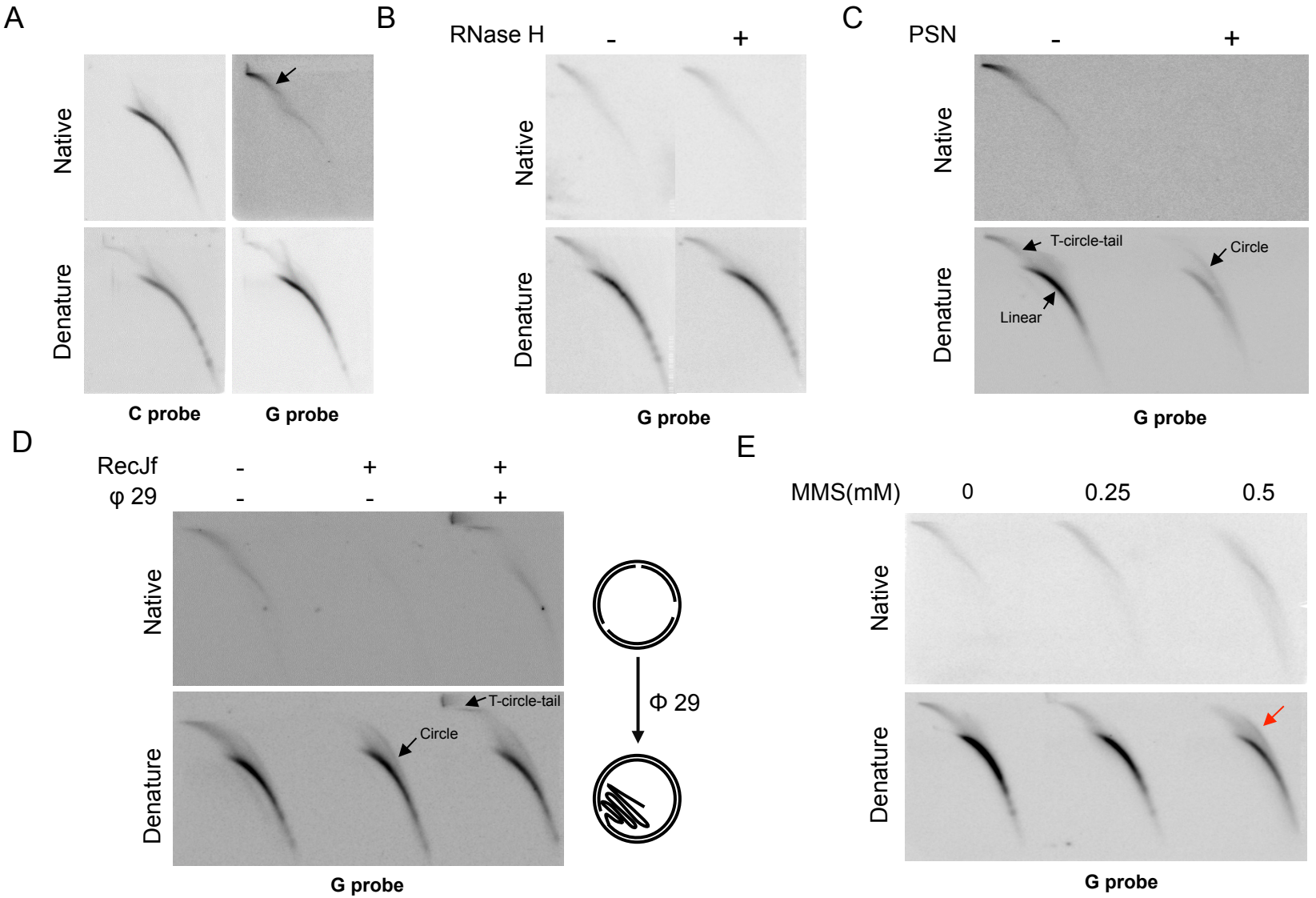
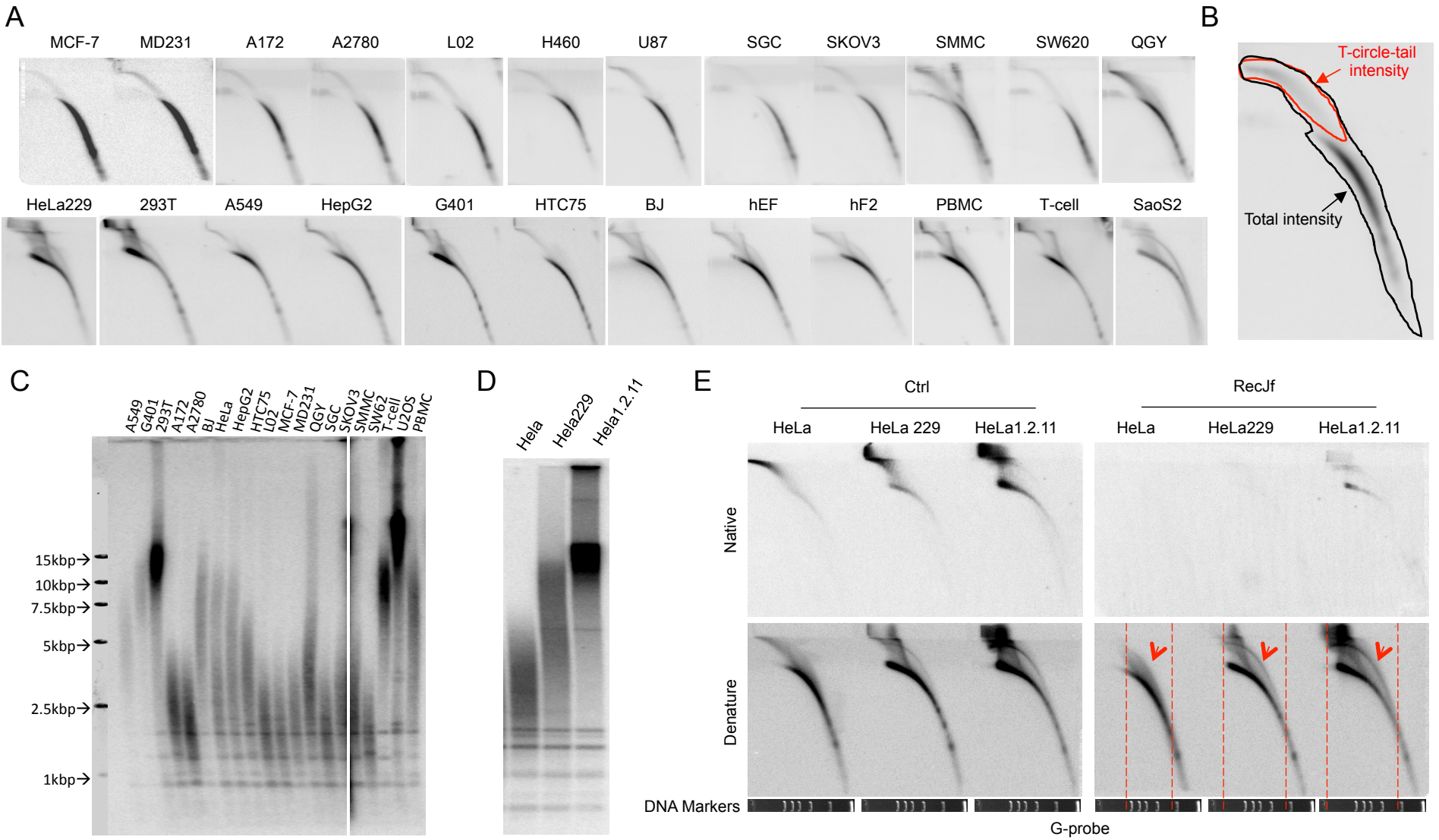
Appendix figures and legends for Zhang TP et al., “Looping-out Mechanism for Resolution of Replicative Stress at Telomeres”

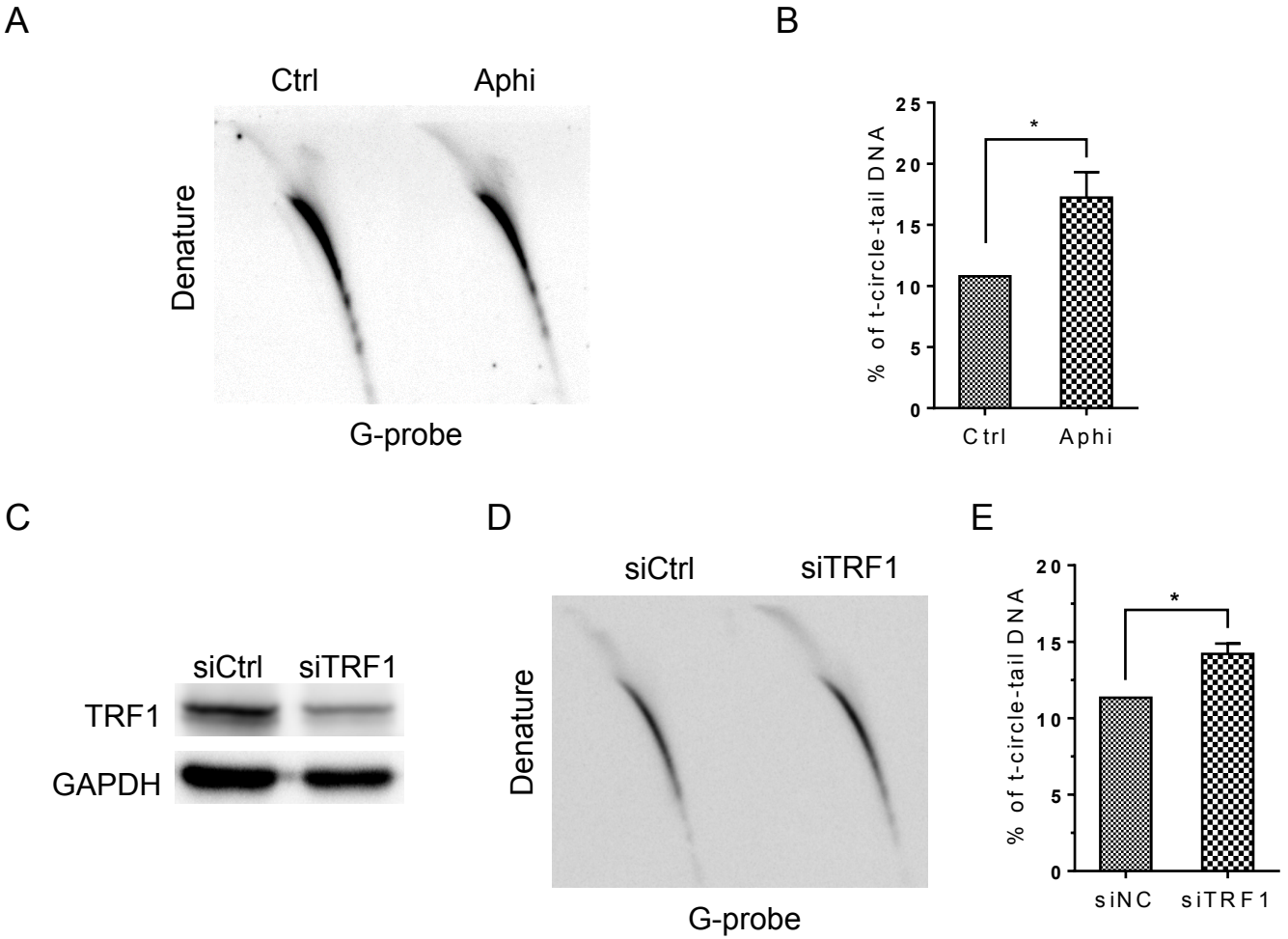
Table of Contents



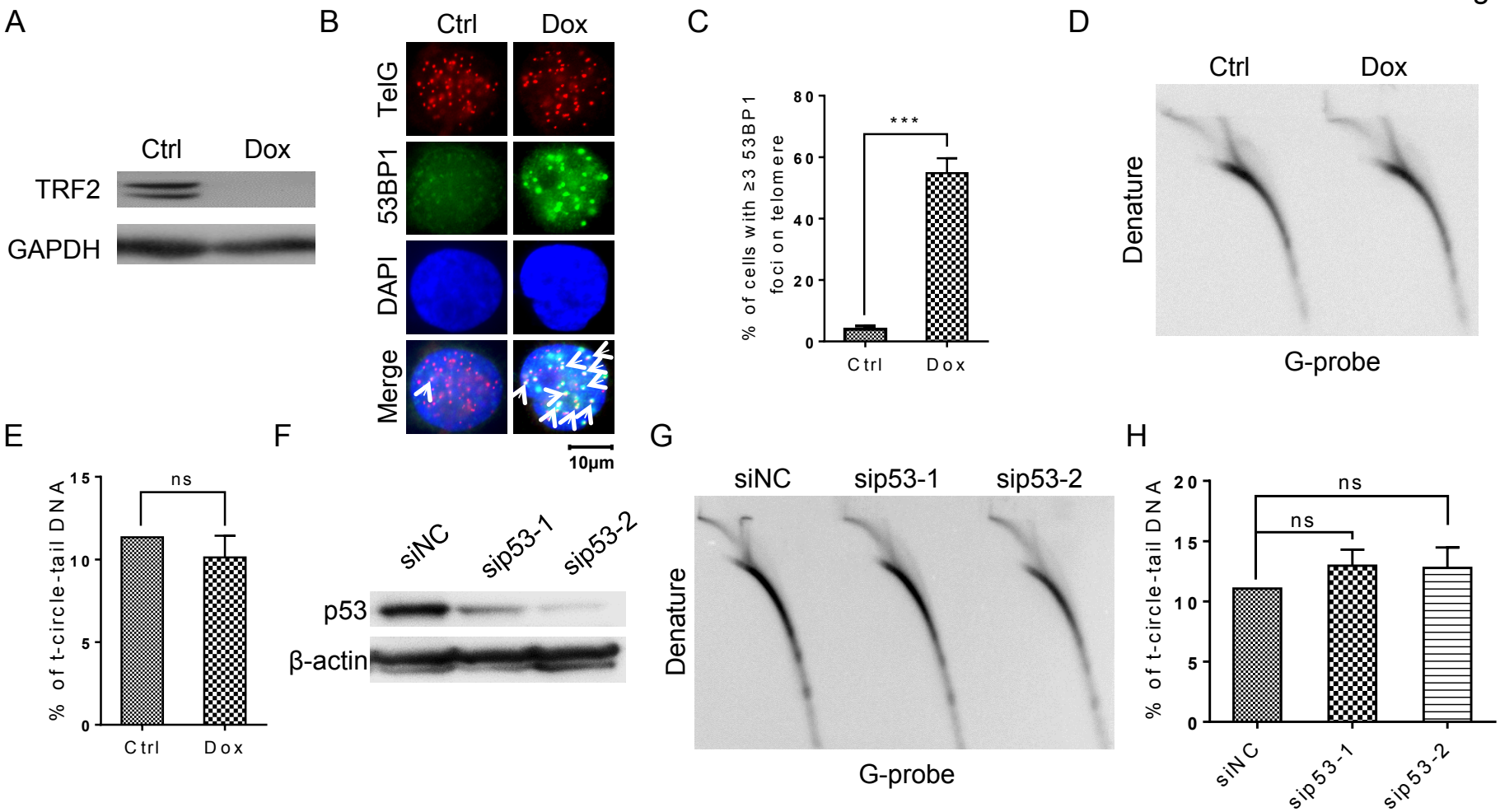
Appendix Fig S1. Identification and characterization of t-circle-tail. (A) Hinf I and Rsa I digested HeLa genomic DNA was subjected to 2D gel electrophoresis and in gel hybridized with G-probe or C-probe at native or denatured condition. The arrow indicates the "slow-mobility structures". (B) T-circle-tail was resistant to RNase H digestion. Genomic DNA was incubated with RNase H prior to 2D gel electrophoresis. (C) Plasmid-safe^{TM} ATP-dependent nuclease (PSN) converted t-circle-tail to closed-circular DNA. T-circle-tail, linear and circle DNA were indicated in images. Untreated DNA was used as a control (-). (D) Rec Jf converted t-cirlce-tail to closed circle DNA and φ29 restored t-circle-tail structure using closed-circular DNA produced by Rec Jf . (E) MMS treatment induces the conversion of t-circle-tail DNA to closed-circular DNA. Cells were treated with indicated concentration of MMS and purified DNA were subjected to 2D gel electrophoresis.



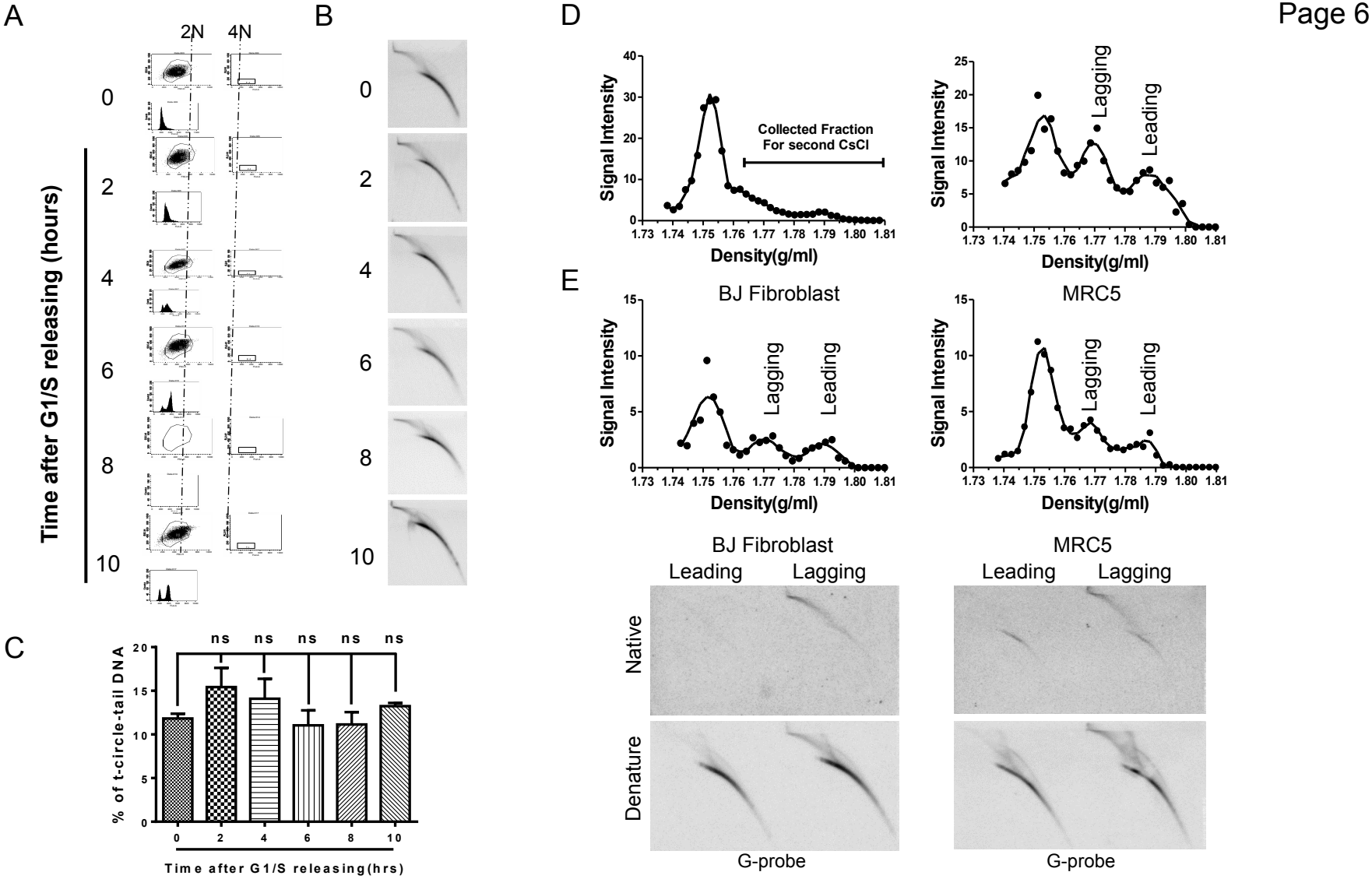
Appendix Fig S2. Abundance of t-circle-tail is positively correlated with telomere length. (A) t-circle-tail DNA were present in all 25 tested cell lines. T-circle-tail was detected by 2D gel electrophoresis and hybridized with G-probe under denatured condition. (B) Schematic of two-dimensional electrophoresis (2D gel) to quantify the relative abundance of t-circle-tail (ratio of t-circle-tail intensity to total intensity). (C) TRF assay to determine the telomere length of tested cell lines. (D) TRF assay of HeLa, HeLa229 and HeLa 1.2.11. (E) 2D gel electrophoresis to determine the abundance of t-circle-tail in three HeLa cell lines. DNA was also treated with Rec Jf that converts t-circle-tail to telomeric circle DNA (indicated by arrow).



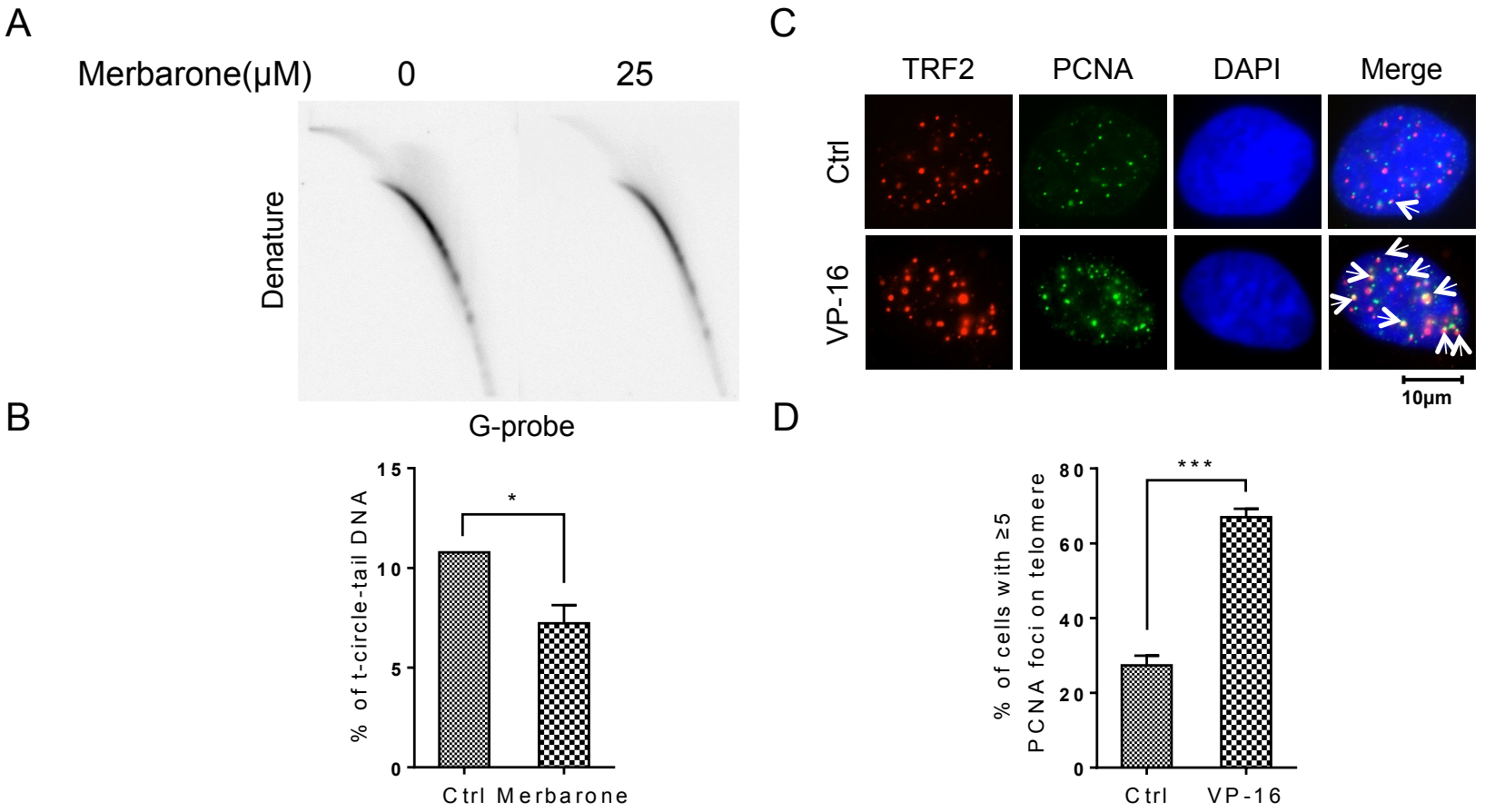
Appendix Fig S3. Telomere replication stress but not deprotection induces formation of t-circle-tail DNA. (A) Genomic DNA from PBS (Ctrl) or aphidicolin-treated HeLa cells was analyzed by 2D gel electrophoresis and hybridized to G-rich telomeric probe under denatured conditions. (B) Quantification of A. Error bars represent the mean ± SEM of three independent experiments. Two-tailed unpaired student's *t*-test was used to calculate P value. *P<0.05. (C) Western blot using antibody to TRF1 in HeLa cells treated with TRF1-targeted or control siRNA, as indicated. anti-GAPDH was used as a loading control. (D) 2D gel of detection of t-circle-tail in HeLa cells treated with siCtrl or siTRF1. (E) Quantification of D. Error bars represent means ± SEM of three independent experiments. Two-tailed unpaired student's *t*-test was used to obtain P value. * P<0.05.



Appendix Fig S4. TIFs and p53 is not involved in formation of t-circle-tail. (A) Western-blot validation of depletion of TRF2 in HeLa cells transfected with inducible (Dox) CRISPR/Cas9 system. Cells were induced by Dox for 5 days before the assay. Cells without Dox induction were used as a control (Ctrl). (B) Depletion of TRF2 leads to the formation of telomere dysfunction induced foci (TIFs). Telomeres and DNA damage response were visualized by FISH (telomeric probe, TelG) and IF (antibody to 53BP1), respectively. (C) Quantification of B. Cells with ⩾3 co-stained foci were counted. >100 cells were scored for each group. Error bars represent the mean ± SEM of three independent experiments. Two-tailed unpaired student's *t*-test was used to calculate P-values. ***P<0.001. (D) 2D gel of detection of t-circle-tail in HeLa cells with or without Dox induction. (E) Quantification of D. Error bars represent the mean ± SEM of three independent experiments. Two-tailed unpaired student's *t*-test was used to calculate P-values. "ns" means P >0.05. (F) Validation of knocking down efficiency of p53 by siRNA in HCT116 Cells. (G) 2D gel detection of t-circle-tail in HCT116 cells treated with control siRNA (siNC) or siRNA to p53 (sip53). (H) Quantification of G. Error bars represents mean ± SEM (n=3). Two-tailed unpaired student's *t*-test was used to obtain P value. "ns" means P >0.05.

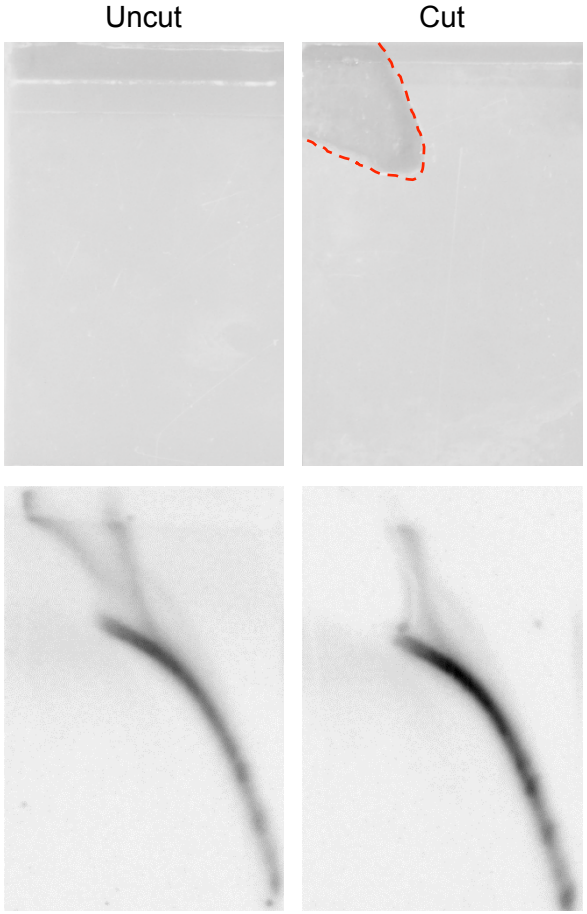


Appendix Fig S5. Formation of t-circle-tail is coupled with telomeric DNA replication. (A) FACS analysis showing that HeLa cells were synchronized at G1/S and released into S phase for different times. (B) Genomic DNA from different time point during S phase was subjected to 2D gel electrophoresis and hybridization with G probe under denatured condition. (C) Quantification of B. Error bars represents mean ± SEM of three independent experiments. Two-tailed unpaired student's *t*-test was used to obtain P value. "ns" means P >0.05. (D) 1h pulse labeling of telomeric DNA in HeLa cells. The first (left) and second round (right) of CsCl gradient centrifugation. (E) Synthesized t-circle-tail was present in human normal BJ fibroblast and MRC5 cells. Two cell lines were incubated with BrdU for 48 hrs and their genomic DNA was subjected to CsCl gradient centrifugation to isolate newly synthesized leading and lagging strands (up) that were used for detection of t-circle-tail by 2D gel electrophoresis (bottom).



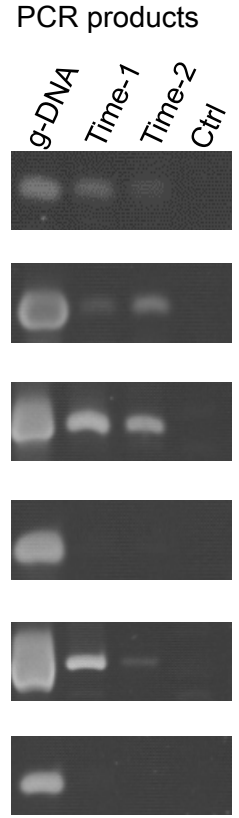
Appendix Fig S6. Inhibition of Topoisomerase II diminishes the formation of t-circle-tail. (A) Topo II inhibitor merbarone suppressed the formation of t-circle-tail. T-circle-tail was determined by 2D gel electrophoresis. (B) Quantification of A. Cells with ⩾5 co-stained foci were counted. >100 cells were scored for each group. Error bars indicated mean ± SEM of three independent experiments. Two-tailed unpaired student's *t*-test was used to obtain P value. * P<0.05. (C) VP-16 treatment induces replication fork stalling at telomeres. Stalled replication and telomeres were visualized by PCNA antibody and telomeric probe, respectively. (D) Quantification of C. Cells with ⩾5 co-stained foci were counted. >100 cells were scored for each group. Error bars indicated mean ± SEM of three independent experiments (> 100 cells each time). Two-tailed unpaired student's *t*-test was used to obtain P value. *** P<0.001.

A

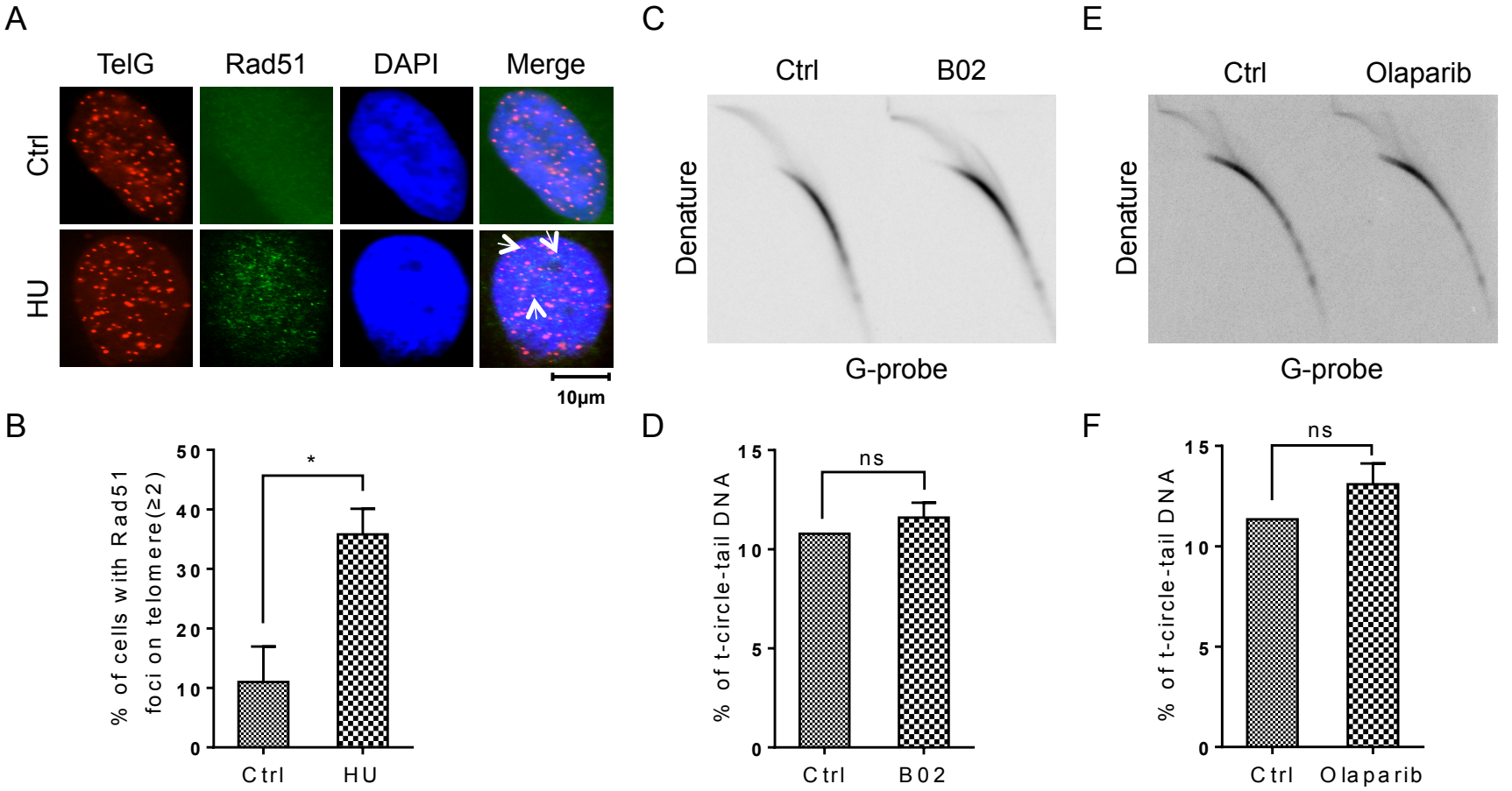


B

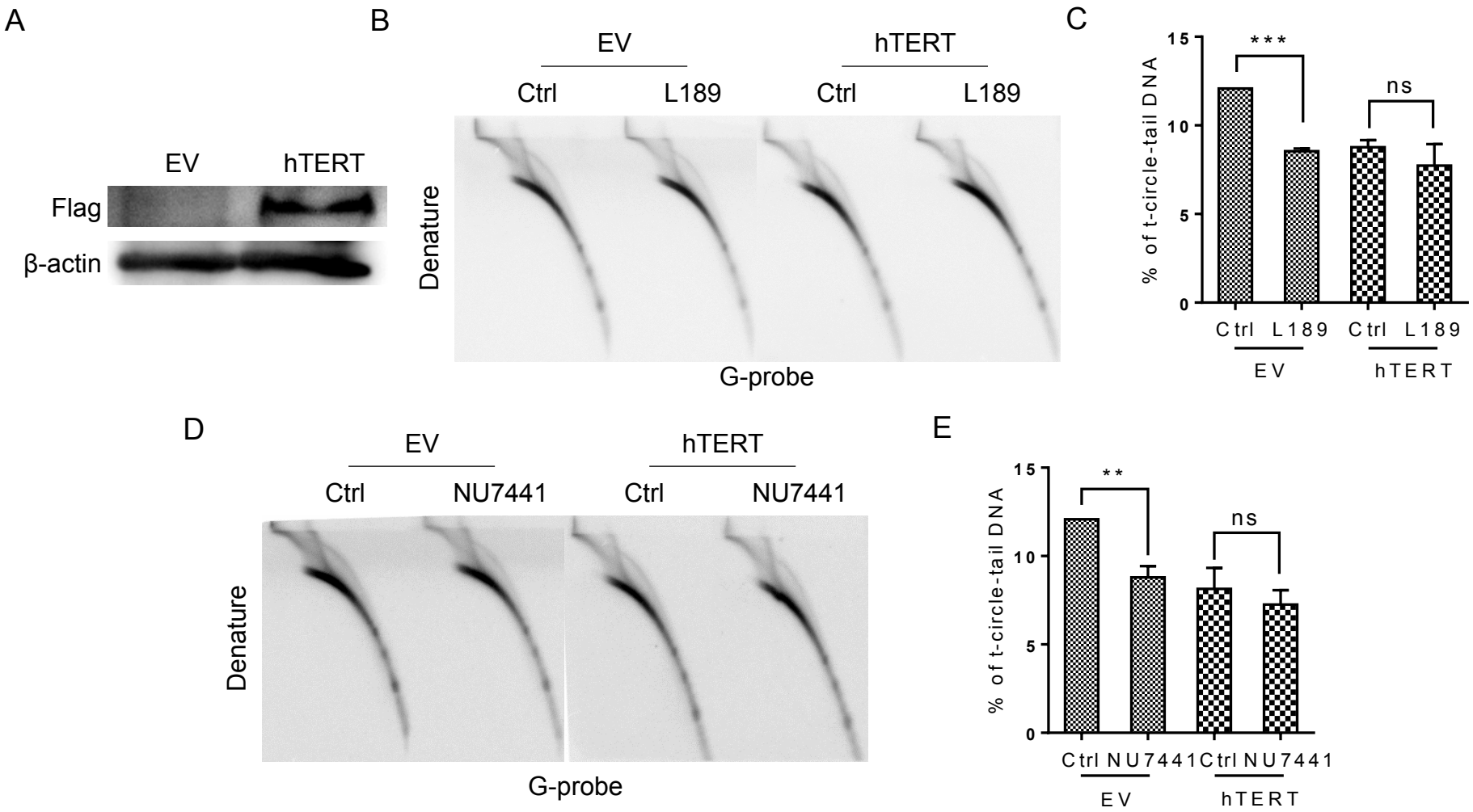
Primer Names	Targeted chromosomes (subtelomeres)	Predicted Size（bp）
XYq-3/ -2733	9p,12p,15q,16p,19p, XYq	55
XYq-1456	9p,12p,15q,16p,19p, XYq	69
10q-2/-1122	1q,2q,4q,5q,6q,8p, 10q, 13q, 16q,19p, 19q, 21q,22q	87
13q-152	1q, 2q, 4q, 5q, 6q, 10q, 13q, 16q, 21q, 22q	59
13q-957 CTCF site	1q, 2q, 4q, 5q, 6q, 10q, 13q, 16q, 19q, 21q	124
15q-941 CTCF site	15q, 16p, 19p	73



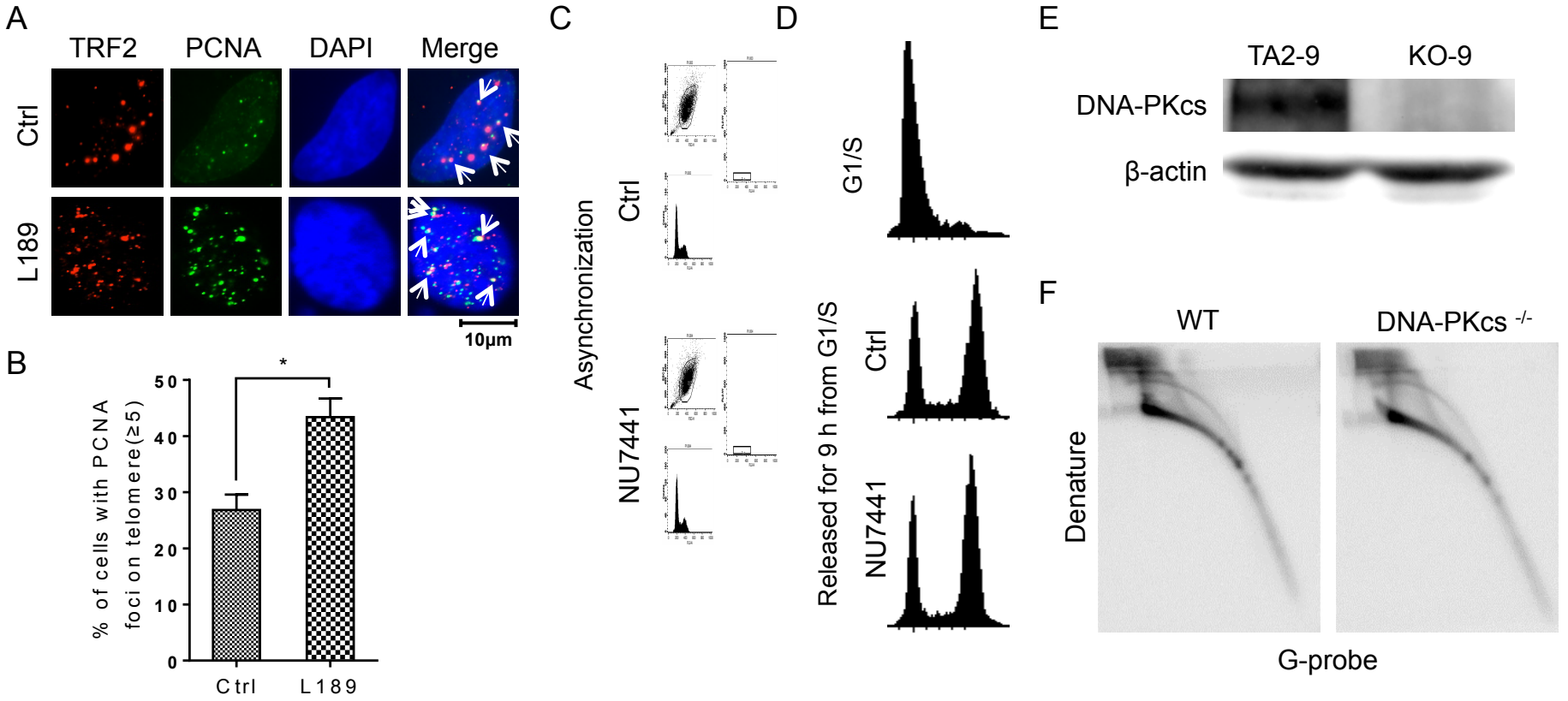
Appendix Fig S7. PCR identification of subtelomeric sequences in T-circle-tail DNA. (A) T-circle-tail DNA were excised from 2-D gel (up panels). The exact cutting was verified by hybridization with telomeric G-rich probe (bottom panels). (B) DNA was purified from excided gel and amplified by PCR using indicated primers. One pair of primers can amplify the sequence at multiple chromosome ends (subtelomeres) (Deng et al., 2012) and predicted size of product was indicated. G-DNA: genomic DNA was used as a positive control; Time-1 and Time-2: two independent experiments; Ctrl: PCR without DNA template was performed as a negative control.



Appendix Fig S8. Homologue recombination is not involved in the formation of t-circle-tail. (A) HU treated cells displayed the recruitment of HR to telomeres. Rad51 was used as a marker of HR. IF of Rad51 and telomeric FISH were used to visualize HR and telomeres, respectively. (B) Quantification of A. The cells with ≥2 co-stained foci were counted. >100 cells were scored for each group. Error bars indicated means± SEM of three independent experiments. Two-tailed unpaired student's *t*-test was used to obtain P value. * P<0.05. (C) 2D gel electropheresis determined the abundance of t-circle-tail in control (Ctrl) and Rad51 inhibitor (B02) treated cells. (D) Quantification of C. Error bars indicated means± SEM of three independent experiments. Two-tailed unpaired student's *t*-test was used to obtain P value. "ns" means P > 0.05. (E) 2D gel electropheresis determined the abundance of t-circle-tail in control (Ctrl) and PARP inhibitor (Olaparib) treated cells. (F) Quantification of E. Error bars indicated means ± SEM of three independent experiments. Two-tailed unpaired student's *t*-test was used to obtain P value. "ns" means P >0.05.



Appendix Fig S9. Overexpression of hTERT attenuates formation of t-circle-tail. (A) Validation of hTERT overexpression in 293T cells by western-blot. EV: empty vector, hTERT: flag-tagged hTERT. (B) 2D gel electrophoresis detection of t-circle-tail in 293T cells with overexpressed EV (EV) or hTERT (hTERT) in the presence (L189) or absence of L189 (Ctrl). (C) Quantification of B. Error bars indicated means± SEM (n=3). Two-tailed unpaired student's *t*-test was used to obtain P value. "ns" means $P > 0.05$, *** $P<0.001$. (D) and (E) same as (B) and (C) except that NU7441 was used to treat the cells. Error bars indicated means± SEM (n=3). Two-tailed unpaired student's *t*-test was used to obtain P value. "ns" means $P > 0.05$, ** $P <0.01$.



Appendix Fig S10. NHEJ deficiency induces decreased formation of t-circle-tail in MEF. (A) L189 treated HeLa cells shows accumulation of PCNA foci on telomere. (B) Quantification of A. The cells with ≥ 5 co-stained foci were counted. >100 cells were scored for each group. Error bars indicated mean± SEM (n=3). Two-tailed unpaired student's *t*-test was used to obtain P value. * P<0.05. (C) FACS analysis of cell cycle of asynchronized HeLa cells treated with NU7441 for 24hrs. Untreated cells were used as a control (Ctrl). (D) FACS analysis of DNA content of HeLa cells at G1/S (G1/S) and cells released for 9 hrs in the presence (NU7441) or absence of (Ctrl). (E) Western blot showing DNA-PKcs in WT (TA2-9) and knockout cells (KO-9). β-actin is used as a loading control. (F) 2D gel assay determined the abundance of t-circle-tail in WT (TA2-9) and knockout cells (KO-9).