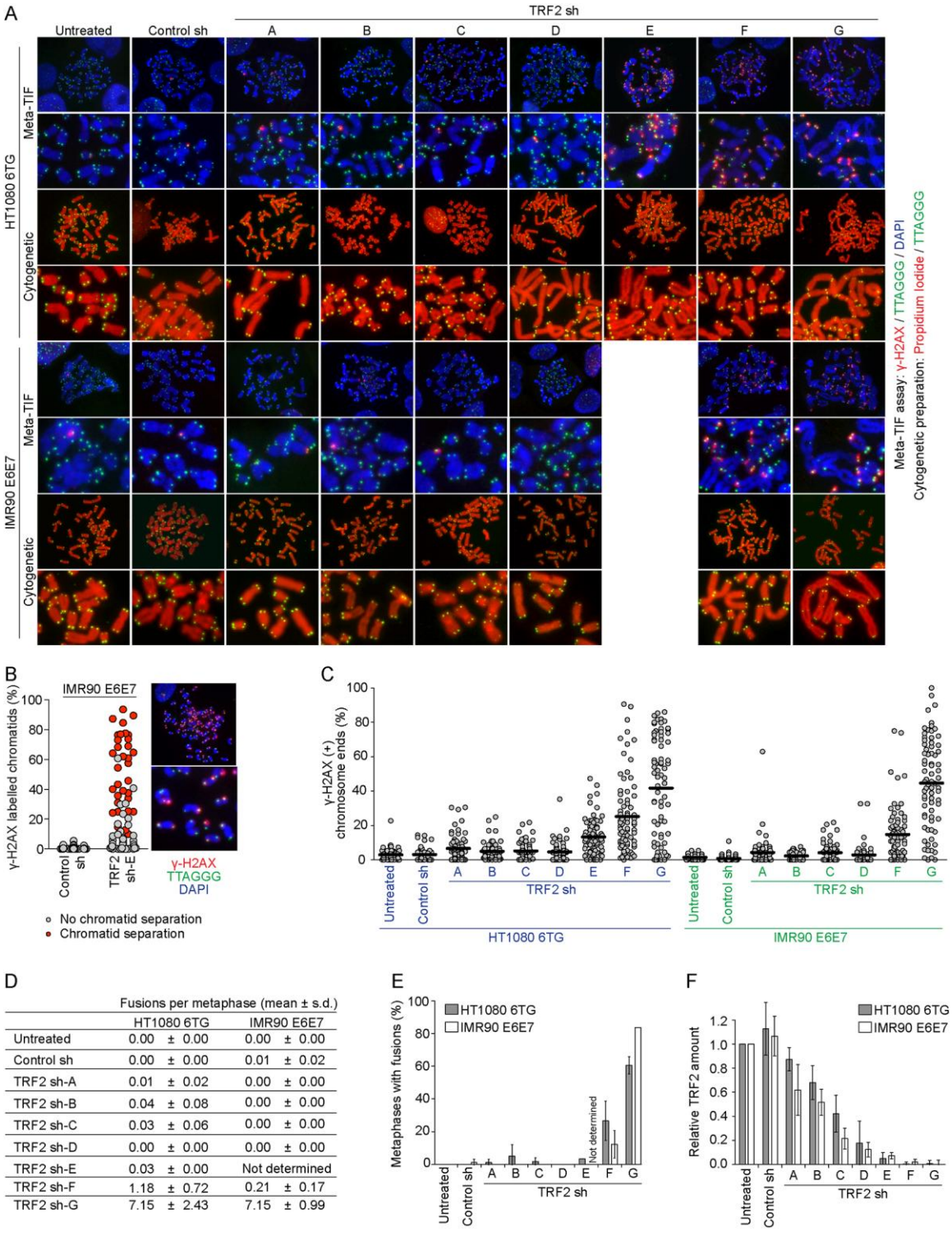
Supplemental Data



	Fusions per metaphase (mean ± s.d.)	
	HT1080 6TG	IMR90 E6E7
Untreated	0.00 ± 0.00	0.00 ± 0.00
Control sh	0.00 ± 0.00	0.01 ± 0.02
TRF2 sh-A	0.01 ± 0.02	0.00 ± 0.00
TRF2 sh-B	0.04 ± 0.08	0.00 ± 0.00
TRF2 sh-C	0.03 ± 0.06	0.00 ± 0.00
TRF2 sh-D	0.00 ± 0.00	0.00 ± 0.00
TRF2 sh-E	0.03 ± 0.00	Not determined
TRF2 sh-F	1.18 ± 0.72	0.21 ± 0.17
TRF2 sh-G	7.15 ± 2.43	7.15 ± 0.99

Figure S1 related to Figure 1: Characterization of TRF2 depletion. A) Examples of Metaphase-TIF (Meta-TIF) and cytogenetic chromosome preparations from untreated, control sh and TRF2 sh transduced cells. Meta-TIF assay samples were stained with DAPI, telomere FISH and γ-H2AX immunofluorescence (IF). Cytogenetic chromosome preparations were stained with telomere FISH and propidium iodide (PI). For each condition and imaging preparation full images are shown above and expanded images are shown below. B) Meta-TIF assay images and quantitation of IMR90 E6E7 cells transduced with TRF2 shRNA E. Images were scored for the number of γ-H2AX labeled telomere chromatids per metaphase. Each metaphase is represented by a single dot (n = 3) C) Quantitation of the Meta-TIF assays depicted in (A). These are the same data as Figure 1D. Each scored metaphase is represented by a single dot and the mean is represented as a black bar (n = 3). D) Quantitation of fusions per metaphase in the cytogenetic chromosome preparations depicted in (A) (n = 3). E) Quantitation of the percentage of metaphases with fusions in the experiments depicted in (A) (n = 3). F) Quantitation of TRF2 knockdown efficiency in TRF2 sh transduced cells as shown in Figure 1B (mean ± s.d., n = 3).

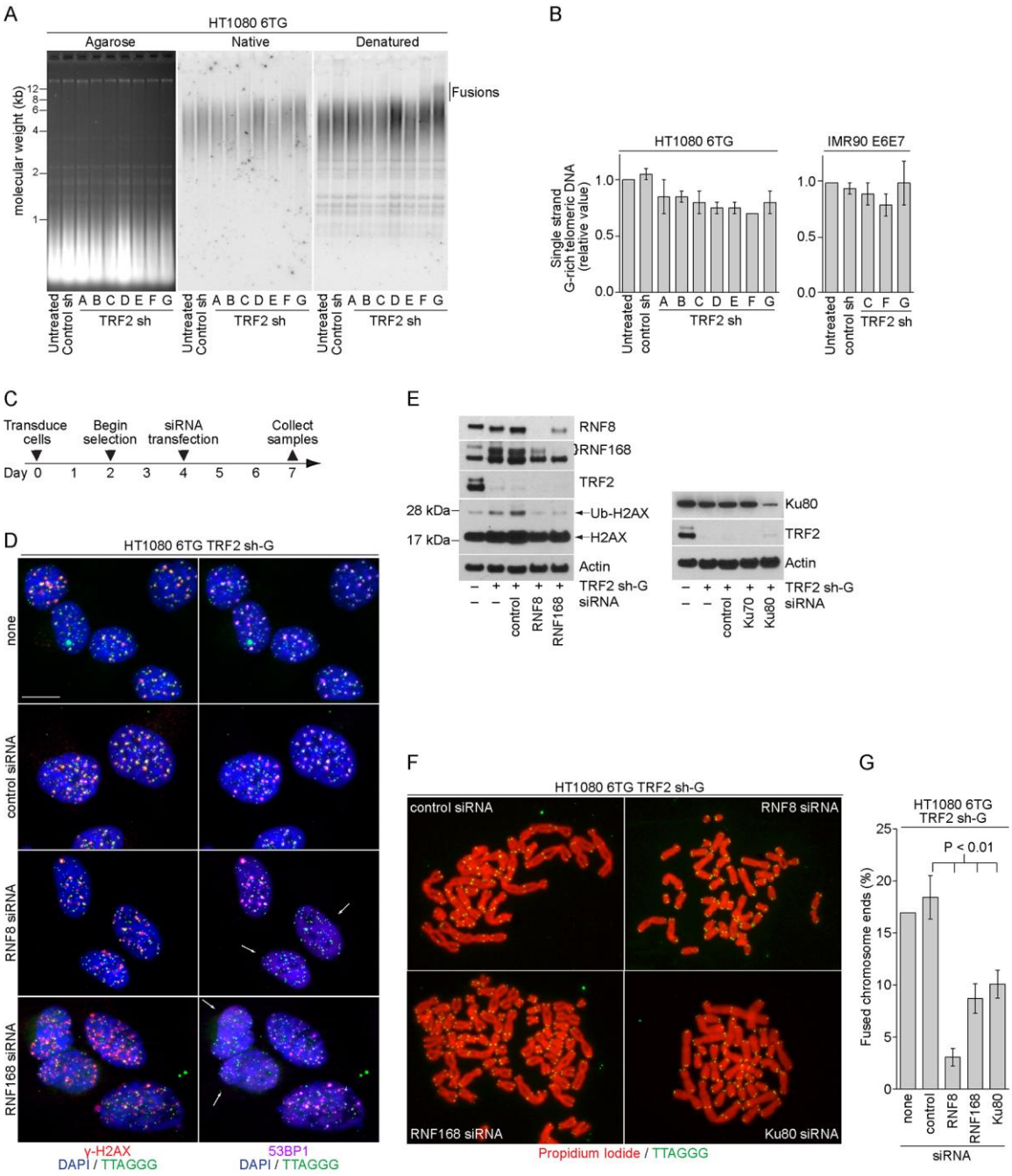

A
HT1080 6TG
Agarose
Native
Denatured
molecular weight (kb)
12
8
6
4
2
1
Fusions
Untreated
Control sh
A B C D E F G
TRF2 sh
B
HT1080 6TG
IMR90 E6E7
Single strand G-rich telomeric DNA (relative value)
1.0
0.5
0.0
Untreated
control sh
A B C D E F G
C F G
TRF2 sh
C
Transduce cells
Begin selection
siRNA transfection
Collect samples
Day 0 1 2 3 4 5 6 7
D
HT1080 6TG TRF2 sh-G
none
control siRNA
RNF8 siRNA
RNF168 siRNA
γ-H2AX
DAPI / TTAGGG
53BP1
DAPI / TTAGGG
E
RNF8
RNF168
TRF2
28 kDa
Ub-H2AX
17 kDa
H2AX
Actin
TRF2 sh-G
siRNA
control
RNF8
RNF168
Ku80
Ku70
Ku80
F
HT1080 6TG TRF2 sh-G
control siRNA
RNF8 siRNA
RNF168 siRNA
Ku80 siRNA
Propidium Iodide / TTAGGG
G
HT1080 6TG
TRF2 sh-G
P < 0.01
Fused chromosome ends (%)
25
20
15
10
5
0
none
control
RNF8
RNF168
Ku80
siRNA

Figure S2 related to Figure 1: Molecular characteristics of intermediate- and uncapped-state telomeres. A) In-gel hybridization with a radiolabeled C-strand telomere probe of native or denatured telomere restriction fragments (5 µg per lane) from untreated, control sh or TRF2 sh transduced HT1080 6TG cells. Loading is shown on the left in the ethidium bromide stained agarose gel, native hybridization in the center and hybridization after denaturation on the right. Higher molecular weight telomeric DNA corresponding to end-to-end chromosome fusions is indicated. B) Quantitation of the relative amount of single-strand G-rich telomeric DNA in the experiments depicted in (A) (mean ± s.d., n = 3). C) Timeline of the shRNA and siRNA co-depletion experiments depicted in panels (D-G). D) Four color imaging of interphase nuclei from HT1080 6TG TRF2 sh-G cells that were not transfected, or transfected with control, RNF8 or RNF168 siRNA pools. Cells were stained with DAPI, telomere FISH, γ-H2AX and 53BP1 IF, with the γ-H2AX IF shown on the left and the 53BP1 IF shown on the right. Arrows indicate nuclei where 53BP1 does not form foci colocalizing with γ-H2AX IF labeled telomeres. E) Western blots on whole cell extracts from HT1080 6TG cells with or without TRF2 sh-G transduction that were either not transfected, or transfected with a control, RNF8, RNF168, Ku70 or Ku80 siRNA pool. F) Cytogenetic chromosome spreads of HT1080 6TG TRF2 sh-G cells transfected with a control, RNF8, RNF168, or Ku80 siRNA pool and stained with PI and telomere FISH . G) Quantiation of the experiments depicted in (F) (mean ± s.d., n = 3 independent siRNA transfections of a single biological replicate of HT1080 6TG TRF2 sh-G, *P* = two-tailed t-test).

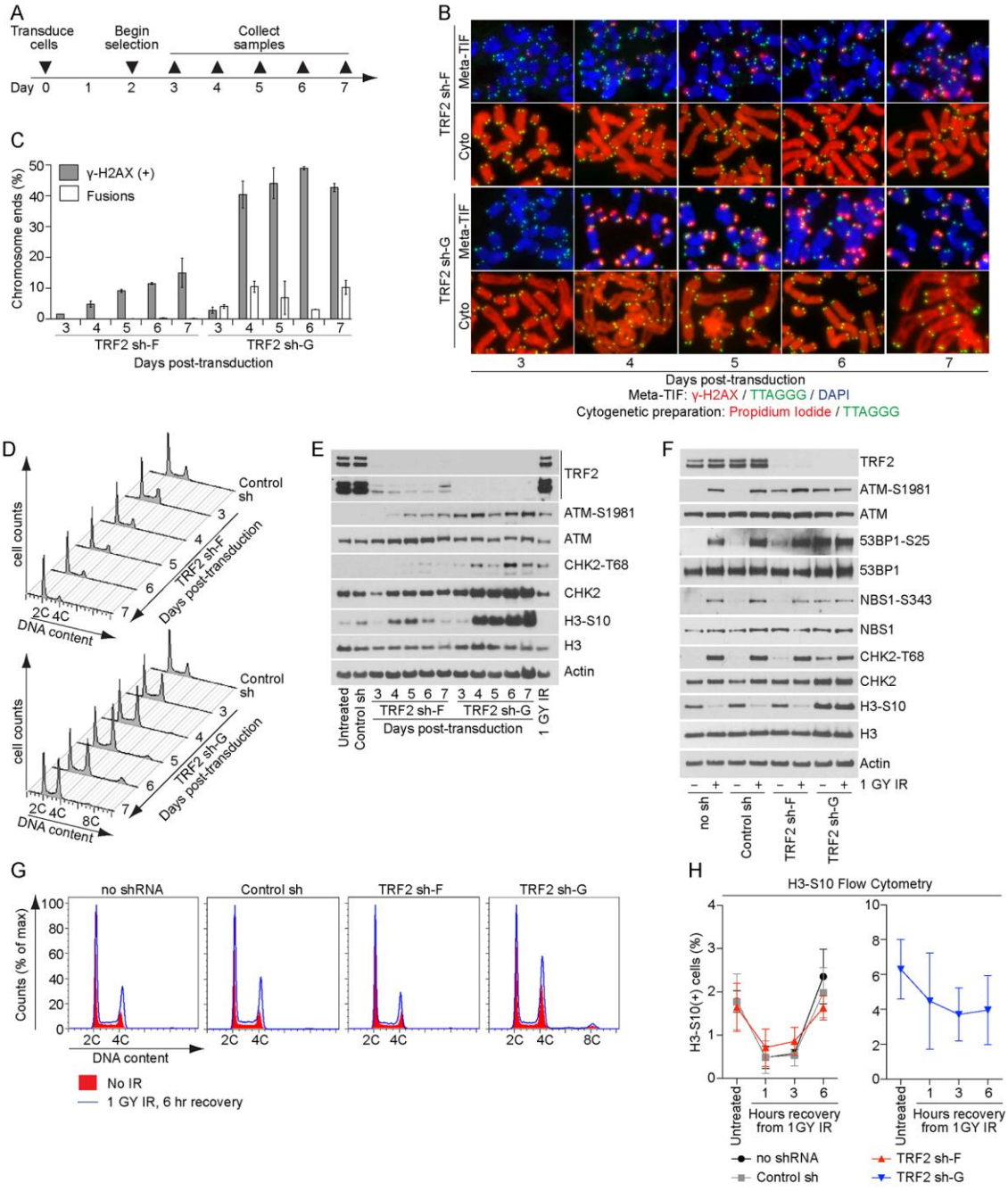
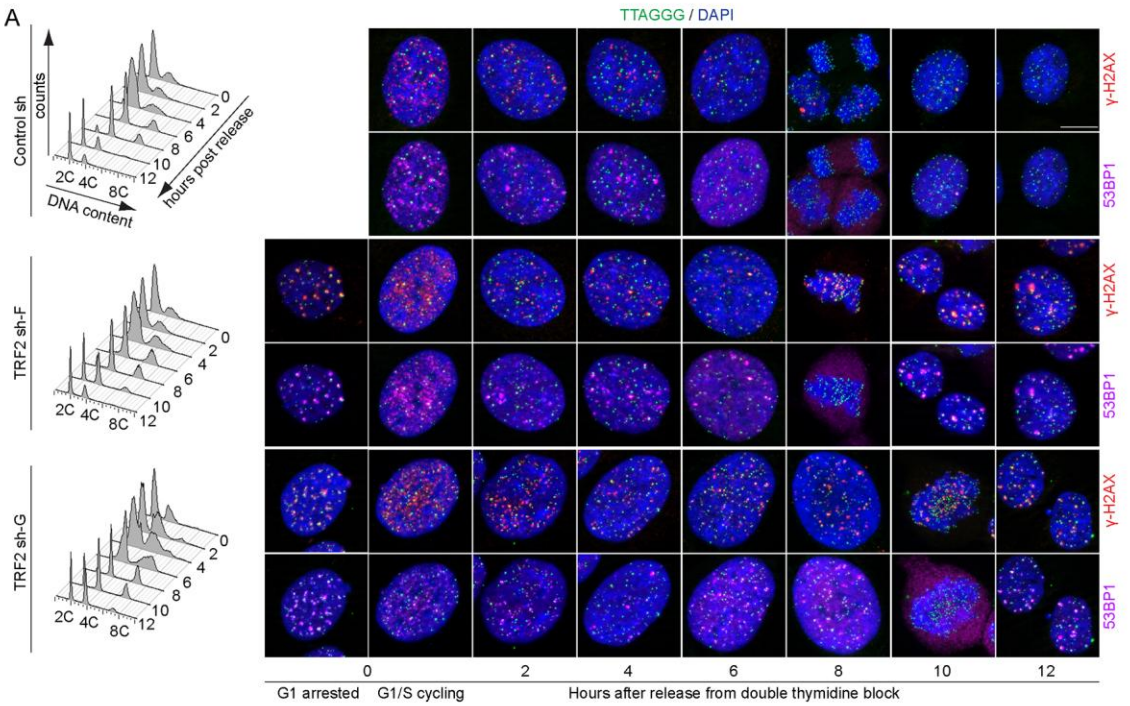

A
Transduce cells
Begin selection
Collect samples
Day 0 1 2 3 4 5 6 7
B
TRF2 sh-F
TRF2 sh-G
Meta-TIF
Cyto
3 4 5 6 7
Days post-transduction
Meta-TIF: γ-H2AX / TTAGGG / DAPI
Cytogenetic preparation: Propidium Iodide / TTAGGG
C
γ-H2AX (+)
Fusions
Chromosome ends (%)
TRF2 sh-F
TRF2 sh-G
Days post-transduction
D
Control sh
TRF2 sh-F
TRF2 sh-G
Days post-transduction
cell counts
DNA content
2C 4C 8C
E
TRF2
ATM-S1981
ATM
CHK2-T68
CHK2
H3-S10
H3
Actin
Untreated
Control sh
TRF2 sh-F
TRF2 sh-G
1 GY IR
Days post-transduction
F
TRF2
ATM-S1981
ATM
53BP1-S25
53BP1
NBS1-S343
NBS1
CHK2-T68
CHK2
H3-S10
H3
Actin
1 GY IR
no sh
Control sh
TRF2 sh-F
TRF2 sh-G
G
no shRNA
Control sh
TRF2 sh-F
TRF2 sh-G
Counts (% of max)
DNA content
No IR
1 GY IR, 6 hr recovery
H
H3-S10 Flow Cytometry
H3-S10(+) cells (%)
Untreated
Hours recovery from 1GY IR
no shRNA
Control sh
TRF2 sh-F
TRF2 sh-G

Figure S3 related to Figure 2: TRF2 depletion does not activate the G2/M checkpoint in IMR90 E6E7 cells. This figure depicts the same set of experiments shown in Figure 2 but performed in IMR90 E6E7 cells. A) Timeline of the experiments in panels (B-E). B) Examples of meta-TIF assays and cytogenetic preparations from IMR90 E6E7 cells. Meta-TIF assays were stained with DAPI, γ-H2AX IF and telomere FISH and the cytogenetic preparations with PI and telomere FISH. C) Quantitation of meta-TIF assays and cytogenetic preparations (mean ± range, n = 2) depicted in (B). D) Cell cycle profiles of IMR90 E6E7 cultures following control or TRF2 sh transduction. A representative experiment quantifying ≥ 15,000 cells per condition is shown. E) Western blots on whole cell extracts prepared from IMR90 E6E7 cultures. The control shRNA sample is from seven days post-transduction and the 1GY IR sample are parental cells that recovered for 1 hour before sample collection. F) Western blots on whole cells extracts from untreated or irradiated IMR90 E6E7 cultures with or without shRNA at seven days post transduction. Irradiated samples recovered for one hour before sample collection. G) Cell cycle profiles of untreated or irradiated IMR90 E6E7 cells with or without shRNA at seven days post transduction. A representative experiment quantifying ≥ 15,000 cells per condition is shown. H) Quantitation of H3-S10 positive cells in untreated or irradiated IMR90 E6E7 cultures with or without shRNA at seven days post transduction (mean ± s.d. n=3 experiments quantifying ≥ 25,000 cells per condition). We note that the percentage of mitotic cells in the IMR90 E6E7 TRF2 sh-G culture is increased. This is due to an unknown mechanism that increases mitotic duration in IMR90 E6E7 cells with chromosome fusions.


A
TTAGGG / DAPI
Control sh
counts
2C 4C 8C
DNA content
hours post release
0 2 4 6 8 10 12
TRF2 sh-F
TRF2 sh-G
γ-H2AX
53BP1
G1 arrested
G1/S cycling
Hours after release from double thymidine block

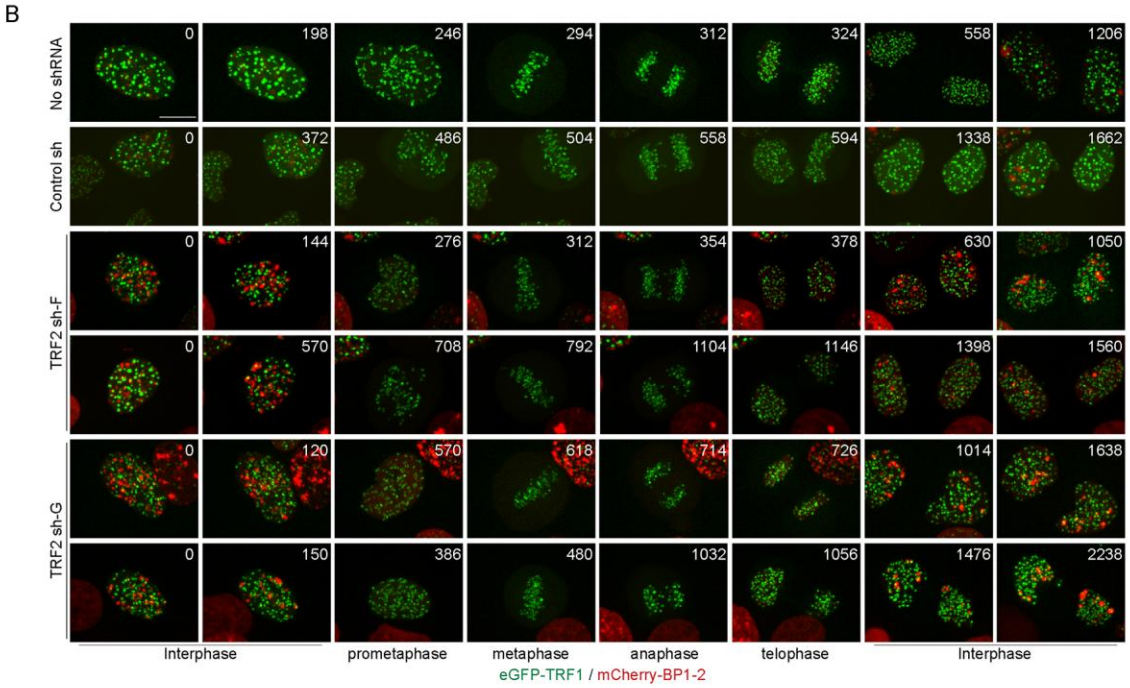

B
No shRNA
Control sh
TRF2 sh-F
TRF2 sh-G
0 198 246 294 312 324 558 1206
0 372 486 504 558 594 1338 1662
0 144 276 312 354 378 630 1050
0 570 708 792 1104 1146 1398 1560
0 120 570 618 714 726 1014 1638
0 150 386 480 1032 1056 1476 2238
Interphase
prometaphase
metaphase
anaphase
telophase
Interphase
eGFP-TRF1 / mCherry-BP1-2

Figure S4 related to Figure 3: Deprotected telomeres do not contribute to the G2/M checkpoint and are passed on between cell cycle phases and through cell division. Panel (A) are the same set of experiments shown in Figure 3A but performed in HT1080 6TG cells. A) shRNA transduced HT1080 6TG cultures were synchronized with a double thymidine block so that at release the cultures were seven days post-transduction. Following release cells were collected every two hours for analysis by flow cytometry and fluorescent imaging. Cell cycle profiles are a representative experiment quantifying ≥ 10,000 cells per condition. Cells were stained for imaging with DAPI, telomere FISH and γ-H2AX and 53BP1 IF and imaged in four-colors. One nucleus is shown for each condition with γ-H2AX IF presented above and 53BP1 IF below. B) Representative examples from live-cell time-lapse imaging using a spinning disk confocal microscope. HeLa 1.2.11 cells expressing exogenous GFP-TRF1 and mCherry-BP1-2 that were not transduced or transduced with either control sh, TRF2 sh-F or TRF2 sh-G were imaged for 36 to 48 hours at six minute intervals. Images are maximum intensity projections of nine z-planes of 1 μm separation. The number in each panel represents the minutes of imaging duration relative to the initial panel. The white bar in (A) and (B) is equivalent to 10 μm.

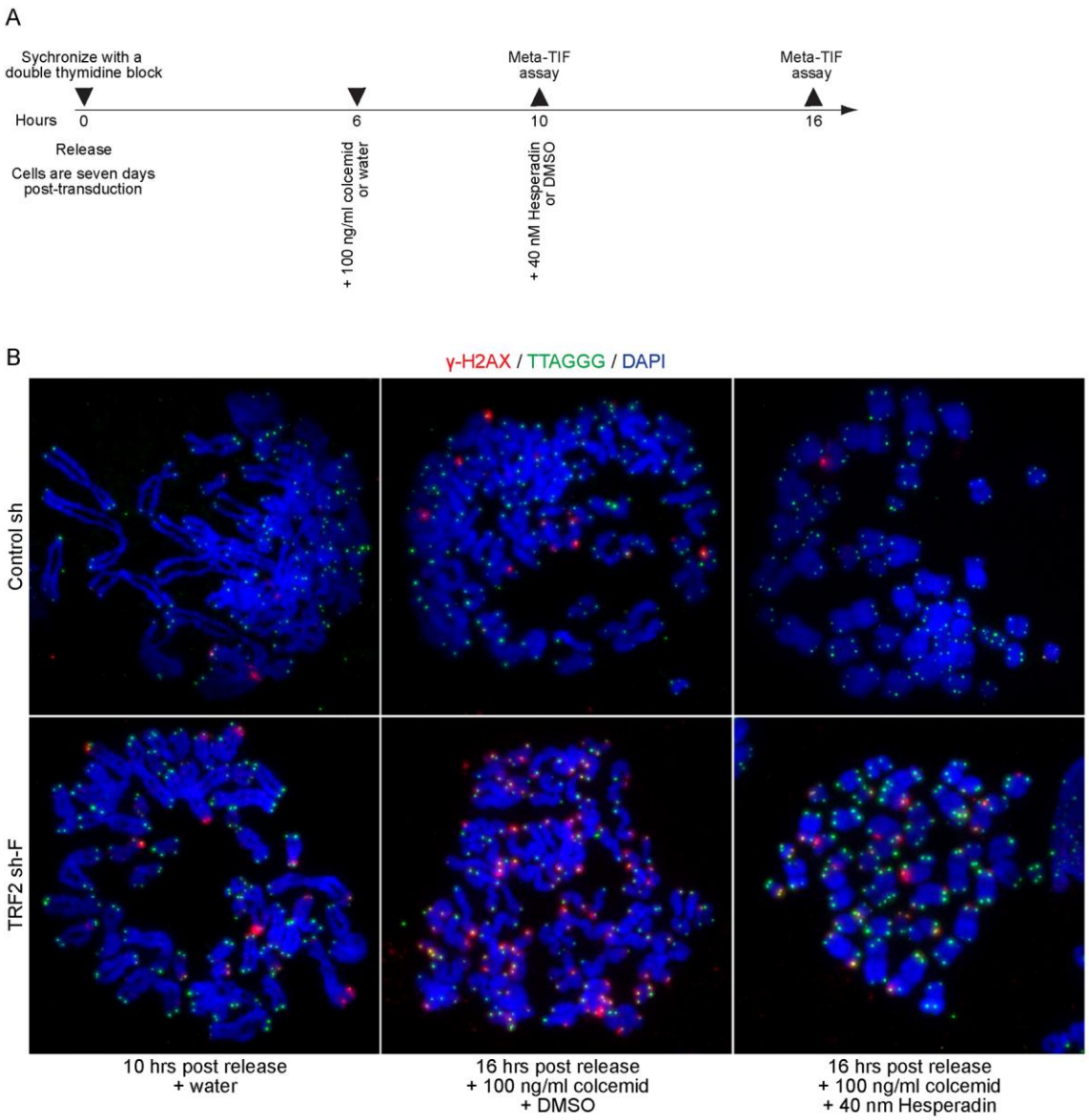


Figure S5 related to Figure 6: Metaphase-TIF assays in IMR90 E6E7 control sh and TRF2 sh transduced cells following prolonged mitotic arrest. A) Time line of the experiment depicted in (B). Cells were synchronized using a double thymidine block so that at release they were day seven post-transduction. 100 ng/ml Colcemid or water vehicle was added six hours after release and 40 nM Hesperadin or DMSO was added ten hours after release. Samples for meta-TIF assays were collected at 10 or 16 hours post-release. B) Representative images of meta-TIF assays from this experiment. The quantitative data are shown in Figure 6E.

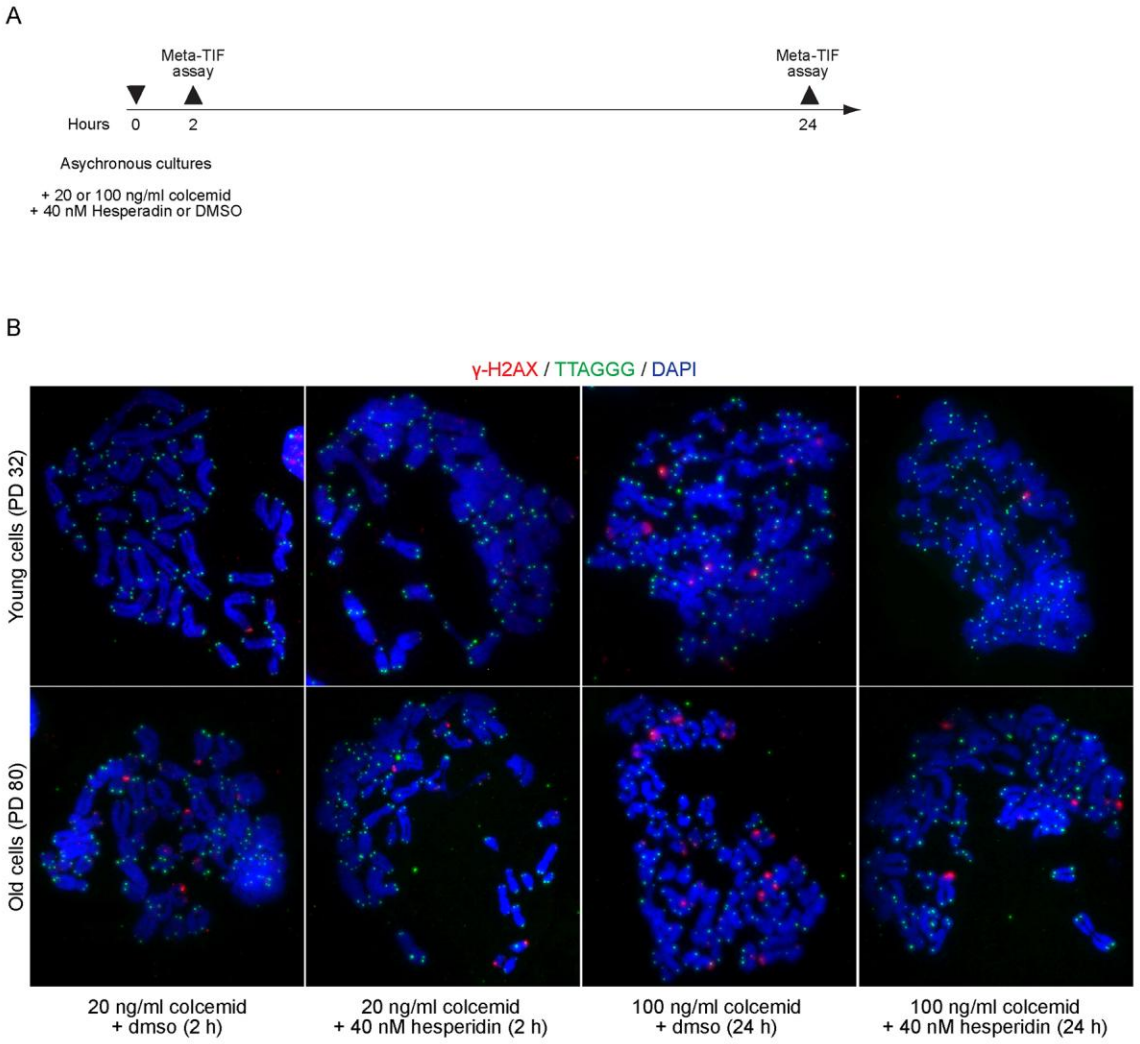


Figure S6 related to Figure 7: Spontaneous TIF in aged human cells are passed into mitosis from G2. A) Time line of the experiment depicted in (B). Asynchronous young or aged IMR90 cells were treated with either 40 nM Hesperadin or DMSO vehicle and either 20 ng/ml colcemid for two hours, which has previously been shown not to induce metaphase-TIF (Hayashi et al., 2012; Kaul et al., 2012), or 100 ng/ml for 24 hours before Meta-TIF assay samples were prepared. B) Representative images of meta-TIF assays from this experiment. The quantitative data are shown in Figure 7D.

Movie S1 related to Figure 3: Time-lapse live cell imaging of a HeLa 1.2.11 cell expressing GFP-TRF1 and mCherry-BP1-2 that was not transduced with an shRNA construct. The movie depicts a maximum intensity projection of nine z-planes separated by 1 μm. Time is depicted in hrs:min and the white bar is equivalent to 10 μm.

Movie S2 related to Figure 3: Time-lapse live cell imaging of a HeLa 1.2.11 cell expressing GFP-TRF1 and mCherry-BP1-2 transduced with the control sh. The movie depicts a maximum intensity projection of nine z-planes separated by 1 μm. Time is depicted in hrs:min and the white bar is equivalent to 10 μm.

Movie S3 related to Figure 3: Time-lapse live cell imaging of a HeLa 1.2.11 cell expressing GFP-TRF1 and mCherry-BP1-2 transduced with TRF2 sh-F. The movie depicts a maximum intensity projection of nine z-planes separated by 1 μm. Time is depicted in hrs:min and the white bar is equivalent to 10 μm.

Movie S4 related to Figure 3: Time-lapse live cell imaging of a HeLa 1.2.11 cell expressing GFP-TRF1 and mCherry-BP1-2 transduced with TRF2 sh-G. The movie depicts a maximum intensity projection of nine z-planes separated by 1 μm. Time is depicted in hrs:min and the white bar is equivalent to 10 μm.

Supplemental Experimental Procedures

Native and denaturing in-gel hybridization

Telomere restriction fragments were generated with *Mbo*I and *Alu*I (New England Biolabs) digestion, separated by gel electrophoresis, hybridized in-gel with a radiolabeled $(CCCTAA)_3$ oligonucletide probe under native and denaturing conditions and the signals quantified as described (Karlseder et al., 2002).

siRNA transfection

Cells were transiently transfected with ON-TARGETplus SMART pool siRNAs (Thermo Scientific) using Dharmafect (Dharmacon) according to the manufactures instructions. siRNA pools used: RNF8 (L-006900-00), RNF168 (L-007152-00), Ku70 (L-005084-00), Ku80 (L-010491-00) and non-targeting control (D-001810-10).

Vectors

pWZL-EGFP-TRF1 was created by cloning EGFP into pWZL-TRF1 by PCR using BamHI-EGFP-For (5'-GGCGGATCCATGGTGAGCAAGGGCGAGG-3') and BamHI-EGFP-Rev (5'-GGCGGATCCGGTGGCGATGCTGCGCTTGTACAGCTCGTCCATGC CG-3'). mCherry-BP1-2-pLPC-Puro was obtained from Titia de Lange via addgene (Plasmid 19835).

Live Cell Imaging

Cells were grown in 35 mm Ibidi dishes and the images were acquired with a 512x512 16bit Evolve EMCCD camera (Photometrics, Tucson, AZ) using a 63x Plan-Apochromat objective and a 1 µm step size every 6 min for at least 36 h on a Cell Observer SD spinning disk confocal microscope (Axio Observer Z1 inverted platform fitted with a Yokagawa CSU-X1 Nipkow spinning disk head, Carl Zeiss, Jena, Germany) equipped with an incubation chamber (37°C, 7.5% CO_2, 3% O_2) as described (Crabbe et al., 2012).

Antibodies

Actin (Sigma AC15); ATM (Epitomics 1549-1); ATM-S1981 (Epitomics EP1890Y); CHK2 (Millipore 05-649); CHK2-T68 (Cell Signaling Technology 2661); H3 (Abcam ab1791); H3-S10 (Cell Signaling Technology 3377); H3-S10 Alexa Flour 488 conjugated (Cell Signaling Technology 3465); H4 (Abcam ab7311); H2AX (Abcam ab11175); γ-H2AX (Millipore 05-636 and Biolegend, 613402); Ku80 (2753, Cell Signaling Tehcnology); NBS1 (GeneTex GTX70222); NBS1-S343 (Cell Signaling Technology 3001); RNF8 (kindly provided by Michael S.Y. Huen); RNF168 (kindly provided by Dan Durocher); TRF2 (Karlseder lab); 53BP1 (Santa Cruz sc-22760) and 53BP1-ser25 (Bethyl A300-652A). We used highly cross-absorbed secondary antibodies conjugated to Alexa Flour 488, Alexa Flour 564 or Alexa Flour 647 (Life Technologies) and secondary antibodies conjugated to HRP (GE healthcare).