SUPPLEMENTAL INFORMATION

Isolation of chromatin from dysfunctional telomeres reveals an important role for Ring1b in NHEJ-mediated chromosome fusions

Cristina Bartocci, Jolene K. Diedrich, Iliana Ouzounov, Julia Li, Andrea Piunti, Diego Pasini, John R. Yates III and Eros Lazzerini Denchi

Supplemental Figure Legends

Figure S1. Validation of novel factors that localize at functional telomeres. Related to Table S2.

A. TRF2$^{F/F}$ Rosa26 CRE-ER MEFs were retrovirally infected with the indicated constructs, treated with 4-hydroxytamoxifen (OHT) and harvested 3 days later. Cells were stained for telomere using an anti-TRF1 antibody (green), myc (red) and DAPI (blue). Scale bar 5 µm. **B.** Quantification of data shown in A: cells with 5 or more foci co-localizing with TRF1 were counted as positive. Error bars represent SD of average of three independent experiments. **C.** TRF2$^{F/F}$ Rosa26 CRE-ER MEFs infected with the indicated shRNA constructs were treated with OHT for 72 hours, fixed and stained for telomeric DNA (green, TTAGGG), 53BP1 (red) and DAPI (blue). Scale bar 5 µm. **D.** Quantification of the data shown in C (cells with 5 or more 53BP1 foci/TIFs were counted as positive). Error bars represent SD of average of three independent experiments. **E.** Quantification of relative mRNA levels of the indicated genes in cells infected with the indicated shRNA constructs.

Figure S2. Nucleoplasmic extraction of cells infected with candidate dysfunctional telomere-binding factors. Related to Figure 2.

A. TRF2$^{F/F}$ Rosa26 CRE-ER MEFs were retrovirally infected with the indicated constructs, treated with 4-hydroxytamoxifen (OHT) and harvested 3 days later. Cells

were either directly fixed (-TRITON) or pre-extracted with TritonX-100 buffer before fixation (+ TRITON, for details see Supplemental experimental procedures), and then stained for γH2AX or 53BP1 (red), myc/flag/GFP (green) and DAPI (blue). Scale bar 5 μm. **B.** Quantification of data shown in A: cells with 5 or more myc/flag/GFP foci colocalizing with DNA damage markers 53BP1 or γH2AX were counted as positive.

Figure S3. Ring1b depletion affects NHEJ-mediated chromosome end-to-end fusions. **A. Related to Figure 3.**

Genomic DNA from TRF2$^{F/F}$ Rosa26 CRE-ER MEFs infected with the indicated shRNA constructs and either treated with OHT for 96 hours (OHT) or untreated (control) was analyzed by in-gel telomere blotting. The left image shows hybridization signal using the TelC probe ([CCCTAA]$_4$) under native conditions detecting the telomeric 3' overhang. The right image shows the total telomeric hybridization signal obtained with the same probe after in-gel denaturation of the DNA. **B.** Cell cycle analysis by FACS of cells treated as in A. **C.** Representative IF image of TRF2$^{F/F}$ Rosa26 CRE-ER MEFs treated as described in A, fixed and stained for γH2AX (red), RIF1 (green) and DAPI (blue). Scale bar 5 μm. **D.** Quantification of experiment described in C. Blue bars represent percentage of cells with more than 5 co-localizing foci of γH2AX and RIF1.

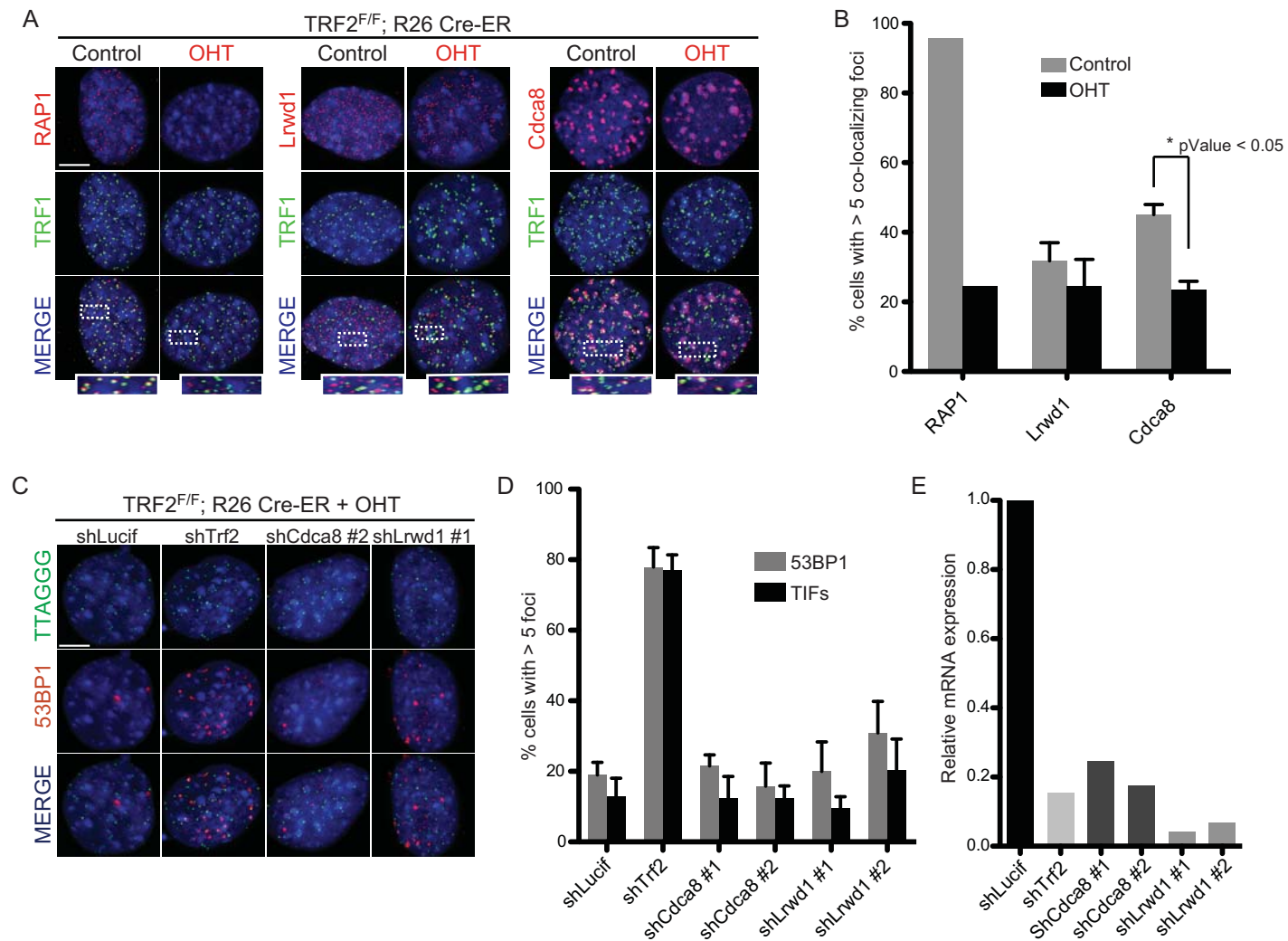**Figure S4. Ring1b depletion inhibits NHEJ-mediated fusions of dysfunctional telomeres. Related to Figure 4.**

A. Western blot for Ring1b on lysates from cells treated as described in Figure 4A. Vinculin (α-Vinc) was used as loading control. **B.** ChIP experiments with the indicated antibodies with chromatin prepared from untreated (control) or OHT-treated (OHT) TRF2$^{F/F}$ Rosa26 CRE-ER MEFs, followed by dot blot southern hybridization with probes

to telomeric repeats (TTAGGG) or Bam repeats. **C.** Quantifications of ChIP analysis in B for the telomeric probe. **D.** Quantifications of ChIP analysis in B for the Bam repeats probe. In both C and D the signal from the IgG ChIP was subtracted from each signal as background. **E.** Western blot for Ring1b on lysates from Ring1b$^{F/F}$ Rosa26 CRE-ER MEFs untreated (-) or treated with OHT for 96 hours (+). Vinculin (α-Vinc) was used as loading control. **F.** Representative IF image of Ring1b$^{F/F}$ Rosa26 CRE-ER MEFs treated as described in E, fixed and stained for Ring1b (red), DAPI (blue). Scale bar 5 μm. **G.** Quantification of 53BP1-positive cells from experiment described in Figure 4E (cells with 5 or more 53BP1 foci were counted as positive).
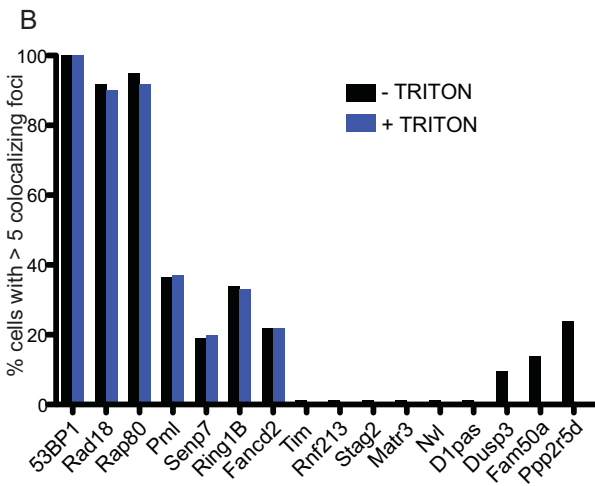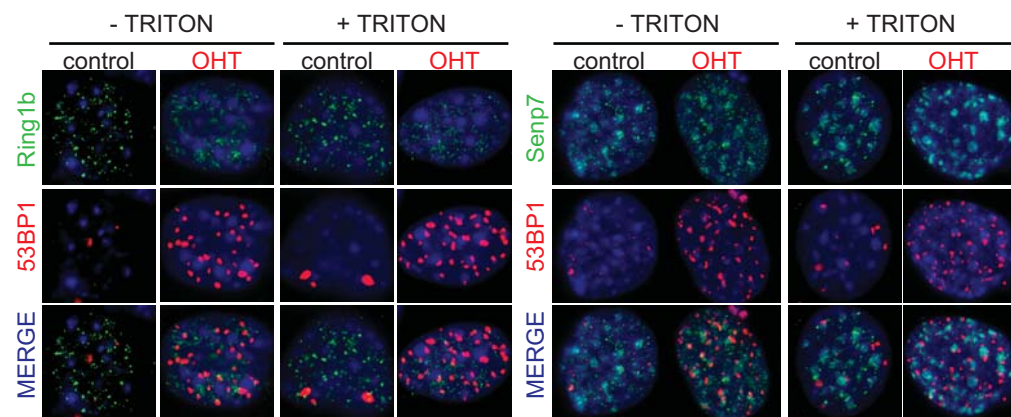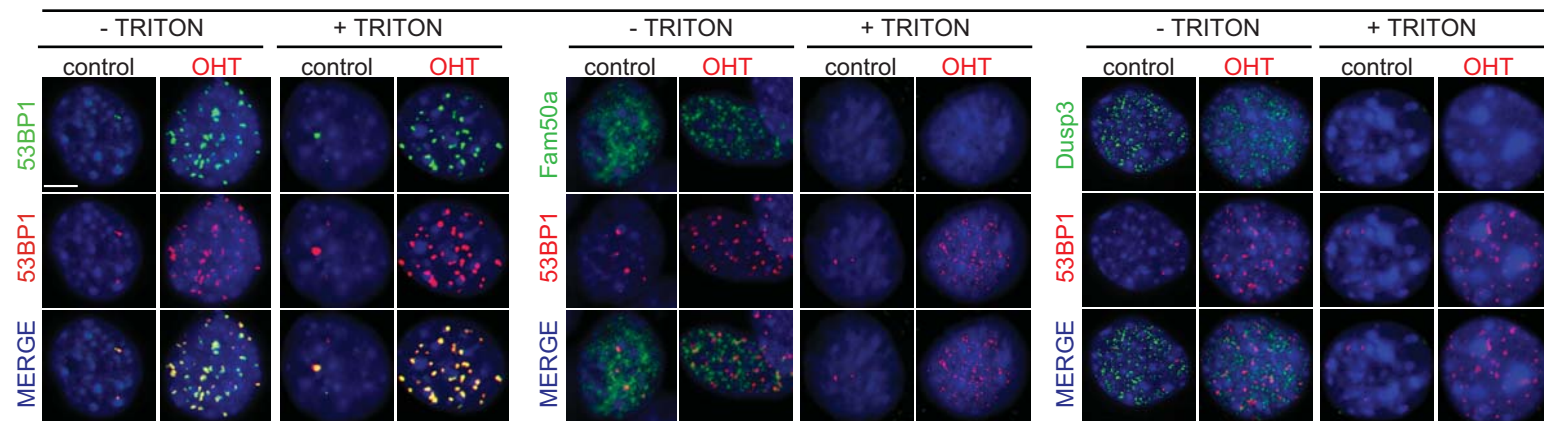
Figure S5. Ring1b depletion does not affect global DNA repair. Related to Figure 5.

 A. Pulse-field gel displaying genome-wide DSB formation visualized by ethidium bromide staining in Ring1b$^{F/F}$ Rosa26 CRE-ER MEFs untreated (control) or treated with OHT for 96 hours (OHT) and irradiated with 20Gy of IR. Ligase4 null MEFs (Lig4$^{-/-}$) were used as control. **B.** Quantification of DNA DSB repair shown in A, measured as the ratio of DNA released in the gel versus total DNA (FAR ratio, Fraction of DNA released). **C.** Quantification of the total number of γH2AX foci in cells treated as described in Figure 5A. Error bars represent SD of average of three independent experiments. **D.** Quantification of 53BP1-positive TRF2$^{F/F}$ Rosa26 CRE-ER MEFs treated with tamoxifen for 72 hours and when indicated with TSA (18 hours). **E.** Cell cycle analysis by FACS of cells treated as in D. **F.** Suv39dn iMEFs and wild type controls (iMEFs) were infected with wild type TRF2 or a TRF2 dominant negative allele (TRF2$^{\Delta B, \Delta M}$) as indicated, fixed and stained for myc (red), 53BP1 (green) and DAPI (blue). Scale bar 5 μm. **F.** Metaphase spreads of Suv39dn iMEFs and wild type controls treated as in F were harvested 96 hours post selection and stained for telomeric DNA (Green) to detect end-to-end chromosome fusions (arrowheads). Number of metaphases analyzed and

percentage of metaphases with at least 1 fusion event are indicated. Scale bar 5 μm. **H.** Quantification of relative Prdm3 mRNA levels in cells infected with the indicated shRNA constructs as described in Figure 5D.

Figure S1. Bartocci et al.

A TRF2^{F/F}; R26 Cre-ER

B % cells with > 5 co-localizing foci

* pValue < 0.05

C TRF2^{F/F}; R26 Cre-ER + OHT

D % cells with > 5 foci

E Relative mRNA expression

Figure S2. Bartocci et al.

A

TRF2$^{F/F}$; R26 Cre-ER

B

Figure S3. Bartocci et al.

A

	control		OHT		control		OHT	
shLucif	+	-	+	-	+	-	+	-
shRing1b	-	+	-	+	-	+	-	+

Telomere Fusions

Bulk Telomere Fragments

100 100 23 30

Native ssTTAGGG

Denatured total (TTAGGG)n

B

PI DNA content

0 2n 4n 6n 8n 10n

	% G1 cells
shLucif control	91.6
shLucif OHT	89.6
shRing1b control	93.7
shRing1b OHT	87.4

C

TRF2$^{F/F}$; R26 Cre-ER

shLucif		shRing1b	
control	OHT	control	OHT

γH2AX

RIF1

MERGE

D

% cells with > 5 foci

γH2AX
RIF1
γH2AX-RIF1

100

80

60

40

20

0

shLucif + OHT   shRing1b + OHT

Figure S4. Bartocci et al.

A

Ring1b-wt*
Ring1b-I53A*

	shRing1b	shLucif		shRing1b	
Ring1b-wt*	-	-		+	-
Ring1b-I53A*	-	-		-	+

α-Ring1b

α-Vinc

B

Control OHT Control OHT

TRF1
H3K27me3
H3K9me3
H2AK119ub
H3
IgG
input 0.5%

TTAGGG Probe Bam Repeats Probe

C

TTAGGG Probe

% of input signal

Control
OHT

TRF1 H3K27ME3 H3K9ME3 H2AK119ub H3

D

Bam Repeats Probe

% of input signal

Control
OHT

TRF1 H3K27ME3 H3K9ME3 H2AK119ub H3

E

Ring1b$^{F/F}$; R26 Cre-ER

OHT   -   +

α-Ring1b

α-Vinc

F

Ring1b$^{F/F}$; R26 Cre-ER

OHT   -   +

α-Ring1b

G

% myc-53BP1 positive (> 5 foci) cells

TRF2
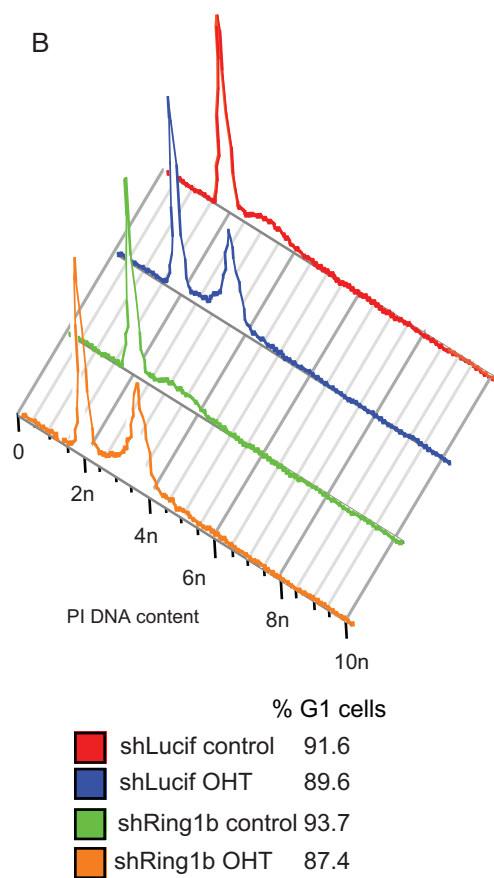TRF2^{ΔBΔM}

OHT   -   -   +   +

Ring1b$^{F/F}$; R26 Cre-ER

Figure S5. Bartocci et al.

A

Ring1b^{F/F}; R26 Cre-ER

Control   OHT   Lig4 ^{-/-}

20 Gy IR   20 Gy IR   20 Gy IR

hours IR   NT   1   6   24   NT   1   6   24   NT   1   6   24

size (kb)
945
915
815

B

Control
OHT
Lig4 ^{-/-}

FAR Ratio unrepaired DNA/ total DNA

NT   1 hr   6 hr   24 hr

C

Control
OHT
Atm ^{-/-}

γH2AX foci/cell

NT   1 hr   6 h   24 h

D

% cells with > 5 53BP1 foci

- TSA   + TSA

E

- TSA
+ TSA

2n
4n

H

Relative Prdm3 mRNA expression

shLuc   shPrdm3

F

wild-type iMEFs   Suv39hdn iMEFs

TRF2   +   -   +   -
TRF2^{ΔBΔM}   -   +   -   +

MYC

53BP1

MERGE

G

wild-type iMEFs

TRF2   TRF2^{ΔBΔM}
n=30   0%*   n=30   60%*

Suv39hdn iMEFs

TRF2   TRF2^{ΔBΔM}
n=30   0%*   n=30   31%*

* % of metaphases with at least 1 fusion event

Supplemental Tables (provided as Excel files):

Table S1. Proteins found by PICh at functional and dysfunctional telomeres. List of proteins identified from both TRF2-proficient (NT) and TRF2-deficient (OHT) telomeric chromatin in at least 2 out of 3 independent purifications. Spectra counts from MS analysis for each individual experiment are indicated.

Table S2. Proteins enriched at TRF2-proficient telomeres. Proteins identified from at least 2 out of 3 independent isolations of TRF2-proficient telomeres were ranked based on their relative enrichment relative to the TRF2-deficient samples and normalized to their absolute expression in mouse cells (see text for details). Factors known to localize to telomeres in a TRF2-dependent manner are in bold.

Table S3. Proteins enriched at TRF2-deficient telomeres. Proteins identified from at least 2 out of 3 independent isolations of TRF2-deficient telomeres were ranked based on their relative enrichment relative to TRF2-proficient samples and normalized based on their absolute expression in mouse cells (see text for details). Factors previously reported to be involved in the DNA damage response are in bold.

Supplemental Experimental Procedures

Plasmids and reagents

Plasmids used in this study were: pLPC-Myc-53BP1 (cloned from pLPC-53BP1-eGFP, T. de Lange, Rockefeller University), pLPC-Myc-Rap1 (A. Sfeir, NYU), pBabe-Myc-TRF2 and pBabe-Myc-TRF2[ΔB,ΔM] (previously described in (van Steensel et al., 1998). Human or mouse cDNAs were cloned into the pLPC retroviral vector with an N-terminal myc, flag or GFP tag from the following sources: pEGFP-C1Senp7 (a gift from R. Hay), pOZ-N-

FH-RAP80 (Addgene ID 27501), pEGFP-STAG2 (Addgene ID 31972), pcDNA4-Flag-Timeless (Addgene ID 22887), pCDNA-Rnf213-Flag (a gift from A. Koizumi, Kyoto University). cDNAs for Rad18, Dusp3, Ppp2r5d, Fam50a Cdca8, Lrwd1 and Ring1b were purchased from OpenBiosystems. The Ring1b-wt* and Ring1b-I53A* alleles were generated from the pLPC-Myc-Ring1b plasmid by site-directed mutagenesis using the Stratagene Quick Change kit according to manufacturer's instructions. TSA was from SIGMA.

shRNA
shRNAs were cloned into the pLKO-puromycin lentiviral vector using annealed primers with the following sequences: shTrf2 5'-CCTTGGAATCAGCTATCAATG-3', shCdca8 #1 TRCN0000177578, shCdca8 #2 TRCN0000178643, shLrwd1 #1 TRCN0000119912, shLrwd1 #2 TRCN0000119914, shMdc1 5'-GCAGCCATCTAGTGGTAAA-3', shRing1b 5'-CCATGACTACAAAGGAGTGTT-3', shRad18 5'-AGGCGAACAGGTTAATGGATA-3', shSenp7 5'-GCTCCCTAAGTTTCCTAGAAA-3', shRap80 TRCN0000125319, shBmi1 #1 5'-TGAGATAATAAGCTTGTCTAT-3', shBmi1 #2 5'-AACAACCAGAATCAAGATCAC-3', shPrdm3 5'-CCAATCACCAAGTGAAGTTAA-3'. pLKO-shLuciferase was a gift from B. Tan (Chang Gung University).

Antibodies
The following antibodies were used: anti myc (9B11, Cell signaling), anti γ-H2AX (Upstate Biotechnology, Lake Placid, NY), anti 53BP1 (Novus, NB 100-304), anti Flag (Sigma M2), anti GFP (Invitrogen, A6455), anti Rap1 (1252, T. de Lange, Rockefeller University), anti Fancd2 (Novus, NB100-182), anti Rad18 (Bethyl Laboratories, A301-340A), anti Ring1b (Y. Koseki, Riken), anti TRF1 (K. Okamoto, Cancer Institute of JFCR for IF; 1449, T. de Lange, Rockefeller University for ChIP), anti Pml (M.N. Boddy, TSRI),

anti vinculin (Sigma V9131), anti Rif1 (S. Buonomo, EMBL), anti H2AK119Ub (Cell Signaling), anti H3K27me3 (Cell Signaling), anti H3K9me3 (pAb, Active Motif), anti H3 (mAb, Active Motif).

Cell permeabilization for immunofluorescence

For IF, where indicated cells were permeabilized with Triton X-100 buffer (0.5% Triton X-100, 20 mM Hepes-KOH pH 7.9, 50 mM NaCl, 3mM MgCl$_2$, 300 mM sucrose) at 4°C for 2 minutes, rinsed twice with PBS1x and then fixed in 3% paraformaldehyde/2%sucrose in 1x PBS for 10 minutes at RT, rinsed 3 times in PBS1x.

FACS

For cell cycle analysis, cells were collected by trypsinization, washed in PBS, fixed with ice-cold 70% ethanol at 4°C for at least 30 minutes. Cells were washed with 0.5% BSA in PBS and resuspended in 0.5% BSA in PBS containing 5 μg PI (propidium-iodide) and RNaseA (10 μg/mL). Samples were analyzed with a FACS calibur flow cytometer (Becton Dickinson). Data was analyzed with FlowJo software.

Analysis of telomeric DNA

Mouse telomeric DNA was analyzed on CHEF gels using previously described protocols (Celli and de Lange, 2005). FISH for telomeric DNA was performed as described (Celli and de Lange, 2005), with the exception that a FITC-TelC (FITC-OO-CCCTAACCCTAAACCCTAA, Applied Biosystems) probe was used to detect telomeric DNA. P values were calculated using the two-sided chi square test.

Pulse-field gel electrophoresis (PFGE)

PFGE to analyze DNA double-strand break formation was performed as described in (Bryant, 2012) using a BioRad CHEFIII system. Cells were irradiated using a Gammacell 40 Exactor (Theratronics).

Supplemental References

Bryant, H.E. (2012). DNA double-strand break damage and repair assessed by pulsed-field gel electrophoresis. Methods Mol Biol *920*, 315-321.

van Steensel, B., Smogorzewska, A., and de Lange, T. (1998). TRF2 protects human telomeres from end-to-end fusions. Cell *92*, 401-413.