Materials and Methods

Cell culture

U2OS, SAOS2, HeLa, SW26, and SW39 cells were cultured in DMEM, 10% FBS, 1% L-Glutamine, and 1% Penicillin/Streptomycin. MG63, SKLU1, CALU6, and NY cells were grown in DMEM F12, 5% FBS, 1% Penicillin/Streptomycin. SJSA1, NOS1, HUO9, and G292 cells were grown in RPMI 1640, 5% FBS, 1% Sodium Pyruvate, 1% Penicillin/Streptomycin. CAL78 was grown in RPMI 1640, 10% FBS, 1% Sodium Pyruvate, 1% Penicillin/Streptomycin. CAL72 was grown in DMEM F12, 10% FBS, and 1% Penicillin/Streptomycin. BJ fibroblasts were grown in EMEM, 10% FBS, 1% penicillin/Streptomycin. MGG119 and MGG4 were grown in neurobasal medium (Invitrogen) supplemented with L-glutamine (3 mM; Mediatech), B27 supplement (Invitrogen), N2 supplement (Invitrogen), heparin (5 mg/ml; Sigma), EGF (20 ng/ml; R and D systems), and FGF2 (20 ng/ml; Peprotec). MG63, SAOS2, SJSA1, NOS1, HUO9, NY, G292 and CAL78 were obtained from the Center for Molecular Therapeutics at Massachusetts General Hospital. SW26 and SW39 were a kind gift of W. Wright (UT Southwestern). BJ fibroblasts were obtained from ATCC.

siRNAs, probes and antibodies

Stealth siRNA targeting ATRX #1 (UUCCAUAGCCGUCUCAAGAUUCUCA) and #2 (UAUAGAAUUCUGAUCAUCA) was obtained from Invitrogen. ATRX knockdown was analyzed by Western blot 72 hr after transfection using RNAi MAX. siRNA for ATR (CCUCCGUGAUGUUGCUUGA) and ATM (GCCUCCAGGCAGAAAAAGAtt) were obtained from Invitrogen and experiments were preformed 72 hr after transfection using RNAi MAX.

The following antibodies and probes were used where indicated, ATR (Bethyl), ATM (Bethyl), ATRX (Santa Cruz), rabbit TRF2 (Bethyl), mouse TRF2 (Millipore), PML (Santa Cruz), RPA32 (Thermo Scientific), RAD52 (Santa Cruz), γH2AX (Millipore), and αTubulin

(Cell Signaling), PNA-TAMRA-(CCCTAA) (Custom Biosynthesis), PNA-FITC-(TTAGGG) (Custom Biosynthesis), 28S (AACGATCAGAGTTTTCACC).

Cell synchronization and FACS

Cells were treated with 2 mM thymidine, 0.1 mg/ml nocodazole, or 7 μM RO3306, for 16-18 hr. Thymidine released cells were either washed three times in PBS, once in growth media, and then collected at the indicated time points or washed and released into 7 μM RO3306 for 20 hr. For FACS, cells were collected by trypsin, washed with PBS, and resuspended in PBS containing 1 mM EDTA. Cells were fixed by addition of ice-cold ethanol overnight. Fixed cells were washed in PBS, and stained with PBS, 0.1 mM EDTA, 1% BSA, 0.25% Tween 20, 10 μg/ml propidium iodide, 0.5 mg/ml RNaseA, for 20 min at 37°C. Samples were analyzed using a FACSCalibur cytometer.

Immunofluorescence analysis

Cells were extracted with 0.25% Triton, fixed in 3% paraformaldehyde, and further permeablized with 0.5% Triton. Cells were subsequently incubated with the primary antibodies (diluted in PBS containing 3% BSA and 0.05% Tween 20) overnight at 4°C in a humidified chamber. Following extensive washing with PBS, cells were incubated with secondary antibodies for 45 min at room temperature, and washed again with PBS. After a 5-min incubation with DAPI, cells were mounted on slides with Vectashield. Slides were analyzed using a Nikon H600L fluorescence microscope or Zeiss LSM 710 confocal microscope. For HeLa cells with ATRX knockdown, 1 x 10^5 cells were reverse transfected with ATRX siRNA using Lipofectamine RNAi Max (Invitrogen), seeded onto coverslips, and incubated for 48 hr. After 48 hr, cells were treated with 2 mM thymidine for 16 hr, washed and released, and processed at the indicated time points. For ATR or ATM knockdown in U2OS cells, 0.75x 10^5 cells were reverse transfected with ATR or ATM siRNA using

Lipofectamine RNAi Max (Invitrogen), seeded onto coverslips, and incubated for 72 hr before APB analysis. To enhance the percentage of cells positive for APB, U2OS cells were seeded at 1.5 x 10^5 and allowed to incubate overnight. The following day methionine free media was added to the cells and they were incubated for an additional 84 hr.

RNA FISH

HeLa or U2OS cells adhered to coverslips were incubated for 7 min on ice, in ice-cold freshly made CSK buffer (100 mM NaCl, 300 mM Sucrose, 3 mM $MgCl_2$, 10 mM PIPES pH 7, 0.5% Triton X-100, 10 mM Vanadyl Ribonucleoside Complex). Cells were then rinsed in 1x PBS and fixed in 4 % Paraformaldehyde for 10 min at room temperature. The coverslips were rinsed in 70% ethanol and dehydrated in a series of ethanol washes (70%, 85%, 100%) for 5 min each at room temperature. After drying the coverslips at 37°C, they were then incubated with 10 nM PNA-TAMRA-(CCCTAA) probe in hybridization buffer (50% formamide, 2x SSC, 2 mg/ml BSA, 10% dextran sulfate, 10mM Vanadyl Ribonucleoside complex) for 16 hr at 37°C. Coverslips were washed in 2x SSC + 50% formamide 3 times at 39°C for 5 min, 3 times in 2xSSC at 39°C for 5 min each, and finally 1 time in 2x SSC + 100 ng/ml DAPI at room temperature for 10 min. Coverslips were mounted on glass microscope slides using VectaShield and sealed with nail polish.

Combined Immunofluorescence FISH

For Combined Immunofluorescence DNA FISH, cells were rinsed with PBS and treated with cytobuffer (100 mM NaCl, 300 mM sucrose, 3mM $MgCl_2$, 10 mM PIPES pH 7, 0.1% Triton-X 100) for 7 min at 4°C. Cells were then rinsed with PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. Cells were then permeabilized in 0.5% NP40/PBS for 10 min and blocked in PBG (0.5% BSA, 0.2% fish gelatin, PBS) for 1 hr at room temperature. Cells were then incubated with the indicated antibodies diluted in PBG and incubated overnight at

4°C. Following incubation with primary antibody, the cells were washed three times with PBST (PBS containing 0.1% Triton) for 10 min each and subsequently incubated with secondary antibody diluted in PBG for 45 min at room temperature. The cells were washed three times with PBST for 10 min each and then fixed in 4% paraformaldehyde for 10 min at room temperature. Fixation was followed by digestion with RNaseA 200 mg/ml in 2x SSC for 30 min at 37°C. Cells were then dehydrated in a series of ethanol washes 70%, 85%, 100% for 2 min each at room temperature, and the coverslips were dried at 37°C for 20 min. 10 nM PNA-TAMRA-(CCCTAA) probe in hybridization buffer (50% formamide, 2x SSC, 2mg/ml BSA, 10% dextran sulfate) was added to coverslips and DNA was denatured at 75°c for 3 min and then place in humidified chamber at 39°C overnight. The following day, the coverslips were washed in 2x SSC +50% formamide three times at 39°C for 5 min each, three times in 2xSSC at 39°C for 5 min each, and finally one time in 2x SSC at room temperature for 10 min. The coverslips were mounted on glass microscope slides with Vectashield mounting medium containing DAPI and analyzed using a Nikon H600L fluorescence microscope.

Combined Immunofluorescence RNA FISH experiments were performed exactly as above except for the following modifications. The initial incubations in cytobuffer included 10 mM Vanadyl Ribonucleoside Complex. The RNaseA digestion step was omitted prior to dehydration. The denaturation step was omitted during probe hybridization.

Telomere sister chromatid exchange assay

Cells were incubated with a 3:1 ratio of bromodeoxyuridine to bromodeoxycytidine (BrdU/BrdC, 10 mM/3.3 mM) for 16 h before nocodazole was added and the cells were incubated for an additional 45 min. Cells were collected by trypsinization, incubated in 75mM KCL at 37°C for 20 min, and then fixed in ice cold 3:1 methanol/acetic acid. Cells were then centrifuged, supernatant aspirated, and resuspended in fresh fixative. This was repeated

twice before fixed cells were dropped onto glass slides. Slides were treated with 0.5 mg/ml 1 RNaseA in 2x SSC at 37°C for 10 min, incubated in 2x SSC containing 10 mg/ml Hoescht 33258 for 15 min, and then exposed to 365-nm UV light (Stratalinker 1800) for 30 min. Slides were then incubated in 10 U/ml ExoIII for 10 min at 37°C to degrade nicked DNA. Slides were dehydrated in an ethanol series [70% (v/v), 85% (v/v) and 100% ethanol] and allowed to air dry overnight. Slides were then incubated with a fluorescein isothiocyanate (FITC)-labeled G-rich telomeric peptide–nucleic acid (PNA) probe PNA-FITC-(GGGATT) in hybridization buffer (50% formamide, 2x SSC, 2 mg/ml BSA, 10% dextran sulfate) for 1 hr at room temperature and then washed in PBS containing 0.02% (v/v) Tween-20 for 10 min at room temperature. Subsequently, slides were incubated in PNA-TAMRA-(CCCTAA) probe for 1 hr at room temperature and washed in PBS containing 0.02% (v/v) Tween-20 for 20 min at 57°C. Finally, slides were incubated with 1 mg/ml DAPI in 2xSSC containing 0.02% (v/v) Tween-20 for 5 min and sealed with coverslips. Telomere sister-chromatid exchange events were imaged using a Zeiss LSM710 Confocal microscope and analyzed using Fiji software.

C-circle assay

C-circle assays were performed as previously described[2]. Briefly, genomic DNA was purified using the Qiagen DNA Blood Mini Kit according the manufacturer's instructions. Purified DNA, was digested with AluI and MboI restriction enzymes at 37°C overnight. The digested DNA was again purified over a Qiagen PCR clean-up column and the DNA was quantified using a Nanodrop spectrophotometer. The DNA (40 ng) was diluted in 10 μl 1× Φ29 Buffer (NEB) containing BSA (NEB; 0.2 mg/ml), 0.1% Tween, 0.2 mM each dATP, dGTP, dTTP, and incubated in the presence or absence of 7.5 U ΦDNA polymerase (NEB) at 30°C for 8 hr, followed by 65°C for 20 min. C-circle amplification products were detected by dot blot using a DIG-labeled probe $(CCCTAA)_4$.

Live-cell imaging

HeLa and RPE-1 cells stably expressing H2B-mRFP, and U2OS cells stably expressing both H2B-mRFP and 53BP1-GFP, were grown on glass-bottom 12-well tissue culture dishes (Mattek) in phenol red-free DMEM:F12 medium (10% FBS, 100 IU/ml penicillin, and 100 mg/ml streptomycin). Cells were treated with 5 μM VE or an equivalent volume of DMSO and imaged on a Nikon Ti-E inverted microscope enclosed within a temperature and CO2-controlled environmental chamber that maintained an atmosphere of 37°C and 5% humidified CO2. Fluorescent images were captured every 15 min for 36 hr with a 20X (0.75 NA) objective and 2 X 2 binning to minimize light exposure. At least 4 separate fields of view were acquired for each condition. Images were subsequently analyzed using NIS-Elements software. Cells were scored as having undergone a "normal" mitosis if no micronuclei were generated following the first anaphase; "slightly abnormal" if 1-3 micronuclei were generated; or "highly abnormal" if 4 or more micronuclei were generated. Only the first mitosis following drug addition was scored.

Quantitative Reverse Transcriptase PCR

HeLa cells were reverse transfected using Lipofectamine RNAiMax and incubated for 48 hr. Following this incubation, either 2 mM thymidine or 0.1 mg/ml nocodazole was added to cells and incubated for an additional 18 hr. The cells were collected and RNA was extracted using the RNeasy Mini kit. Following quantification, 1 mg of total RNA was reverse transcribed using the oTEL primer and SuperScript III Reverse Transcriptase for 1 hr at 55°C, followed by a 70°C incubation for 15 min. cDNA was amplified using the SYBR green master mix with the indicated primers and analyzed using the Roche Light Cycler 480 with the following PCR conditions, 95°C 10 min, (98°C 15 sec, 60°C 20 sec, 72°C 1 min) x 39, 72°C 5 min. Primer sequences for oTEL, 15q, and Xp/Yp are as follows,

oTEL 5' $(CCCTAA)_5$ 3'

15q Forward 5'-CAGCGAGATTCTCCCAAGCTAAG-3'

15q Reverse 5'-AACCCTAACCACATGAGCAACG-3'

Xp/Yp Forward 5' GCAAAGAGTGAAAGAACGAAGCTT-3'

Xp/Yp Reverse 5'-CCCTCTGAAAGTGGACCAATCA-3'

Telomere-repeat amplification protocol

TRAP assays were performed using the TRAPeze telomerase detection kit (Millipore) according to the manufacturers recommendations. For TRAP assay on HeLa cells, HeLa cells were reverse transfected with siRNA against ATRX using Lipofectamine RNAiMax and incubated for 72 hr. HeLa, osteosarcoma, and glioblastoma cell lines were collected by trypsinization and counted to obtain 1 x 10^6 cells. Cells were resuspended 1x CHAPS Lysis buffer and incubated on ice for 30 min. Lysates were centrifuged at 12,000x g for 20 min at 4^oC and protein concentration was determined using Bradford reagent. Approximately, 150 ng of total protein was used in each reaction and PCR amplification reactions were performed as recommended. DNA products were separated by 10% PAGE in 0.5x TBE run at 200 V for 2 hr and visualized using SYBR gold.

Cellular viability

For cell viability assays, between 250-1,500 cells were seeded per well, in triplicate, of a 96-well plate and incubated overnight. The following day cells were either left untreated, or treated with increasing concentrations of VE-821, KU-55933, AZ20, or Gemcitabine. The cells were incubated for 4-6 days and cell viability was analyzed using CellTiter Glo and a SpectraMax M5 plate reader. IC50s were calculated using Excel or Prism software.

For analysis of cell death, cells were seeded in a 6-well dish at 0.5 x 10^5 and allowed to incubate for 8 hr. Cells were either left untreated, or treated with 3 mM VE-821 and incubated for 6 days. Cells were collected by trypsin and stained for FACS analysis using

the Annexin V assay kit (Life Technologies) per the manufacturers recommendations. Cell death was analyzed using FACS Diva Software.

For analysis of population doubling, U2OS cells were seeded at 0.6 x 10^5 and RPE were seeded 0.3 x 10^5 every 3-4 days in growth media with, or without, 1.5mM VE821. Cells were collected by trypsinization and counted using a hemacytometer for a total of 21 days. Population doubling was calculated using the standard formula PD= $\log(N_{final}/N_{initial})/\log(2)$.

DNA-protein binding assay using biotinylated ssDNA

Biotinylated ssTEL [$(TTAGGG)_8$] was attached to streptavidin-coated magnetic beads in 10 mM Tris-HCl (pH 8.0), 100 mM NaCl at room temperature for 30 min. The biotinylated ssTEL (0.4 pmol) was first incubated with purified RPA (1.2 pmol) for 30 min at room temperature. Recombinant RPA complex was purified from *E. coli* as previously described[1]. The RPA pre-coated ssTEL was retrieved with a magnet and subsequently mixed with increasing concentrations of whole cell extracts (WCE) for 30 min at room temperature. The RPA coated ssTEL was again retrieved using a magnet and the amount of RPA still bound to the ssTEL was analyzed by Western blot with the indicated antibody. To generate WCE, cells were lysed in binding buffer [10mM Tris-HCl (pH 7.5), 600 mM NaCl, 10 mg/ml BSA, 10% glycerol, 0.05% NP-40] and sonicated for 10 sec at a power of 3, 3 times. Cell lysates were normalized with a binding buffer containing no salt and then added to the RPA-coated ssTEL reactions. For binding assays on HeLa cells, HeLa cells were either mock treated or reverse transfected with siRNA against ATRX (Lipofectamine RNAiMax) and incubated for 48 hr. Cells were then left untreated or incubated with 2 mM thymidine or 0.1 mg/ml nocodazole for 16-18 hr and then collected with trypsin prior to lysis.

Dot blot

RNA was purified using the RNAeasy purification kit and 2-10 μg was denatured in 50% formamide, 2.5 mM EDTA for 15 min at 65°C. The denatured RNA was loaded onto Hybond XL membrane using a BioRad dot blot vacuum manifold. The membrane was crosslinked for 35 sec at 125 J and washed briefly in 2x SSC. The membrane was then incubated in Ultra-Hyb hybridization buffer (Ambion) for 1 hr at 50°C. Telomeric $(CCCTAA)_4$ or 28S (AACGATCAGAGTTTTCACC) probes were labeled using the DIG oligonucleotide 3'-End labeling Kit, 2nd Generation (Roche), according to the manufacturers instructions. DIG-labeled probe (10 pmole) was added to the Ultra-Hyb hybridization buffer and incubated with the membrane overnight at 50°C. The following day, the membrane was washed twice with 2x SSC + 0.1% SDS at room temperature for 5 min each and twice with 0.5x SSC + 0.1% DS at 55 ^{o}C for 15 min. The membrane was then developed using the DIG CDP-STAR detection system (Roche) according to the manufacturer's instructions. When necessary, membranes were stripped by boiling in 0.1% SDS for 15 min at room temperature and reprobed. Fold change in TERRA was calculated after normalization to 28S using ImageLab software.

References

34. Henricksen, L. A. & Wold, M. S. Replication protein A mutants lacking phosphorylation sites for p34cdc2 kinase support DNA replication. J Biol Chem 269, 24203-8 (1994).
35. Henson, J. D., Cao, Y., Huschtscha, L. I., Chang, A. C., Au, A. Y. M., Pickett, H. a, & Reddel, R. R. (2009). DNA C-circles are specific and quantifiable markers of alternative-lengthening-of-telomeres activity. Nature Biotechnology, 27(12), 1181–5.

Fig. S1

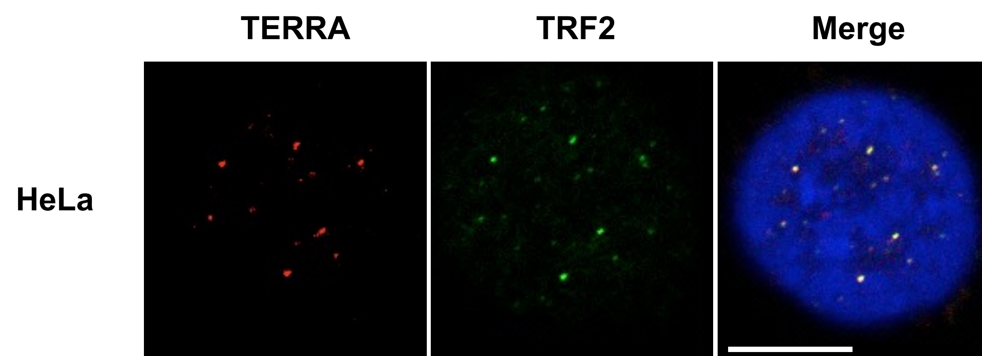

Figure S1. TERRA colocalizes with TRF2 in telomerase-positive HeLa cells. TERRA foci were analyzed in HeLa cells by combined RNA FISH and immunofluorescence using telomere probe $(CCCTAA)_4$ and TRF2 antibodies. Scale bar: 10 μm.

Fig. S2

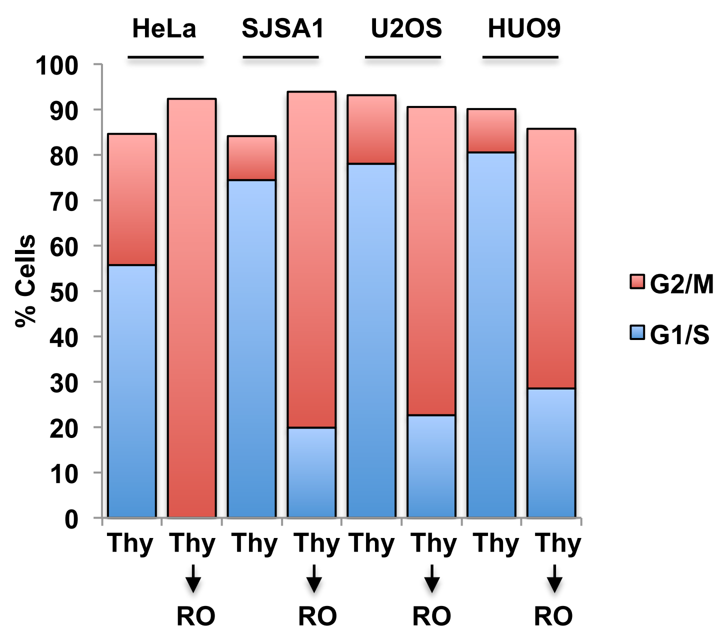

Figure S2. Synchronization of cells in S phase and G2. HeLa, SJSA1, U2OS, and HUO9 cells were treated with thymidine alone for 18 hrs, or treated with thymidine for 18 hrs and then released into 7 μM of the CDK inhibitor RO3306 for 18 hrs. The percentage of cells in G1/S and G2/M was analyzed by FACS and quantified using FlowJo software. These synchronized cell populations were analyzed in Fig. 1A-B.

Fig. S3

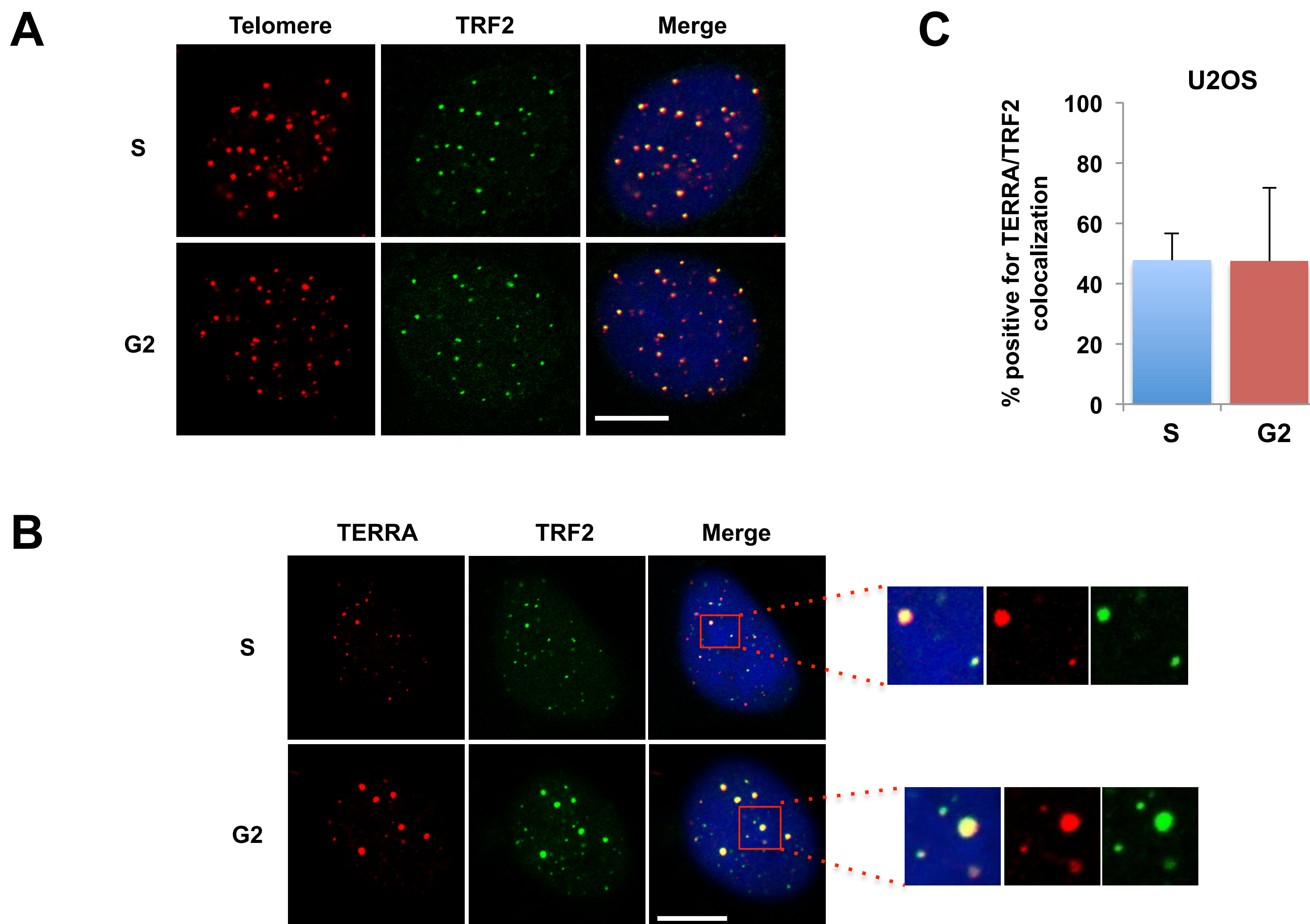

Figure S3. TERRA persistently associates with telomeres in ALT$^+$ cells. (A) U2OS cells synchronized in S and G2 were analyzed using telomere DNA FISH and TRF2 immunofluorescence. Scale bar: 10 μm. (B) U2OS cells synchronized in S and G2 were analyzed using TERRA RNA FISH and TRF2 immunofluorescence. Scale bar: 10 μm. (C) The percentage of cells positive for TERRA/TRF2 colocalization was graphed as the mean of experiments performed in triplicate, with error bars representing one standard deviation.

Fig. S4

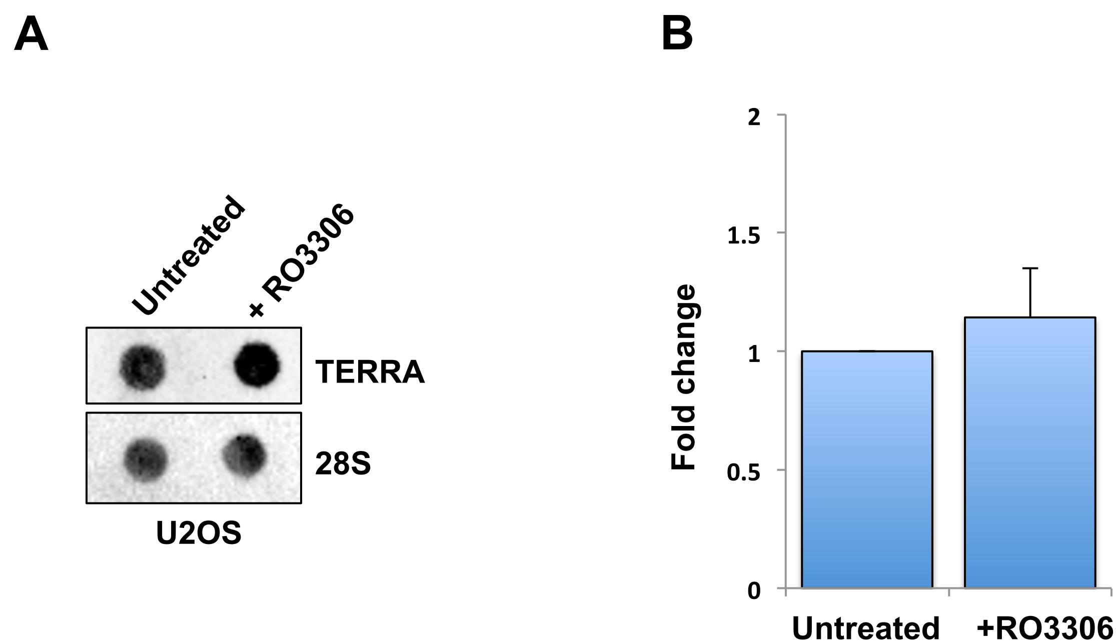

Figure S4. TERRA levels in U2OS cells do not decline in G2. U2OS cells were arrested in G2 with the CDK1 inhibitor RO3306. (A) The levels of TERRA were analyzed by dot blot. The levels of input RNA were normalized to 28S RNA. (B) The relative levels of TERRA were quantified in untreated and RO3306-treated cells. Error bar: standard deviation (n=2).

Fig. S5

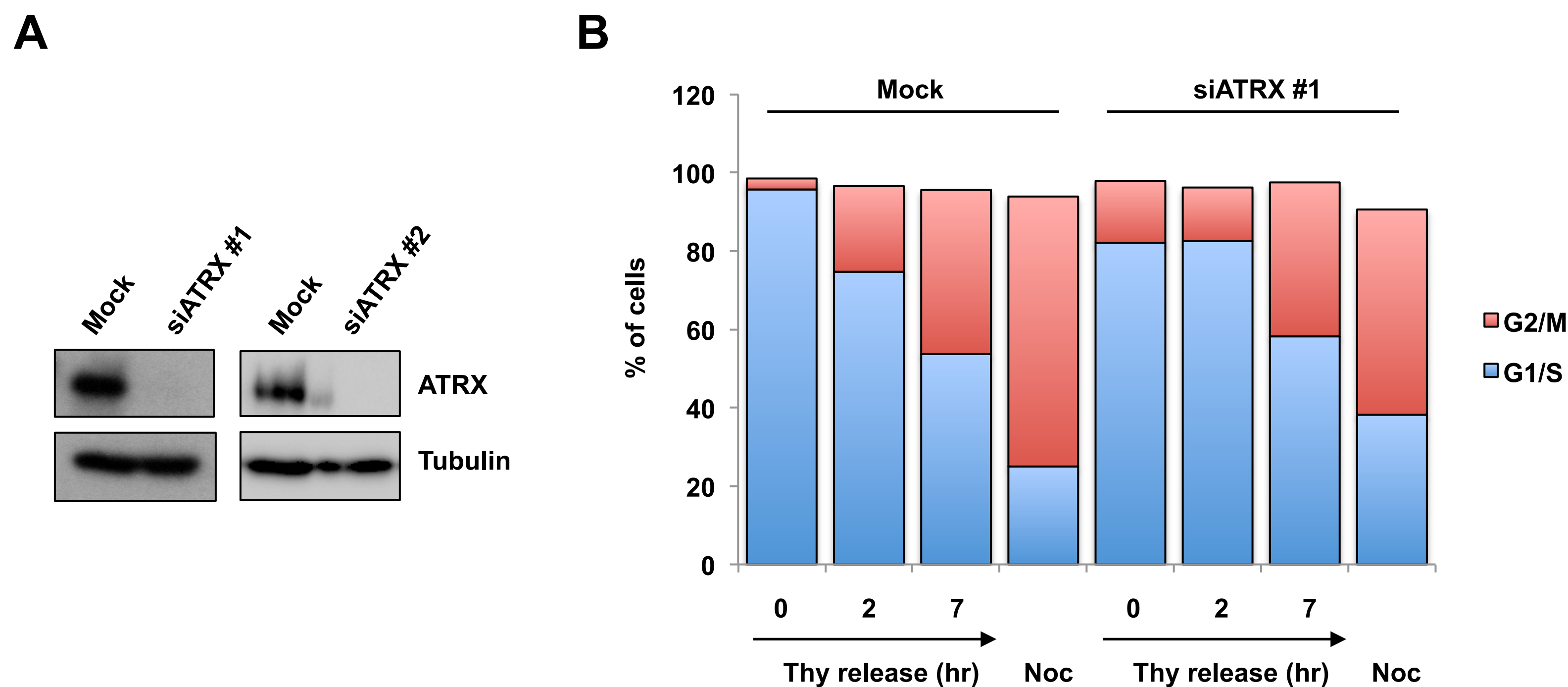

Figure S5. Cell-cycle characterizations of ATRX knockdown cells. (A) HeLa cells were either mock treated or treated with two independent ATRX siRNAs. Three days after transfection, ATRX protein levels were analyzed by Western blot with tubulin as a loading control. (B) HeLa cells were either mock treated or treated with ATRX siRNA #1. Two days after transfection, cells were incubated with thymidine (Thy) or nocodazole (Noc) for 16-18 hrs. Cells in thymidine were released and collected at the indicated time points. Cell cycle analysis was performed using FACS Diva software.

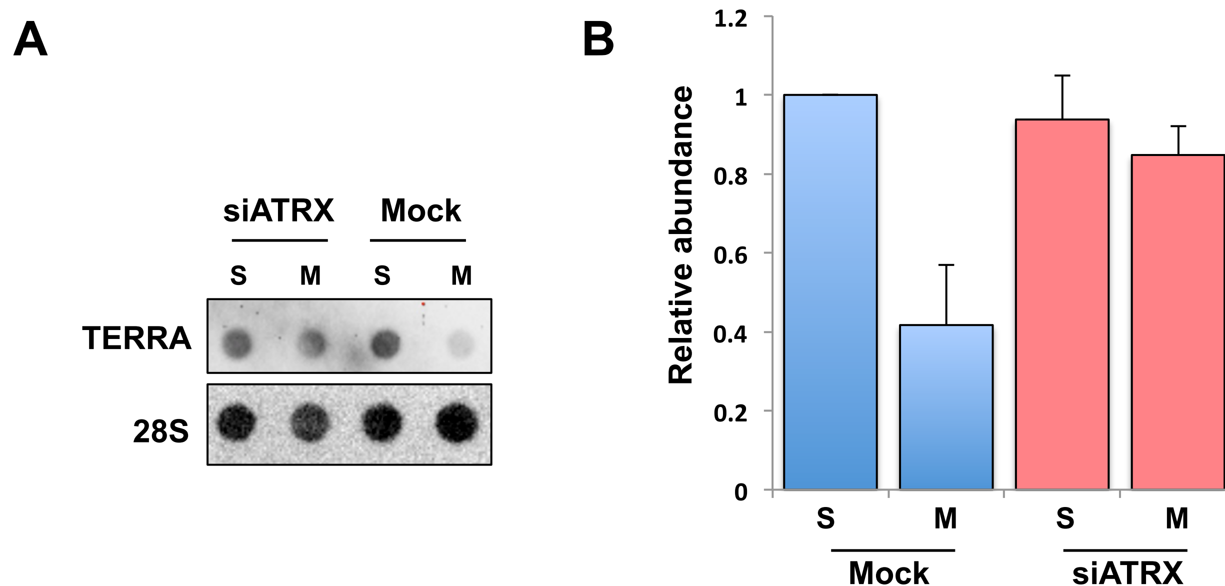

Figure S6. ATRX is a cell cycle-regulated repressor of TERRA . (A) HeLa cells were either mock transfected or transfected with siATRX #1. Two days after transfection, cells were treated with either thymidine or nocodazole for an additional 18 hrs. The cells were collected and RNA was extracted using the RNeasy RNA purification kit according to the manufacture's instructions. Approximately 15 μg of total RNA was analyzed by dot blot using DIG-labeled TERRA and 28S RNA probes. **(B)** Dots detected by probes were quantified using ImageLab software and normalized to 28S. Error bars represent the standard deviation from experiments preformed in duplicate (n=2).

Fig. S7

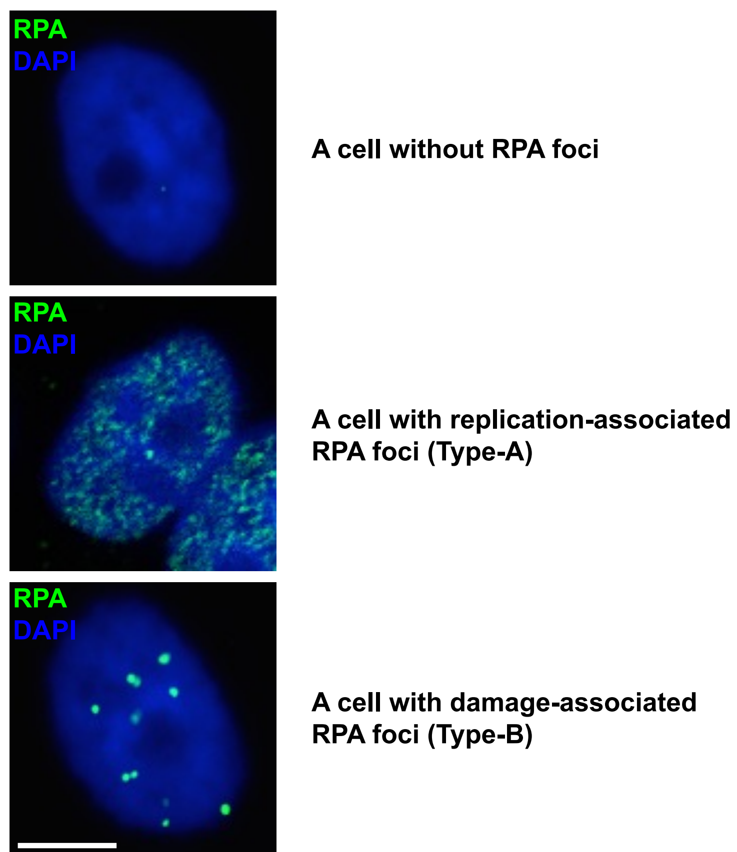

Figure S7. Cells with type-A and type-B RPA foci. Representative images of cells with no RPA foci, type-A RPA foci, or type-B RPA foci are shown. Scale bar: 10 μm.

Fig. S8

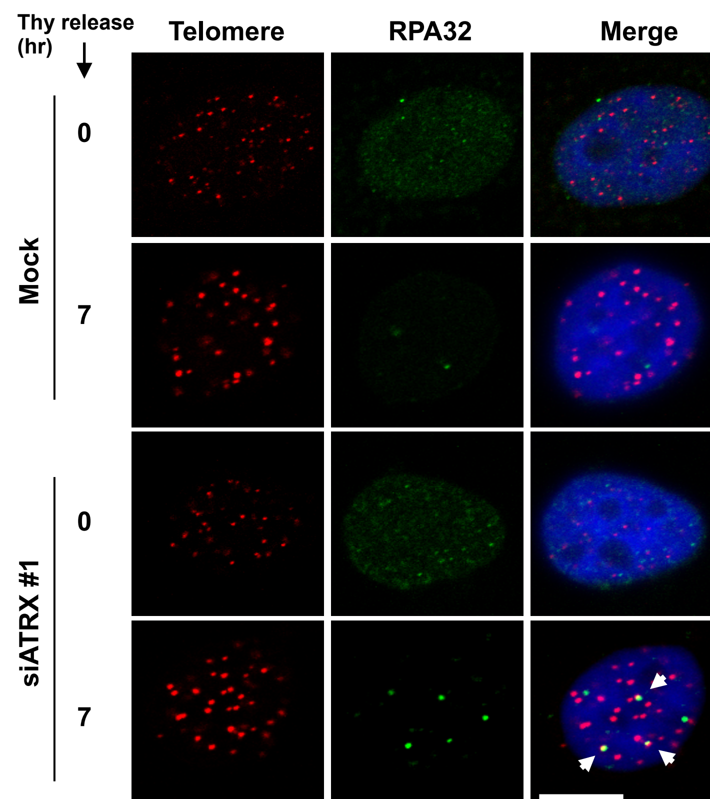

Figure S8. Loss of ATRX compromises RPA release from telomeres. (A) HeLa cells were mock treated or treated with ATRX siRNA #1 and cells were enriched in S and G2 phases with thymidine block and release. Following thymidine release cells were analyzed using a combined IF-DNA FISH approach with RPA32 antibody and the telomeric probe $(CCCTAA)_4$. Scale bar: 10 μm.

Fig. S9

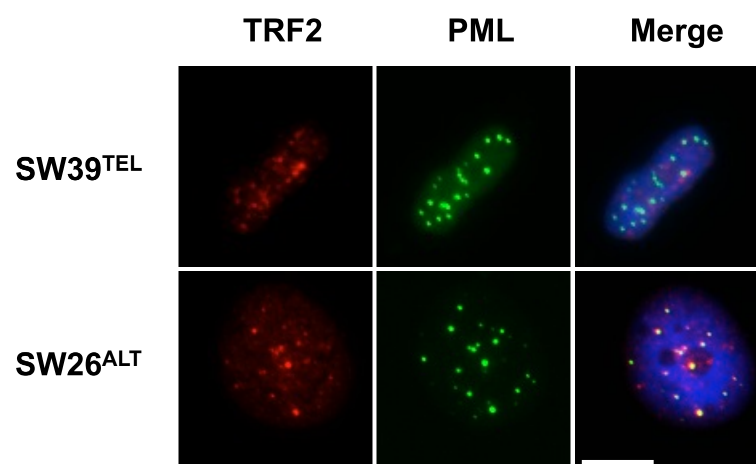

Figure S9. SW26^{ALT} cells but not SW39^{TEL} cells display ALT-associated PML bodies (APBs). SW26^{ALT} and SW39^{TEL} cells were immunostained for TRF2 and PML. APBs were detected by the colocalization of TRF2 and PML. Scale bar: 10 μm.

Fig. S10

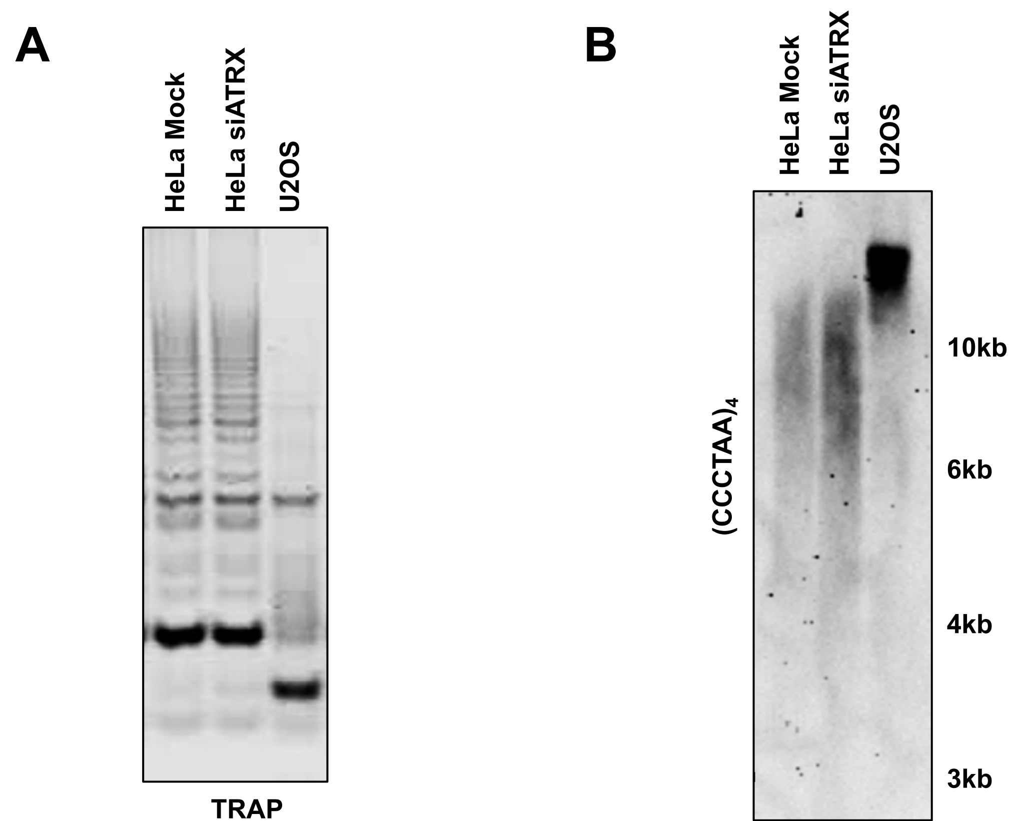

Figure S10. Loss of ATRX does not affect telomerase activity or induce telomere lengthening. HeLa cells were either mock transfected or transfected with siATRX #1 and incubated for 4 days. (A) Telomerase activity was analyzed by the TRAPeze assay kit and (B) telomere length was assessed by TRF analysis following isolation of genomic DNA. U2OS cells were used as an ALT-positive control.

Fig. S11

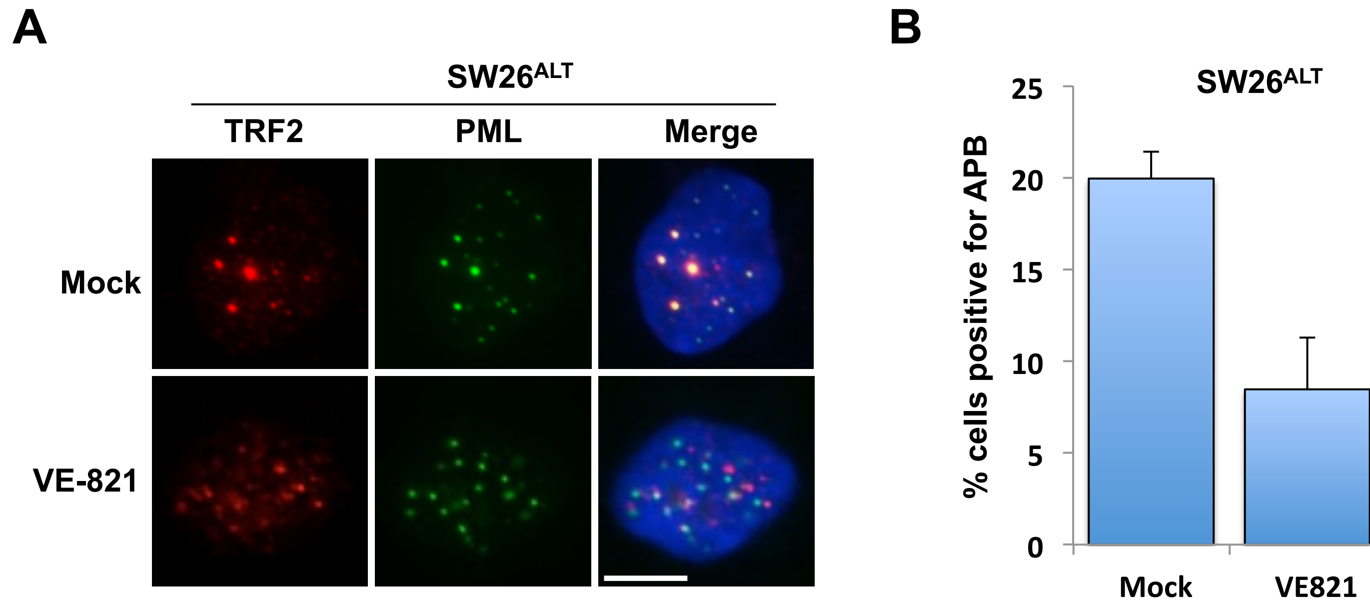

Figure S11. ATR inhibitor disrupts APBs in ALT cells. (A) SW26^{ALT} cells were either mock treated or treated with 5 μM VE-821 for 3 hrs and then immunostained for APB using TRF2 and PML antibodies. Scale bar: 10 μm. (B) The percentage of cells positive for APB was graphed as the mean with error bars representing the standard deviation (n=2).

Fig. S12

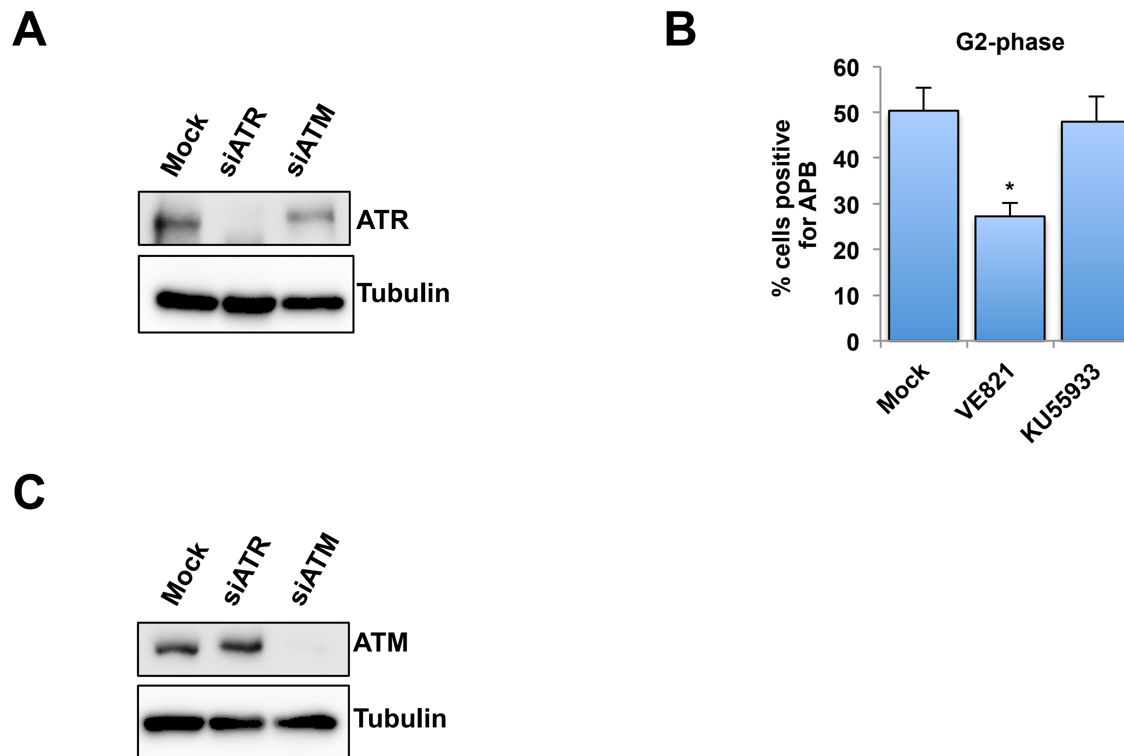

Figure S12. The effects of ATR and ATM ablation on APBs. (A, C) U2OS cells were transfected with ATR siRNA, ATM siRNA, or mock transfected. The knockdown of ATR and ATM was confirmed by Western blot. The ATR and ATM knockdown cells were analyzed in Figs. 3A-B. (B) U2OS were synchronized in G2 in methionine-free media before addition of VE-821 and KU-55933. Cells positive for APBs were graphed as the mean with error bars representing the standard deviation, experiment performed in triplicate (n=3).

Fig. S13

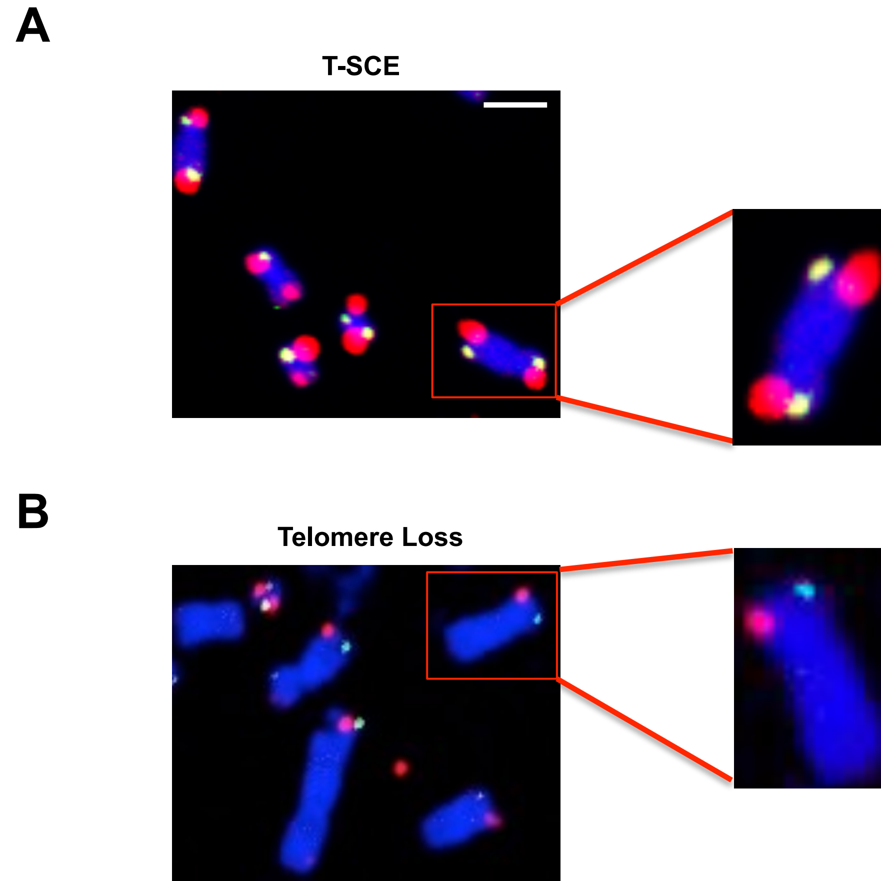

Figure S13. Analyses of ALT phenotypes in U2OS cells. (A) Representative chromatin spread from mock treated U2OS cells quantified in Fig. 3C is shown. A T-SCE event is depicted in red box and enlarged. (B) Representative chromatin spread from VE-821 treated U2OS cells quantified in Fig. 3F is shown. A telomere loss event is depicted in red box and enlarged. Scale bar: 5 μm.

Fig. S14

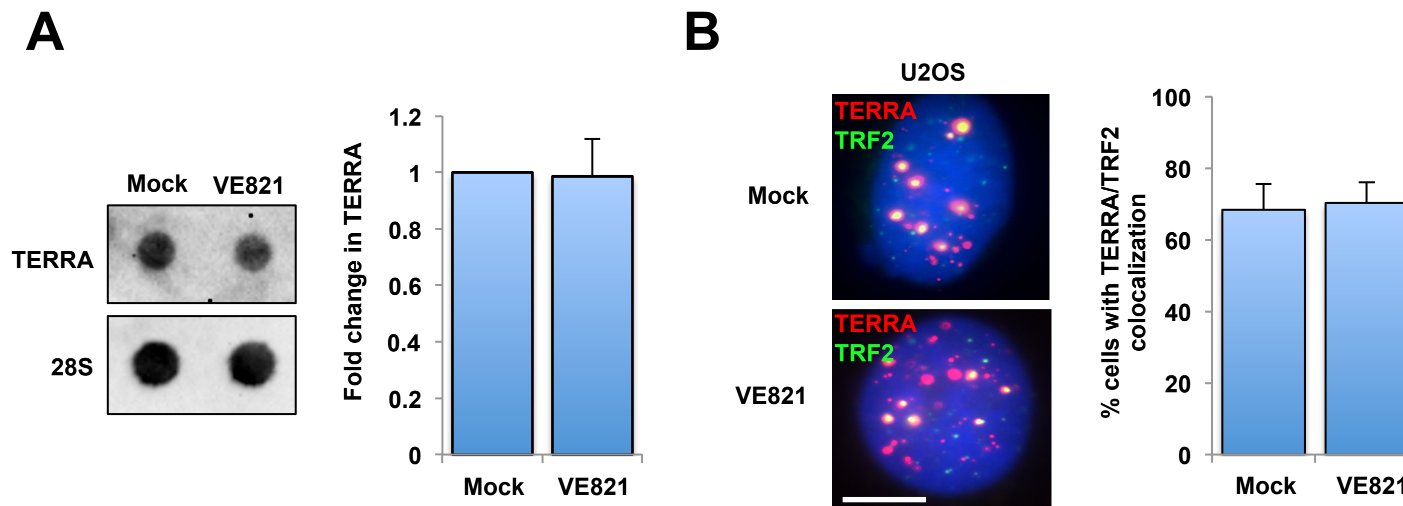

Figure S14. VE821 does not affect TERRA abundance or localization. (A) U2OS cells were treated with VE-821 for 24 hrs and TERRA was analyzed by dot blot using TERRA and 28S probes. Fold change in TERRA was analyzed using ImageLab software and normalized to 28S. Error bar: standard deviation (n=2). (B) TERRA localization was analyzed using combined IF-FISH using TERRA specific probes and TRF2 antibody. Positive cells had $\geq$5 TERRA foci that colocalized with TRF2 were quantified. Error bar: standard deviation (n=2). Scale bar: 10 μm.

Fig. S15

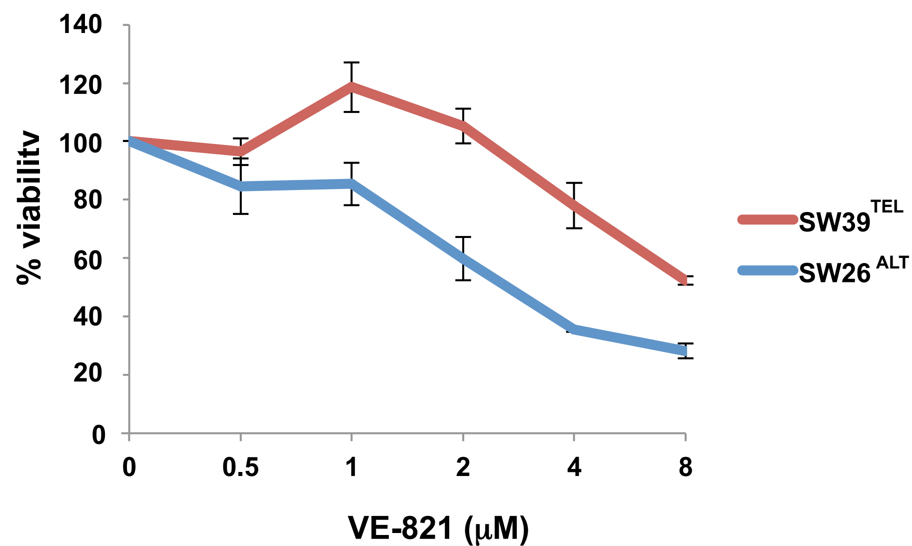

Figure S15. SW26^{ALT} cells are more sensitive to ATR inhibitor than SW39^{TEL} cells. SW26^{ALT} and SW39^{TEL} cells were treated with increasing concentrations of VE-821 for 6 days. Cell viability was analyzed using CellTiter Glo. Mean viability is representative of experiments preformed in triplicate with error bars representing the standard deviation (n=3).

Fig. S16

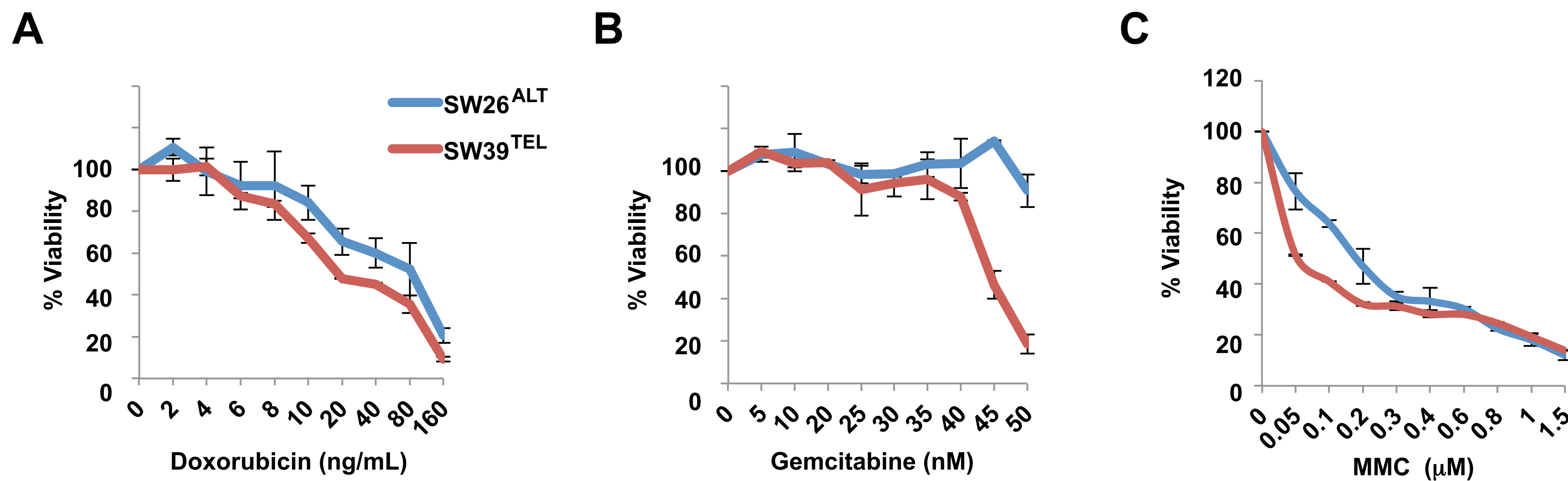

Figure S16. SW26^{ALT} and SW39^{TEL} cells are similarly sensitive to general DNA damage. SW26^{ALT} and SW39^{TEL} were treated with increasing concentrations of (A) doxorubicin, (B) gemcitabine, or (C) mitomycin C. All cells were treated for 4-6 days and cell viability was analyzed using Cell Titer Glo. Error bar: standard deviation (n=2).

Fig. S17

Figure S17. ATR inhibitor induces higher levels of genomic instability in SW26^{ALT} cells than in SW39^{TEL} cells. SW39^{TEL} and SW26^{ALT} cells were treated with 2.5 μM VE-821, collected at the indicated time points, and H2AX phosphorylation was analyzed by Western blot using the anti-γH2AX antibody

Fig. S18

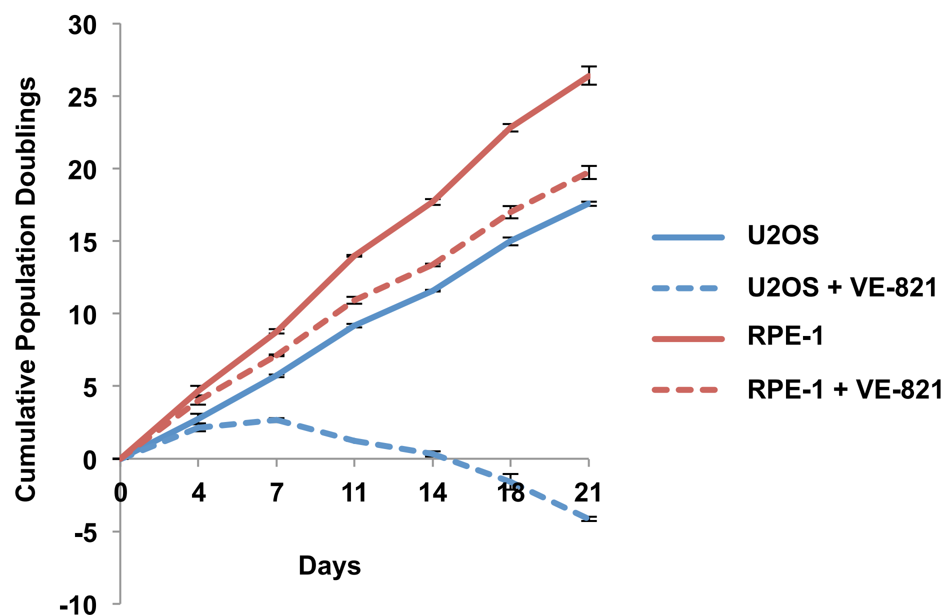

Figure S18. VE-821 has modest affects on untransformed REP-1 cells. RPE-1 and U2OS cells were either left untreated or treated continuously with 1.5 μM VE821 for 21 days. Population doublings were calculated using the standard formula (see Methods) and results were graphed as cumulative population doubling over time in days. Error bar: standard deviation (n=2).

Fig. S19

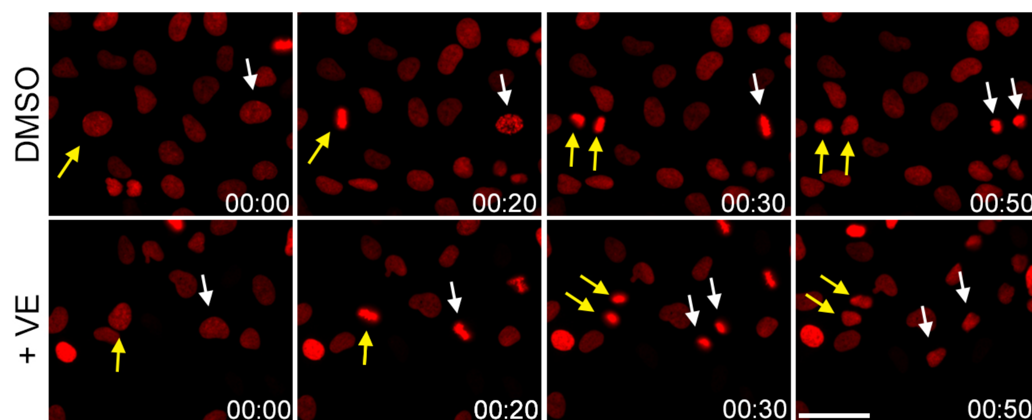

Figure S19. VE-821 does not impair anaphase chromosome segregation in RPE-1 cells. Stills from a time-lapse live-cell imaging experiment of RPE-1 cells stably expressing H2B-mRFP following treatment with either DMSO or 5 μM VE-821. Colored arrows mark individual cells as they progress through mitosis. Scale bar: 20 μm.

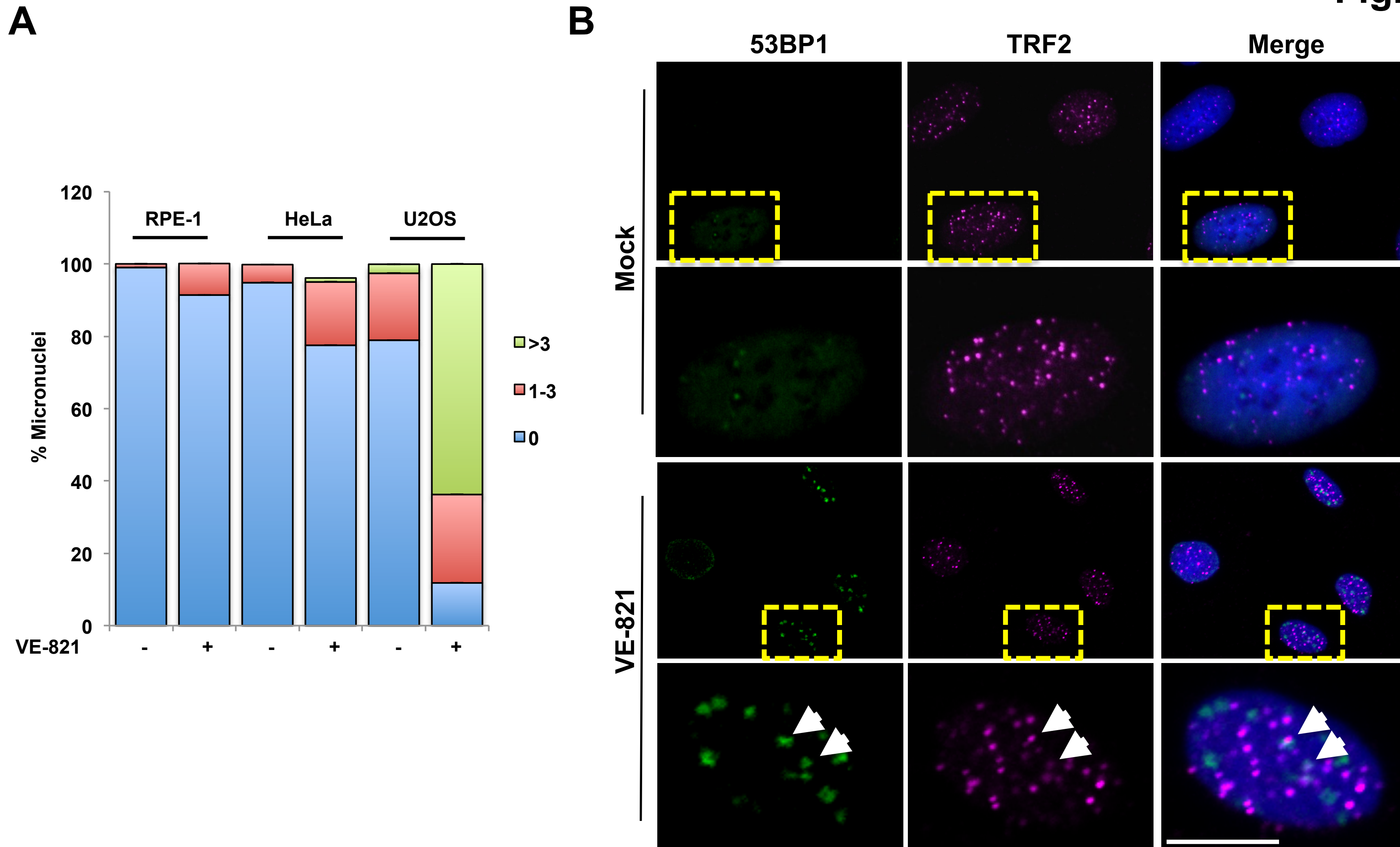

Figure S20. VE-821 induces micronucleation and 53BP foci in U2OS cells. (A) Cells expressing H2B-mRFP were either left untreated or treated with 5 μM VE-821 for 24 hr, and scored as having undergone a "normal" mitosis if no micronuclei were generated following the first anaphase; "slightly abnormal" if 1-3 micronuclei were generated; or "highly abnormal" if 4 or more micronuclei were generated. Only the first mitosis following drug addition was scored. (n=2) (B) U2OS cells were treated as in A and analyzed by IF using 53BP1 and TRF2 antibodies. Scale bar: 10 μm. The same enlarged VE-821-treated cell was shown in Fig. 4C.

Fig. S21

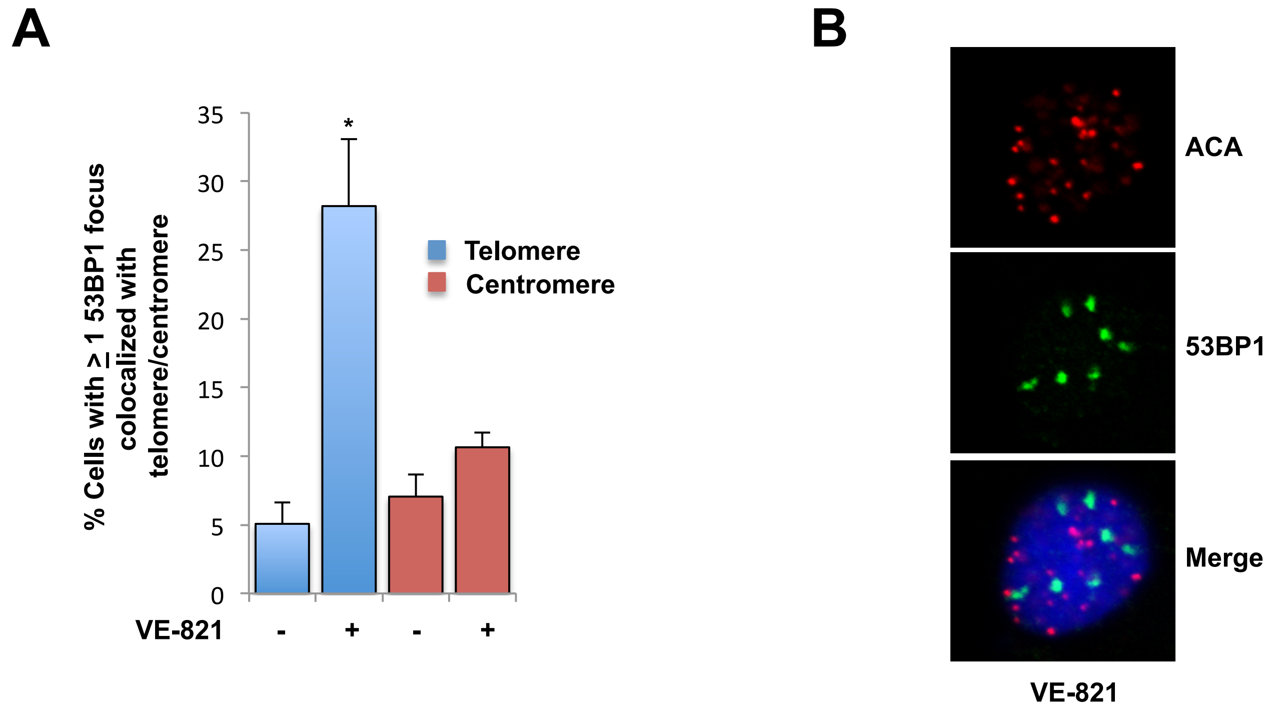

Figure S21. ATR inhibition promotes DNA damage at telomeres. U2OS cells were either mock treated, or treated with 5μM VE-821 for 24 hrs and analyzed using either IF-DNA FISH (53BP1 antibody and telomeric probe) or standard immunofluorescence using 53BP1 and ACA antibodies. The percentage of cells positive for Telomere/53BP1 or ACA/53BP1 colocalization was graphed as the mean of experiments performed in triplicate, with error bars representing one standard deviation. * P-Value = .001. (B) Representative image of U2OS cells treated with VE821 and analyzed by immunofluorescence using ACA and 53BP1 antibodies.

Fig. S22

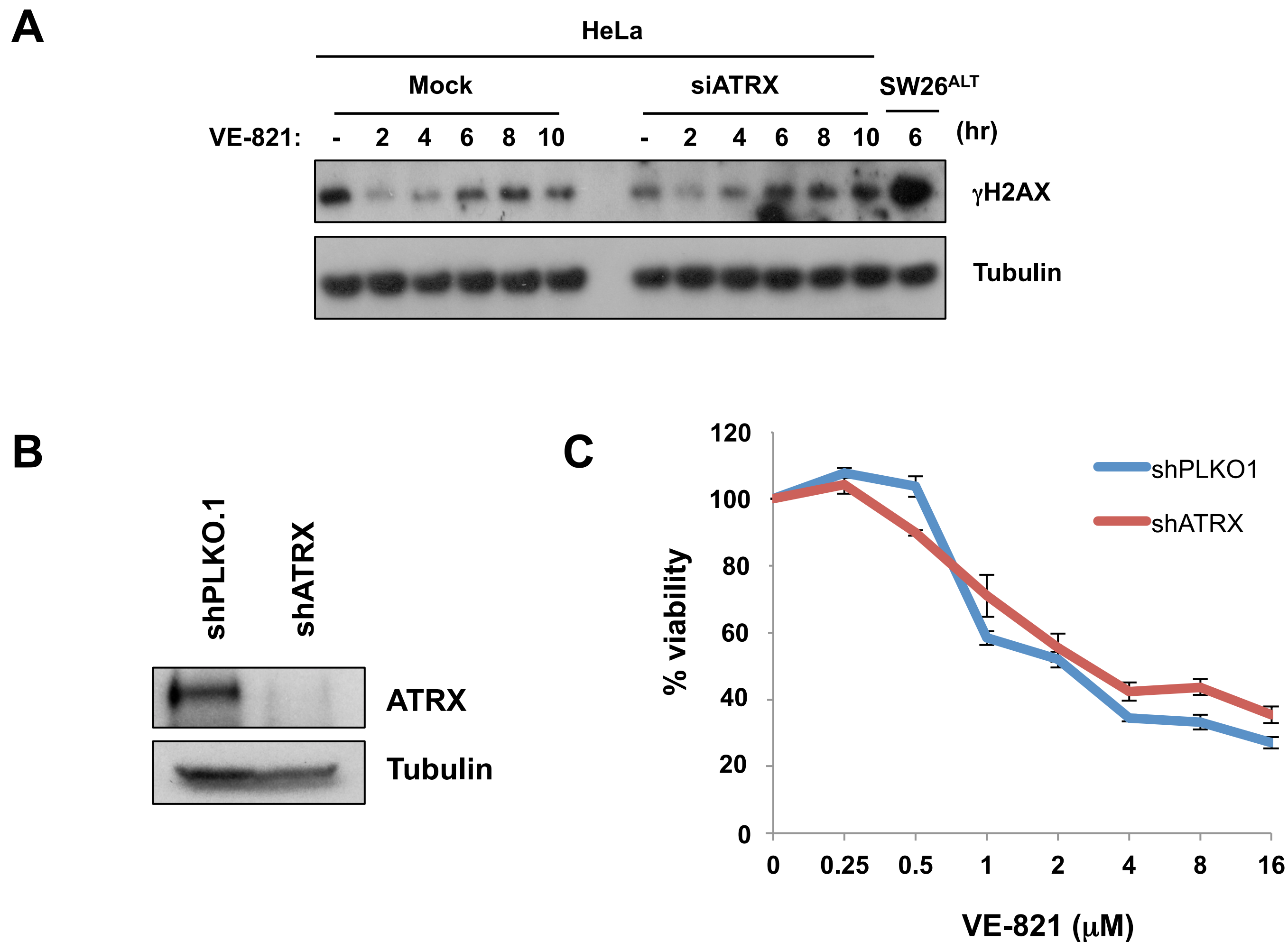

Figure S22. Knockdown of ATRX does not render HeLa and BJ cells hypersensitive to VE-821. (A) HeLa cells were either mock treated or treated with ATRX siRNA for 72 hrs and then incubated with 2.5 μM VE-821. Cells were collected at the indicated time points and analyzed by Western blot. $SW26^{ALT}$ cells treated with VE-821 for 6 hrs were included as an ALT-positive control. **(**B) BJ fibroblasts stably expressing control or ATRX shRNA were analyzed for ATRX levels by Western blot. (C) BJ cells stably expressing control or ATRX shRNA were treated with increasing concentrations of VE-821 for 6 days, and cell viability was analyzed by CellTiter Glo. Mean viability is representative of experiments preformed in triplicate with error bars representing the standard deviation (n=3).

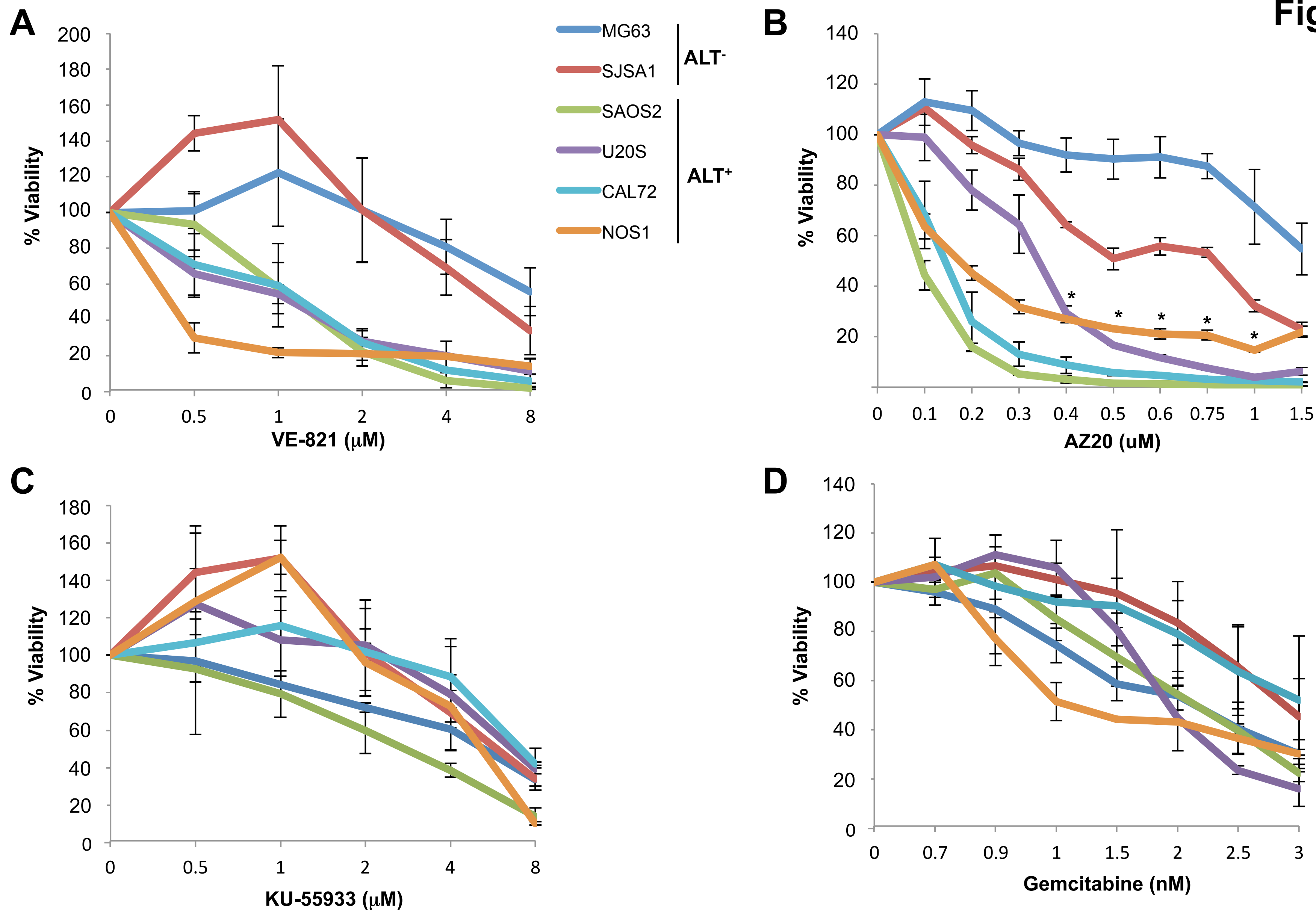

Figure S23. Selective killing of ALT cells by two different ATR inhibitors, but not ATM inhibitor or gemcitabine. The indicated panel of osteosarcoma cell lines were treated with increasing concentrations of (A) VE-821 (n=3), (B) the ATR inhibitor AZ20 (n=3), (C) the ATM inhibitor KU-55933 (n=3), or (D) Gemcitabine (n=2) for 4-6 days. Cell viability was analyzed using CellTiter Glo. Error bar: standard deviation. * P-Value < .01.

Fig. S24

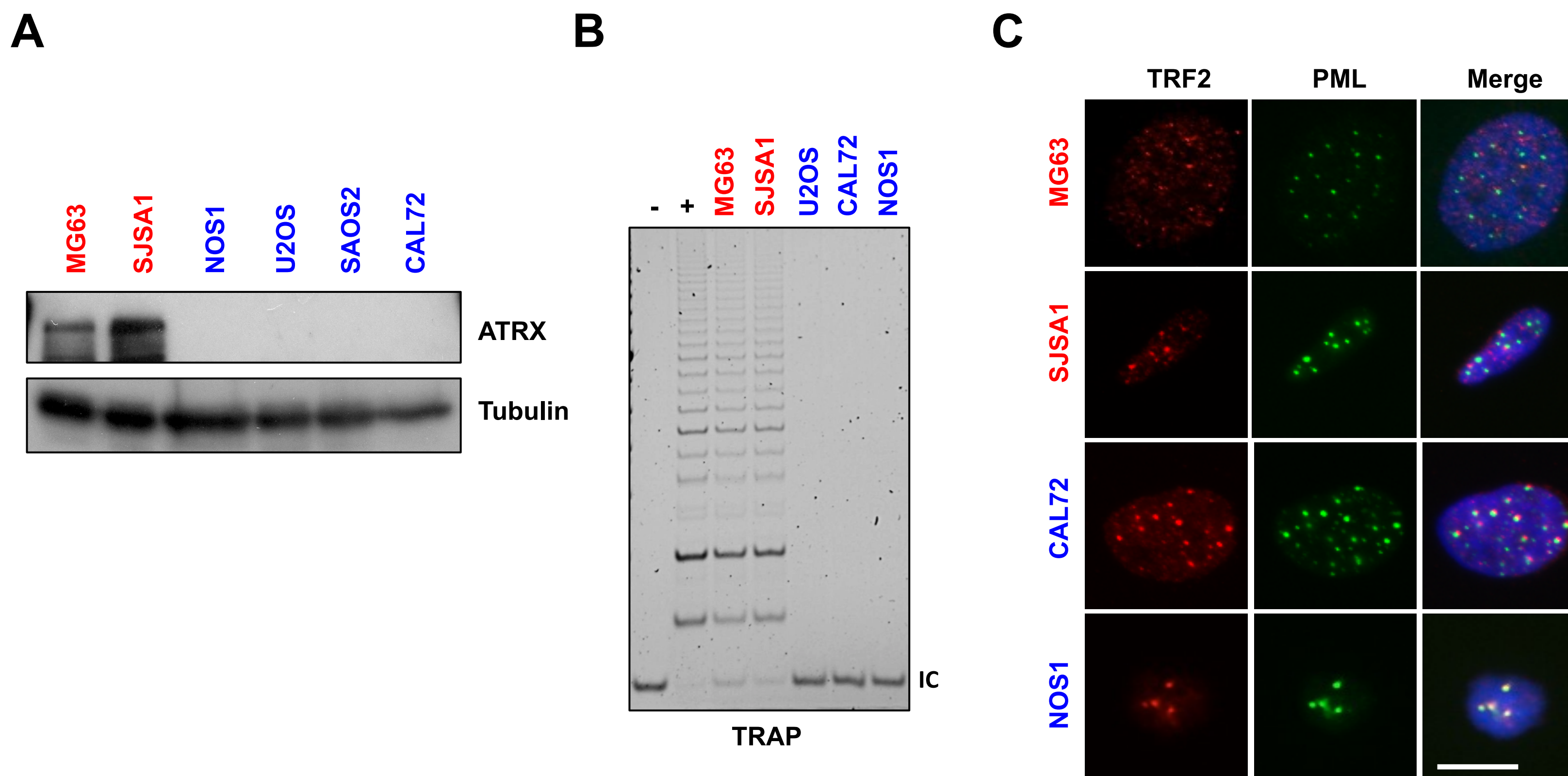

Figure S24. Characterization of the ALT status of osteosarcoma cell lines. (A) MG63, SJSA1, U2OS, CAL72, and NOS1 osteosarcoma lines were analyzed for ATRX protein. (B) The indicated cell lines were analyzed for telomerase activity using the TRAPeze assay kit. + refers to the positive control included in the kit and – refers to CHAPS lysis buffer alone, IC denotes internal control band. (C) The indicated cell lines were analyzed for APB formation by immunostaining for both TRF2 and PML proteins. Scale bar: 10 μm.

Fig. S25

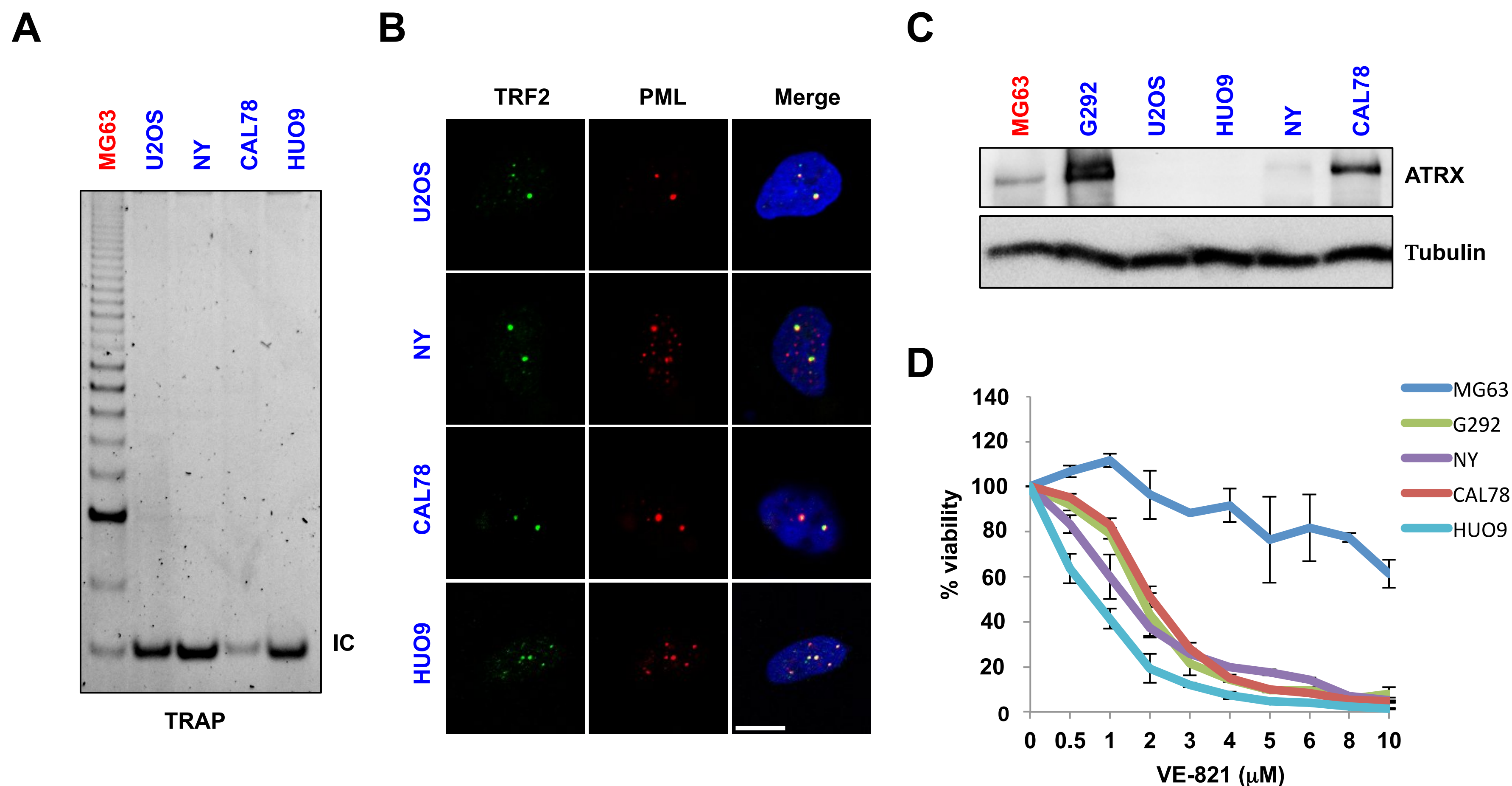

Figure S25. ALT-positive osteosarcoma cell lines are hypersensitive to ATR inhibition. (A) Telomerase activity was analyzed in MG63, U2OS, NY, CAL78, and HUO9 osteosarcoma cell lines using the TRAPeze assay kit. (B) APBs were analyzed in U2OS, NY, CAL78, and HUO9 cell lines using immunofluorescence staining with TRF2 and PML antibodies. Scale bar: 10 μm. (C) ATRX protein was analyzed by Western blot in the indicated cell lines. (D) The indicated osteosarcoma cell lines were treated with increasing concentrations of VE-821 and cell viability was analyzed using Cell Titer Glo 4-6 days after treatment. Error bar: standard deviation (n=3).

Fig. S26

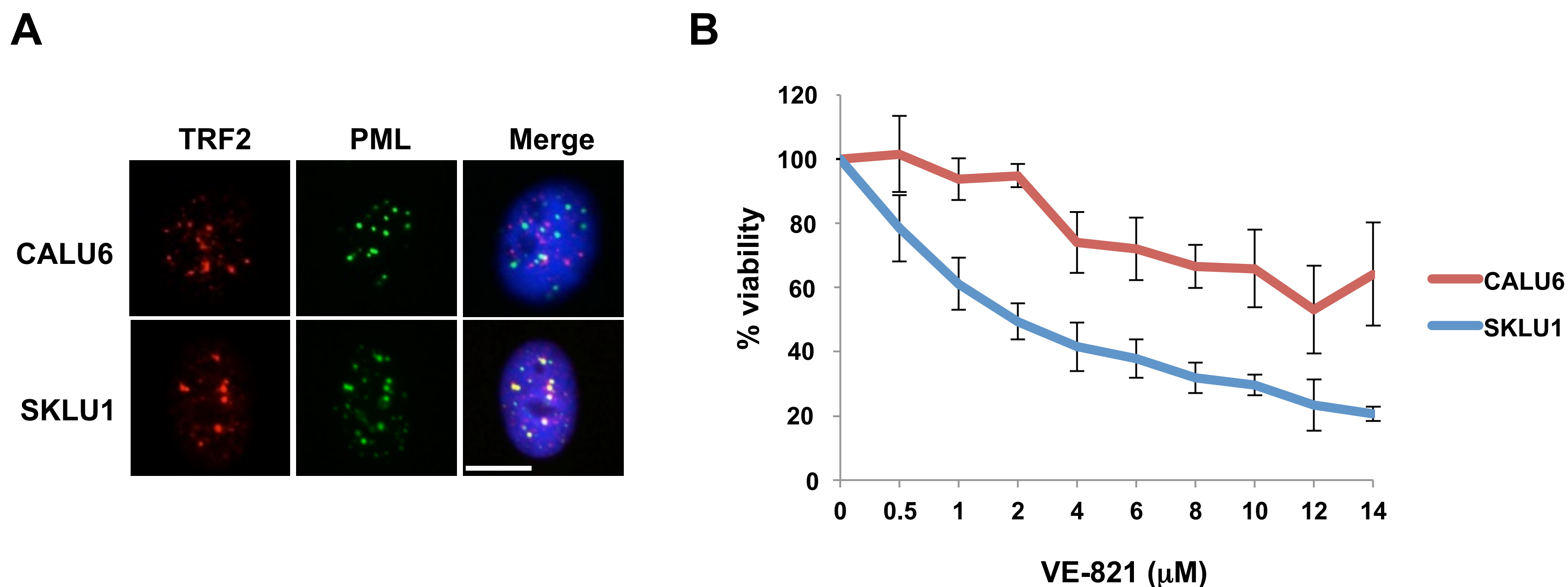

Figure S26. ATRX-positive ALT lung cancer cells are hypersensitive to ATR inhibition. (A) The CALU6 and SKLU1 lung cancer cell lines were analyzed for APBs by immunofluorescence with TRF2 and PML antibodies. Scale bar: 10 μm. (B) Cells were treated with increasing concentrations of VE-821 for 5 days. Cell viability was analyzed by CellTiter Glo. Mean viability is derived from experiments preformed in triplicate with error bars representing the standard deviation (n=3).

Fig. S27

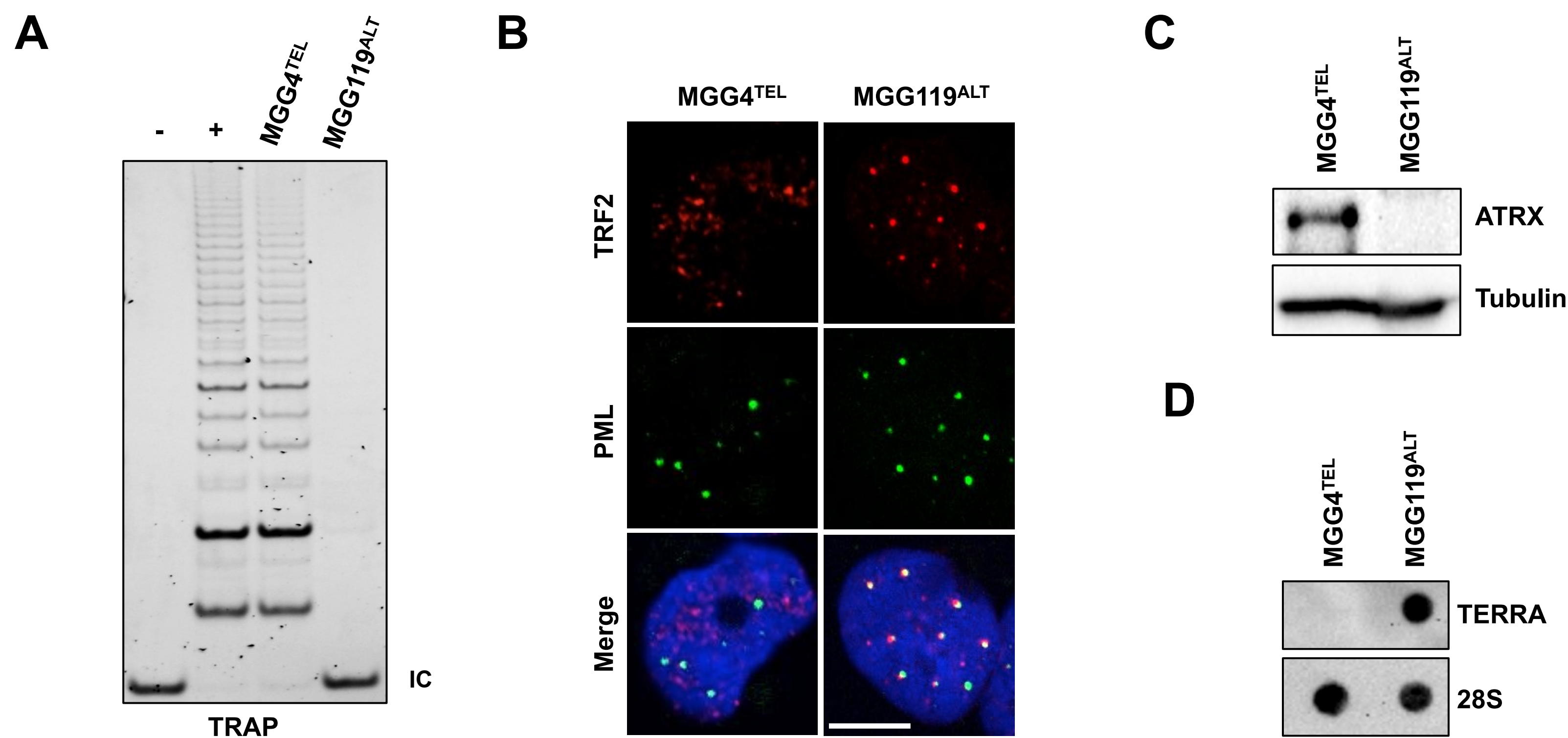

Figure S27. Characterization of the ALT status of GSC lines. (A) $MGG4^{TEL}$ and $MGG119^{ALT}$ GSC lines were analyzed for telomerase activity using the TRAPeze assay kit. + refers to the positive control included in the kit and – refers to CHAPS lysis buffer alone, IC denotes internal control band. (B) APBs were analyzed by immunostaining with TRF2 and PML proteins, Scale bar: 10 μm. (C) ATRX protein was analyzed by Western blot. (D) TERRA levels were analyzed by dot blot with the indicated probes.

Fig. S28

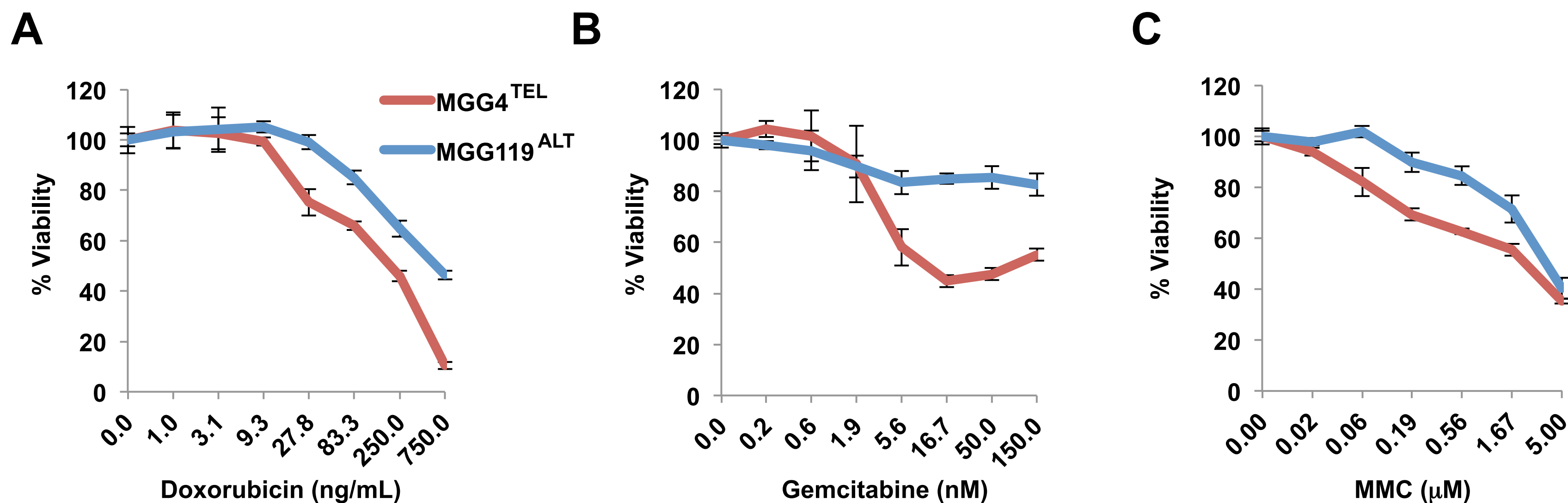

Figure S28. MGG118^{ALT} and MGG4^{TEL} are similarly sensitive to general DNA damage. MGG119^{ALT} and MGG4^{TEL} cells were treated with increasing concentrations of (A) doxorubicin, (B) gemcitabine, or (C) mitomycin C. All cells were treated for 4-6 days and cell viability was analyzed using Cell Titer Glo. Mean viability is representative of experiments preformed in triplicate with error bars representing the standard deviation (n=3).

Fig. S29

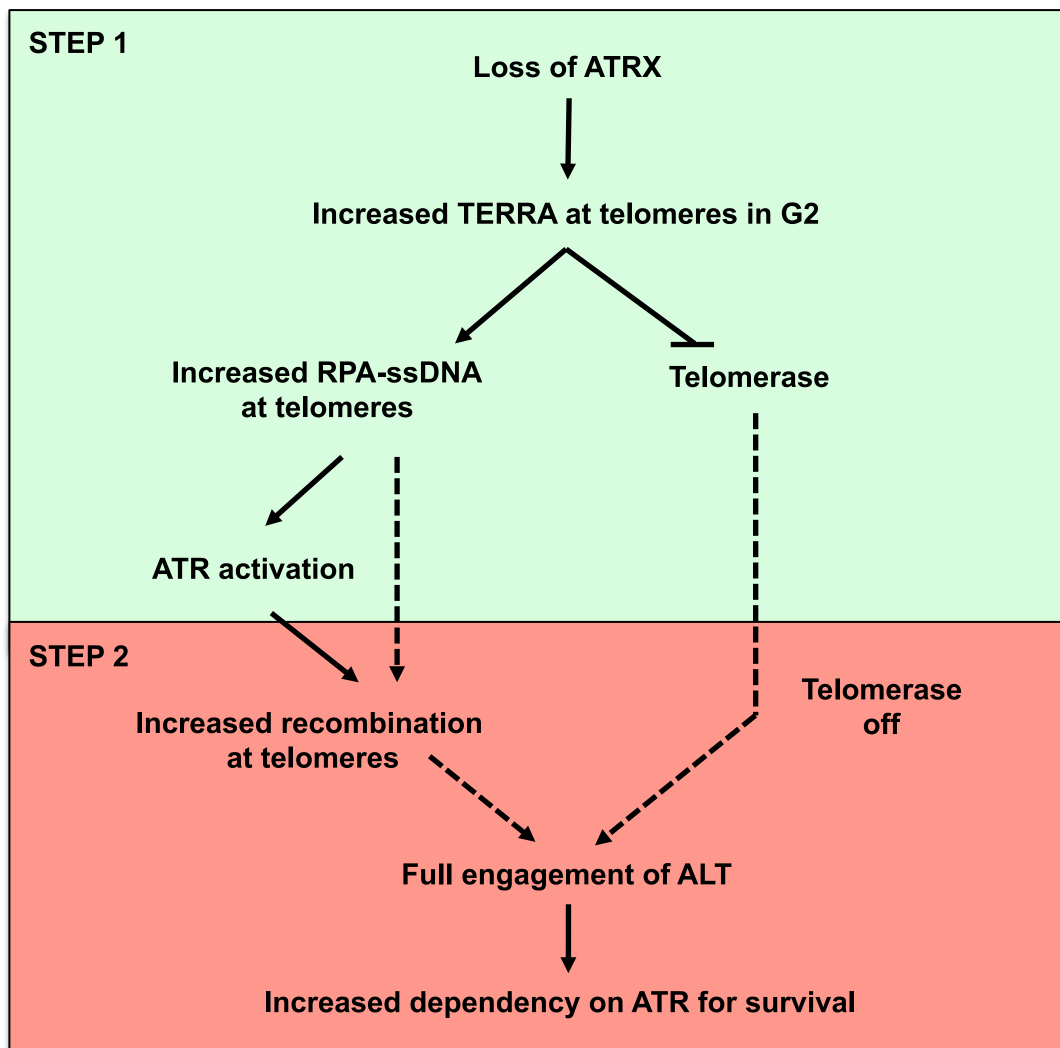

Figure S29. A two-step model for the establishment of ALT and ATR dependency. In step 1, loss of ATRX compromises the cell-cycle regulation of TERRA, leading to increased RPA retention at telomeres. Increased RPA-ssDNA at telomeres promotes ATR recruitment and telomere recombination. If telomerase is active, it may be inhibited by increased TERRA at telomeres. In step 2, additional genetic or epigenetic changes at telomeres allow telomeres to fully engage ALT, rendering cells increasingly dependent on ATR for survival.