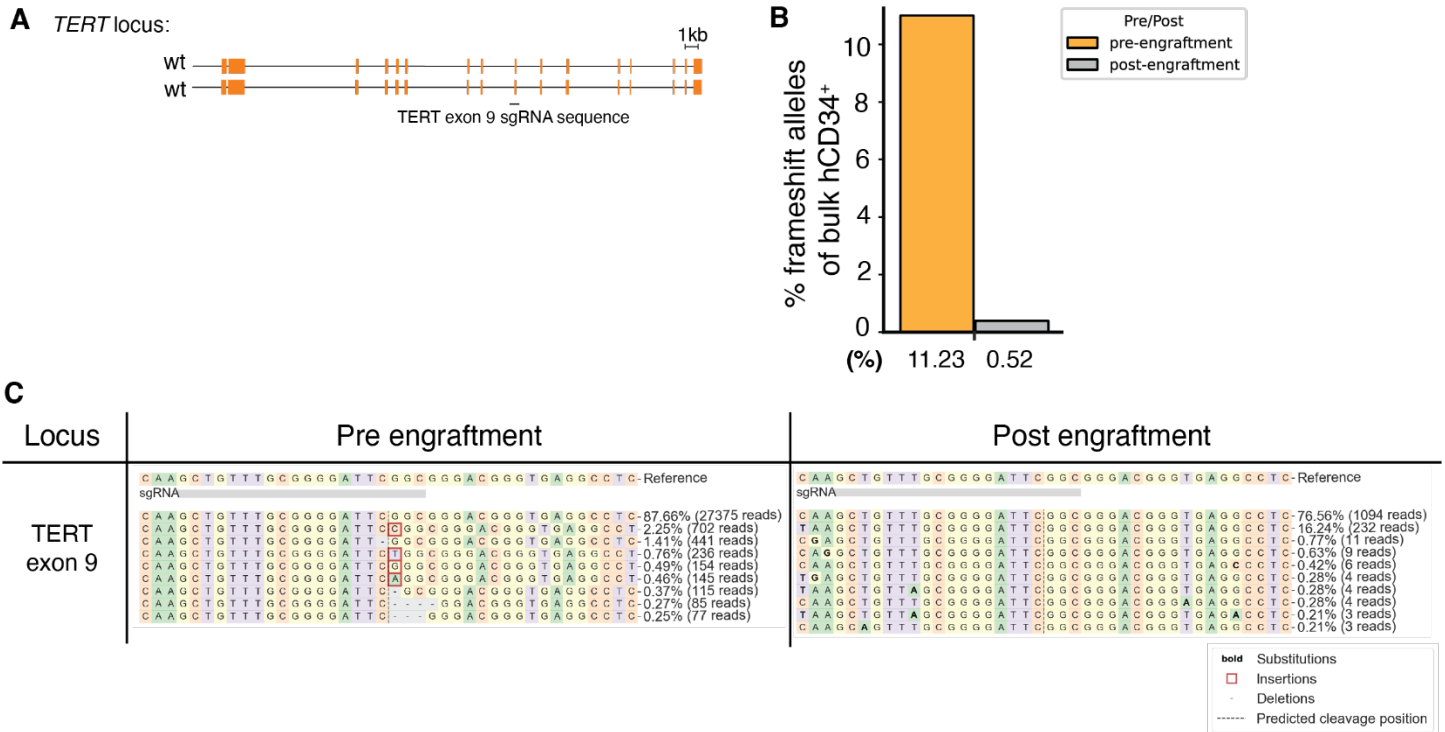
Supplemental Figure 7: *TERT* knock-out using a different sgRNA sequence. (A) A schematic of the *TERT* locus with the TERT exon 9 sgRNA sequence marked. (B) Percentages of frameshift alleles of bulk hCD34⁺ cells before and after xenotransplantation. (C) Allele distributions of bulk hCD34⁺ cells before and after xenotransplantation.
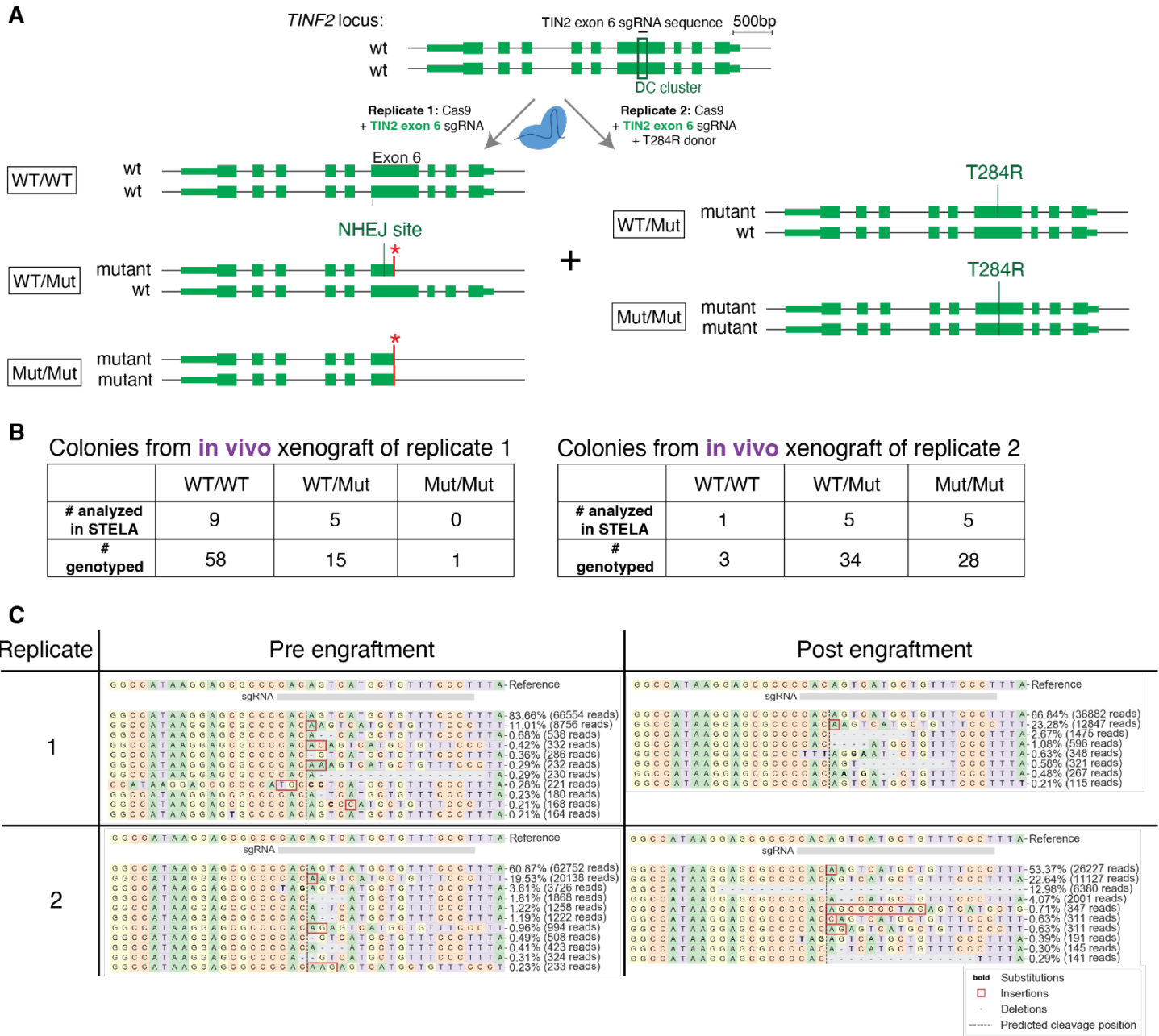
Supplemental Figure 8: Introduction of *TINF2*-DC mutations in donor HSPCs. (A) Possible outcomes of CAS9-mediated editing at the *TINF2* locus. In replicate 2, a single-stranded T284R donor DNA was used to introduce *TINF2* missense T284R mutation via HDR. (B) Numbers of colonies of each genotype derived from replicates 1 and 2. (C) Allele distributions of bulk hCD34+ cells before and after engraftment in NBSGW mice from two replicates of *TINF2* editing. One of the biological replicates per each replicate is shown for post engraftment. 1-nt A insertion allele was generated in high frequencies in both replicates (11.01% and 19.53% respectively) which would lead to an early stop at amino acid 291 in exon 6. All the mutant colonies we obtained had the 1-nt A insertion allele and they still had shorter telomeres compared to the wild-type colonies (Figure 5B). Notably, the T284R allele generated in replicate 2 seemed to have depreciated in vivo, whose frequency was decreased to 0.39% in the post-engraftment population from 3.61% in the pre-engraftment population.
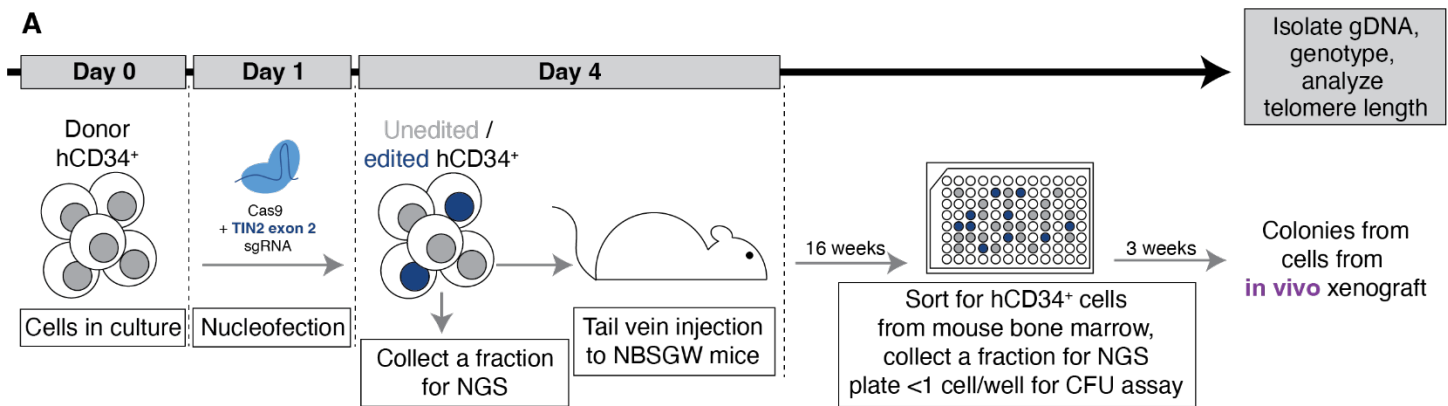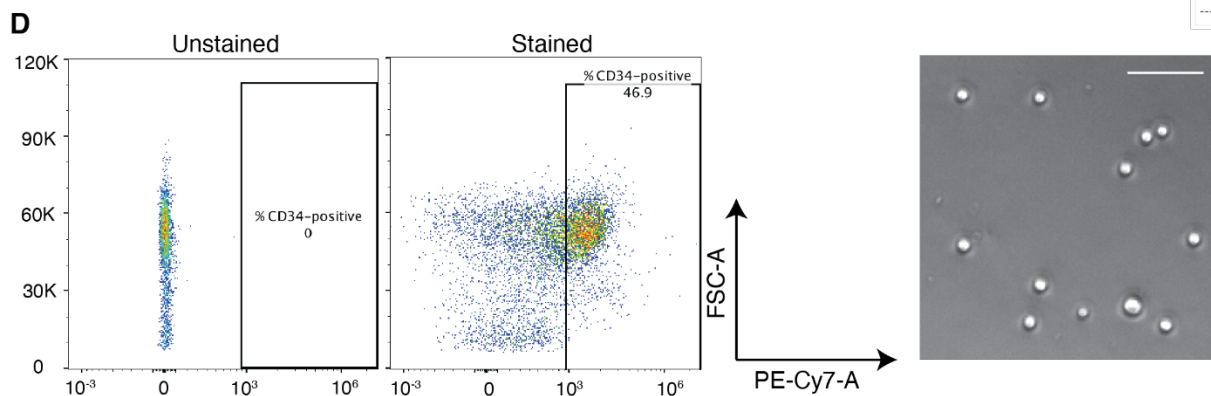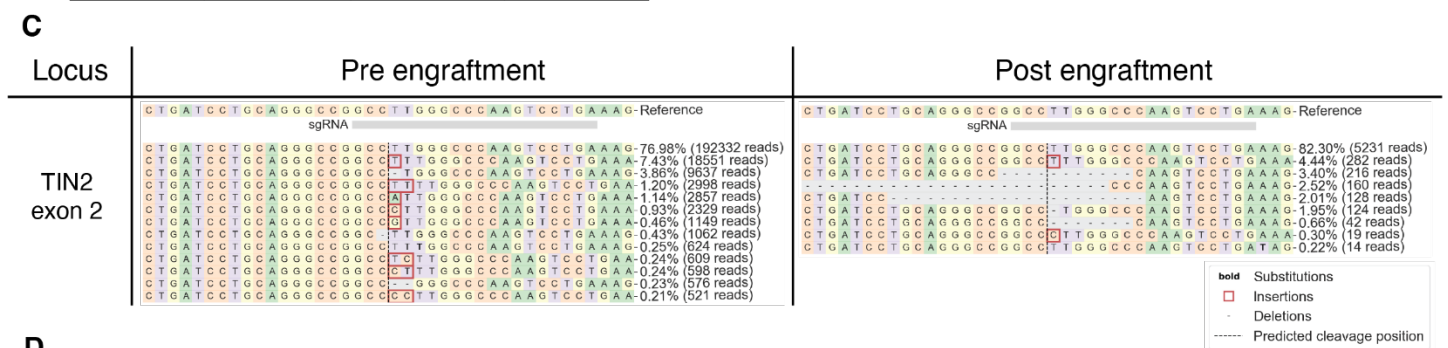
Supplemental Figure 9: Disrupting *TINF2* at exon 2 in donor and patient HSPCs. (A) A schematic of the in vivo humanized mouse model to study the effect of *TINF2* disruption at exon 2 in donor HSPCs. (B) Numbers of colonies of each genotype after xenotransplantation of donor HSPCs nucleofected with TIN2 exon 2 sgRNA. (C) Allele distributions of bulk hCD34⁺ cells before and after engraftment in NBSGW mice. (D) Flow cytometric analysis of CD34⁺ cells from the patient marrow (left). A representative image of CD34⁺ cells sorted from the patient marrow. Scale bar = 100 μm (right).

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