G-quadruplexes mark Alternative-Lengthening-of-Telomeres

Sunny Y. Yang[1], Emily Y.C. Chang[2], Joanne Lim[1], Harwood Kwan[1], David Monchaud[3], Stephen Yip[4], Peter C. Stirling[2] and Judy M.Y. Wong[1,*]

[1] Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, Canada
[2] Terry Fox Laboratory, BC Cancer, Vancouver, Canada
[3] Institut de Chimie Moléculaire, ICMUB CNRS UMR 6302, UBFC Dijon, France
[4] Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, Canada

* To whom correspondence should be addressed. Tel: 604-827-3314 ; Fax: 604-822-3035 ; Email: judy.wong@ubc.ca

EXTENDED METHODS

Production and purification of BG4

BL21-DE3 bacteria was transformed using the plasmid pSANG10-3F-BG4 (Addgene #55756) using standard quick transformation (1) in TSS buffer (1X Luria Broth (LB), 10% polyethylene glycol 8000, 5% DMSO, 50mM Mg^{2+}, pH 6.5, sterile-filtered). Colonies were checked using restriction enzyme digestion by NcoI/HindIII (0.9 and 5.2 kb bands). Selected clone was grown in 500mL LB in 2L flasks until OD_{600} 0.5-0.6 was reached. Then, Isopropyl-β-D-thiogalactopyranoside (IPTG) (Calbiochem, 420322) was added to culture to the final concentration of 0.5 mM. The flask was incubated overnight at 17°C while shaking at 120 rpm. Cells were collected and sonicated using the Branson Sonifier. Ni-NTA agarose beads (Qiagen, #30210) was used for affinity chromatography for capturing His-tagged BG4 antibody. Western blotting and ICC were used to check for FLAG-tag integrity. ICC in cells treated with G4 ligands was performed to test for ligand-induced changes in BG4 signals.

Telomere length measurements

Genomic DNA was harvested using QIAamp DNA kit (Qiagen cat #56304). Telomere restriction fragment (TRF) by Southern Blotting was performed as described previously (2). Southern blot images were collected using a storage phosphor screen and Typhoon Scanner (GE Healthcare) and quantified using ImageQuant. For Tel-qPCR, PCR reaction was set up using tel1b primer (5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') with final concentration of 100nM and

tel2b primer (5’-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3’) with final concentration of 500 nM. Alu repeat was used as genome background control. Alu DNA was quantified using Alu forward primer (5'-GACCATCCCGGCTAAAACG-3’) and reverse primer (5'-CGGGTTCACGCCATTCTC-3’) at a final concentration of 100nM. The ratio of qPCR signal of Tel to Alu was used as relative telomere length score.

Sequential ChIP (ChIP-ReChIP)

1st ChIP was performed as previously mentioned. After the 1st ChIP washing step, the beads were resuspended in 1% SDS/10 mM DTT/1X PIC/TE and incubated for 30 min at 37°C. The tube was spun down and the supernatant was transferred to a new tube and diluted 20 times in 1X IP buffer (0.1% SDS, 1 mM EDTA, 10 mM Tris, pH 8, 1% Triton-X, 150 mM NaCl). BSA was added to 0.1% final concentration. A small aliquot was saved from the 1st ChIP eluate for input. 5 µL of 2nd ChIP antibody was added to each tube and incubated overnight at 4°C on the rotator. The next day, 20 µL of 50% slurry of the appropriate pre-blocked agarose beads was added and incubated for 2h on rotator at 4°C. Beads were wash 3 times in ReChIP Wash Buffer (0.1% SDS, 1% NP40, 2mM EDTA, 500mM NaCl) and one time in TE buffer for 5 min each. Beads were incubated in 1% SDS/15mM DTT/TE at 65°C overnight for elution. Extraction was performed the next day as described above. Normalization of the signal was performed against beads only control for each corresponding sample.

Transfection for ICC and ChIP

Cells were transfected using JetPrime transfection reagent (Polyplus). Cells were seeded 20000-60000 cells per coverslips in a 24-well plate and incubated for 24h at 37°C. The next day, the cells were transfected with 250 ng of vector DNA using a ratio according to the manufacturer's recommended protocol. Cells were then incubated for 6h at 37°C. Then, the media were replaced with fresh media and incubate for another 24h. Cells were then harvested for ICC or ChIP, as mentioned above.

Transfection for PLA

For experiments with in vitro RNaseH1 treatment, RNaseH1 treatment was done after permeabilization for 2h at 37°C before blocking. For experiments with GFP or GFP-RNH1 overexpression, transfection

was done 24h post-seeding, and cells were fixed 24h post-transfection. For RNA interference, cells were transfected with siGENOME-SMARTpool siRNAs from Dharmacon (MQ-0219550-01-0005)：Non-targeting siRNA Pool #1 as si-Control and si-FANCM. The set of 4 siRNA sequences has the following sequences: [GUACUGCACUUGAGAAUUU], [CAAACCAUGUUCACAAUUA], [CAACAGUGGUGAAUAGUAA], [GAACAAGAUUCCUCAUUAC]. Transfections were done with Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's protocol, and cells were fixed 48h after the siRNA administration.

ChIP for nuclease-treated samples

ChIP was performed as described above with the following modifications. Two plates of cells were sonicated and pooled. The polled sonicates were precleared then split into three fractions: one for mock control (same conditions without any nuclease addition) and the two others with nuclease of interest added. A small aliquot was saved as input control. DNase I (NEB) and RNase H (NEB) were used at 50U in 1X reaction buffer/ IP buffer. RNase A was used at 50 µg/mL. Samples were then incubated at 37°C for 1h. The proceeding steps followed those of the ChIP protocol described above.

SUPPLEMENTARY FIGURES AND TABLES

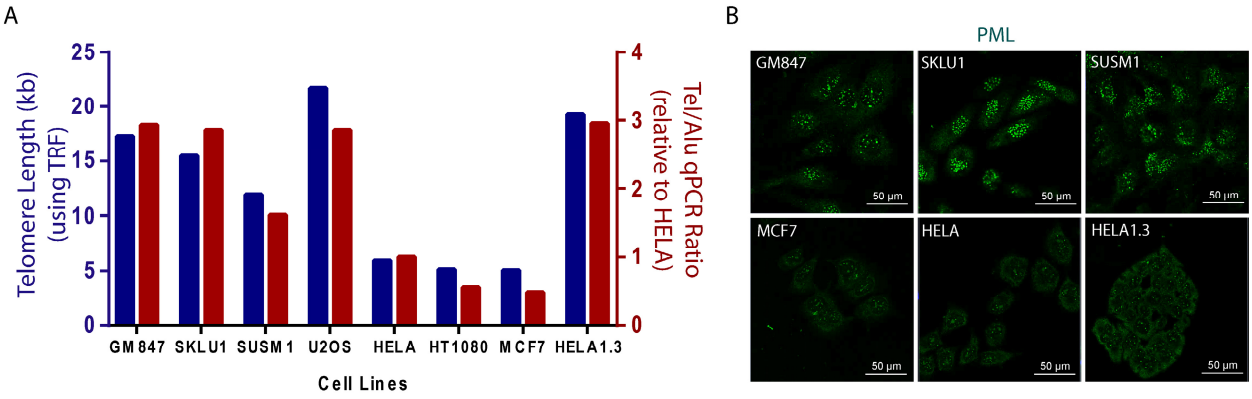


Figure 1. Confirmation of ALT status in a panel of ALT+ and TERT+ cell lines. (A) Mean telomere length as measured by TRF (blue) or Tel-qPCR (red) in ALT+ cell lines (GM847, SKLU1, SUSM1, U2OS) and TERT+ cell lines (HELA, HT1080, MCF7, HELA1.3) **(B)** ICC staining for PML in a panel of ALT+ (top) and TERT+ (bottom) cell lines.

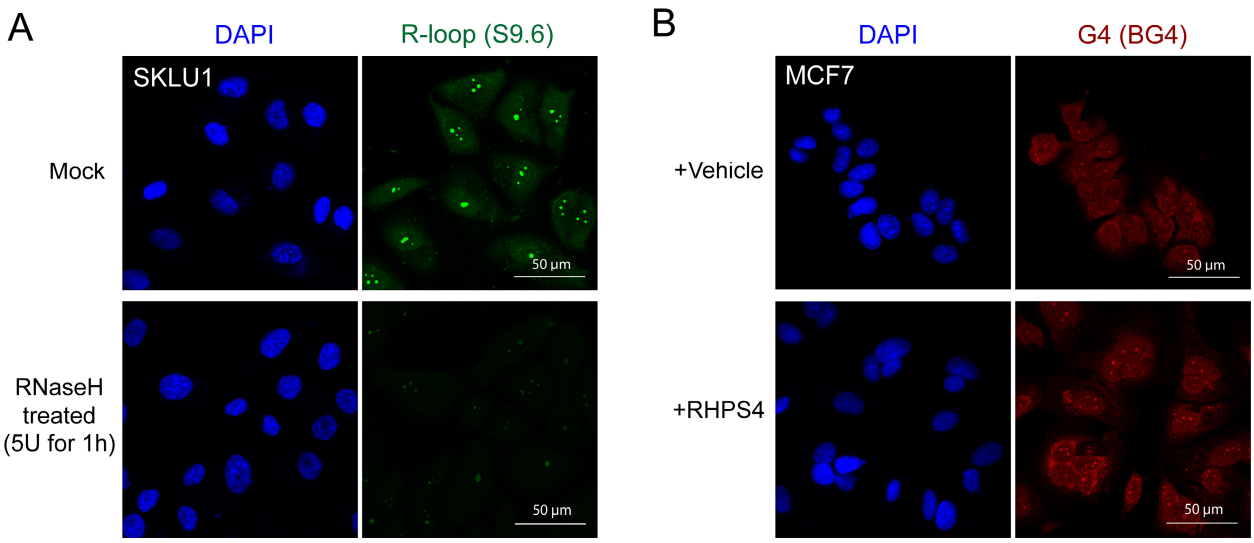


Figure 2. Antibody specificity test. A) R-loop antibody, S9.6, staining in SKLU1 undergoing either the mock condition or RNAseH digestion (5U) for 1h. B) G4 antibody, BG4, staining in either untreated RHSP4-treated MCF7.

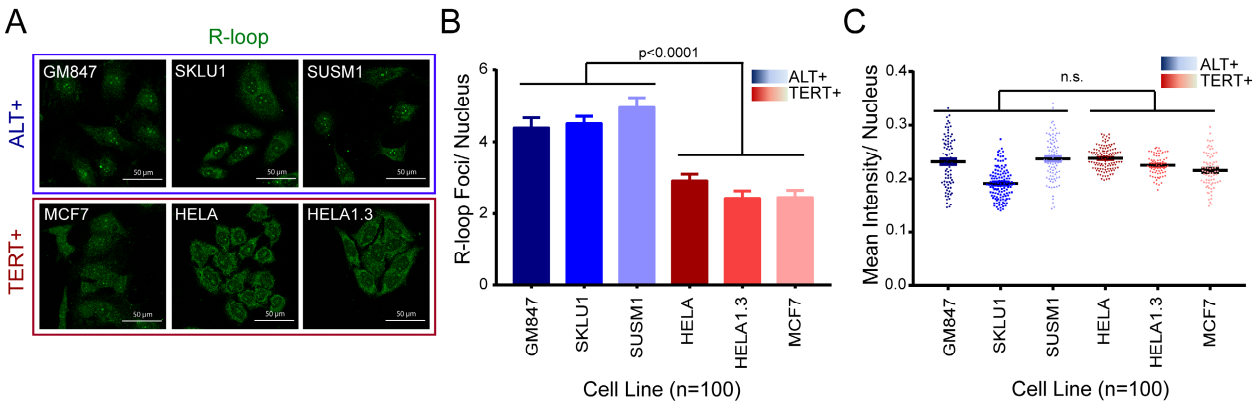


Figure 3. ALT+ cells harbour higher level of telomeric R-loops than TERT+ cells. (A) Immunocytochemistry (ICC) straining experiments for R-loop in ALT+ cells lines (within blue frame) versus TERT+ (within red frame). (B) Average number of R-loop foci per nucleus. Automated quantification of 100 cells per cell line was performed in parallel (C) Mean R-loop foci intensity per nucleus as quantified. Automated quantification of 100 cells per cell line was performed in parallel. Two-tailed t-tests were performed for comparing ALT+ versus TERT+ groups.

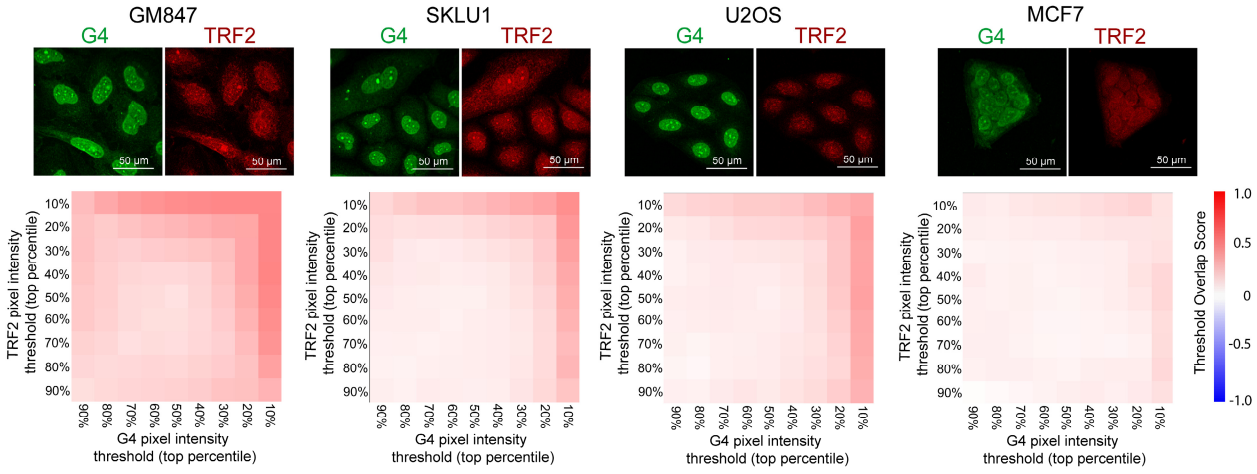


Figure 4. ICC colocalization analysis of G4 and TRF2. ICC co-staining for G4 (green) and TRF2 (red) for ALT+ cell lines (GM847, SKLU1, U2OS) and TERT+ cell line (MCF7). Below is the metric matrix of the threshold overlap score (TOS) for the top percentile of pixel (by intensity) of nuclear G4 signal (x-axis) and TRF2 signal (y-axis). Darker red colour corresponds with higher TOS.

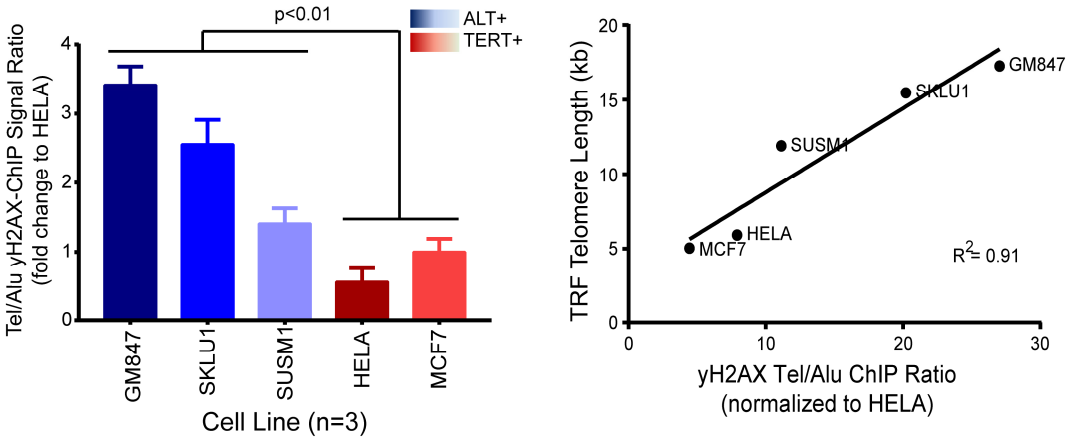


Figure 5. Level of DNA damage signal, γH2AX, is higher in ALT+ cells and are broadly associated with telomere length. γH2AX-ChIP assay in ALT+ cell lines (blue shades) versus TERT+ cell lines (red shades). Data shown are the mean from 3 biological repeat experiments. T-test (two-tailed) was performed for the combined ALT+ versus TERT+ datasets. Bottom; linear regression plot of Tel/Alu ChIP signal ratio against telomere length (as measured by telomere restriction fragment (TRF) analysis). Line of best fit is plotted along with R^2 value.

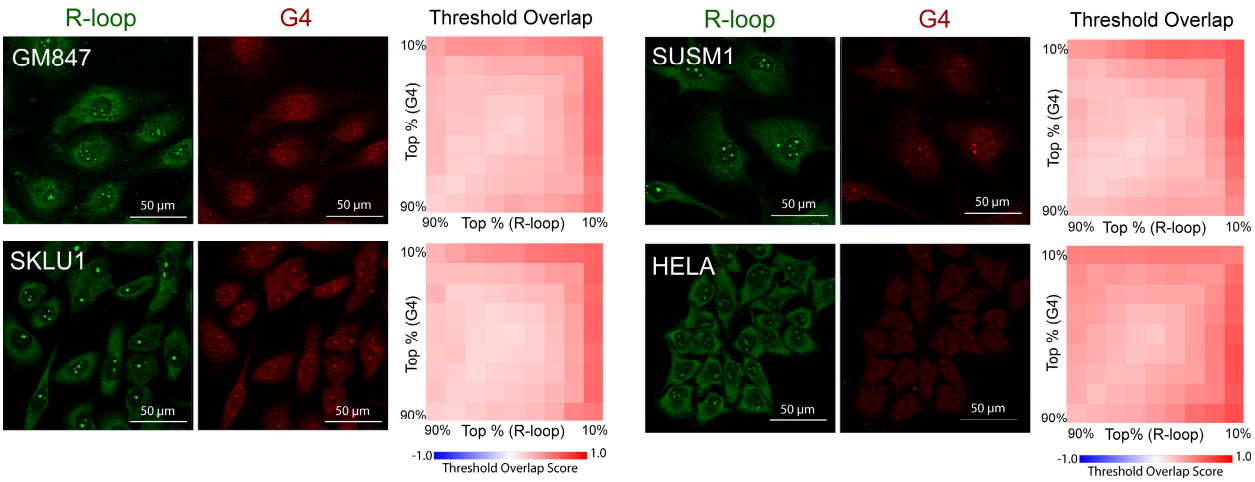


Figure 6. ICC colocalization analysis of R-loop and G4. ICC co-staining for R-loop (green) and G4 (red) in ALT+ (GM847, SKLU, SUSM1) and TERT+ (HELA). Right is the corresponding metric matrix of the threshold overlap score (TOS) for the top percentile of pixel (by intensity) of nuclear R-loop signal (x-axis) and G4 signal (y-axis).

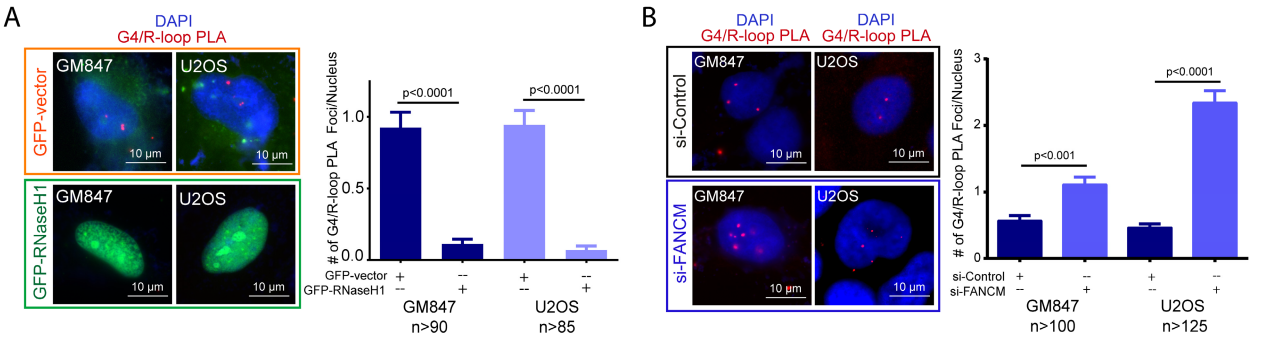


Figure 7. PLA analysis in ALT+ cells with overexpressed RNaseH1 or FANCM-knockdown. (A) Top; Proximity ligation assay (PLA) for G4 and R-loop (red foci) in GM847 and U2OS transfected with either GFP-vector (within orange frame) or GFP-RNaseH1 (within green frame). Bottom; Quantification for mean PLA foci count per nucleus. (B) Left; Proximity ligation assay for G4 and R-loop (red foci) in GM847 and U2OS transfected with either si-Control (within orange frame) or si-FANCM (within green frame). Right; Quantification for mean PLA foci count per nucleus. Two-way ANOVA with Sidak multiple comparisons was performed. Error bars represent the SEM.

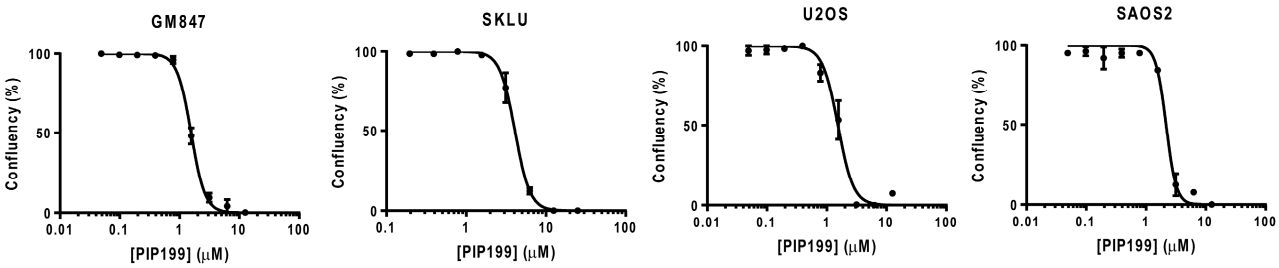


Figure 8. Cytotoxicity profiles of PIP-199 in ALT cell lines. Dose response curves after treatment with PIP-199 for 72h was generated for GM847, SKLU1, U2OS, and SAOS-2 cell lines. Curves were generated by 3 biological replicates. Error bars represent the SEM.

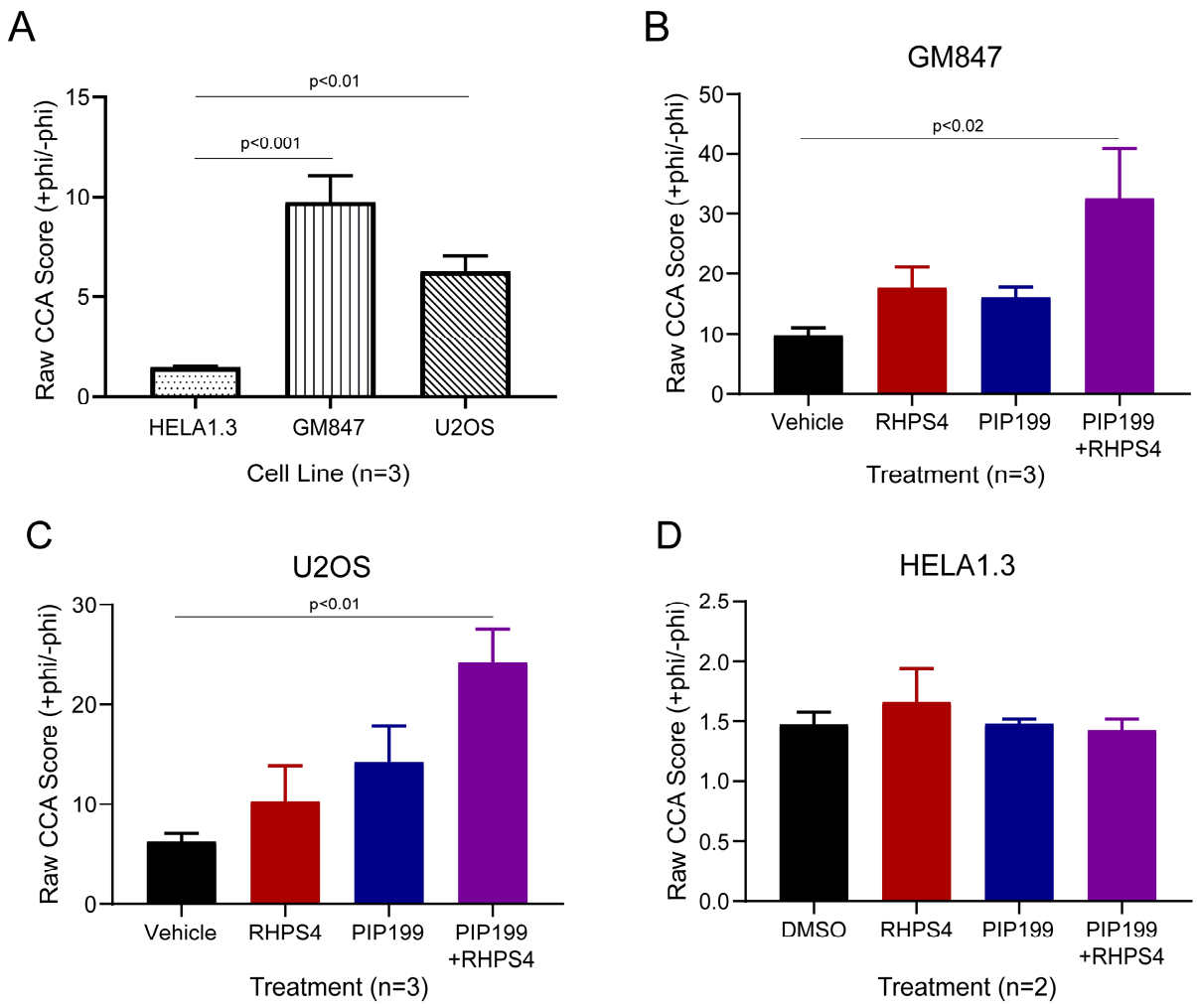


Figure 9. Raw C-circle Assay (CCA) score. A) Baseline CCA score in TERT+ (HELA 1.3) and ALT+ (GM847 and U2OS) cells B) GM847; C) U2OS; or D) HELA1.3 treated with either vehicle, RHPS4, PIP-199 or RHPS4+PIP-199. Raw CCA scores were derived from the ratio of Tel DNA in sample with phi polymerase (+phi) to sample without phi polymerase (-phi). One-way ANOVAs with Dunnett's multiple test correction were performed. Error bars represent the SEM.

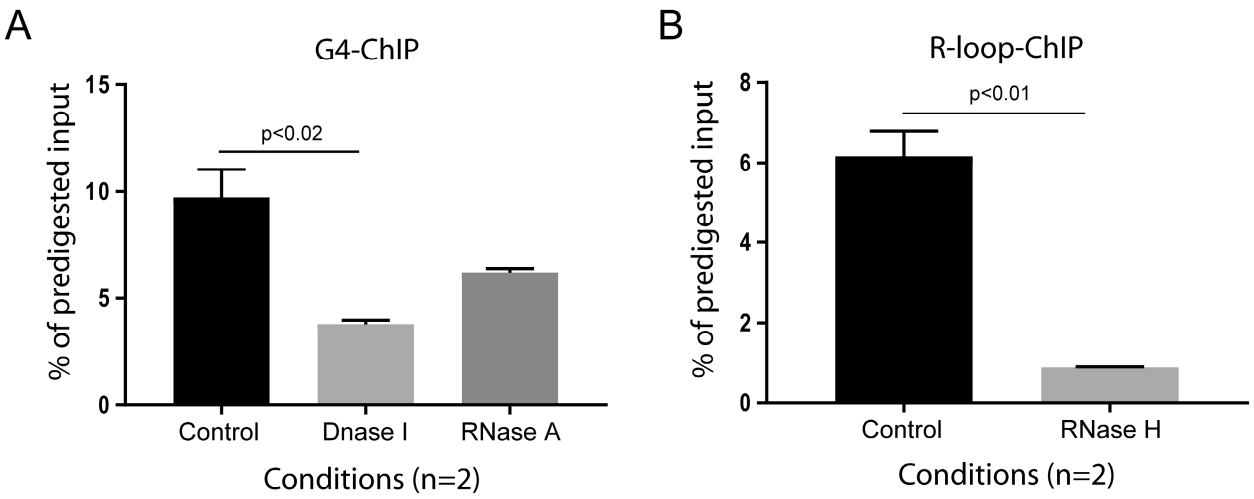


Figure 10. ChIP in nuclease-treated samples. A) G4-ChIP in mock control (with same condition), DNase I-treated sample and RNase A-treated sample harvested from GM847. One-way ANOVA with Dunnett's multiple test correction was performed. B) R-loop-ChIP in mock control (with same condition) and RNase H-treated sample harvest from GM847. Two-tailed t-test was performed. Telomeric DNA was quantified using qPCR. Error bars represent the SEM.

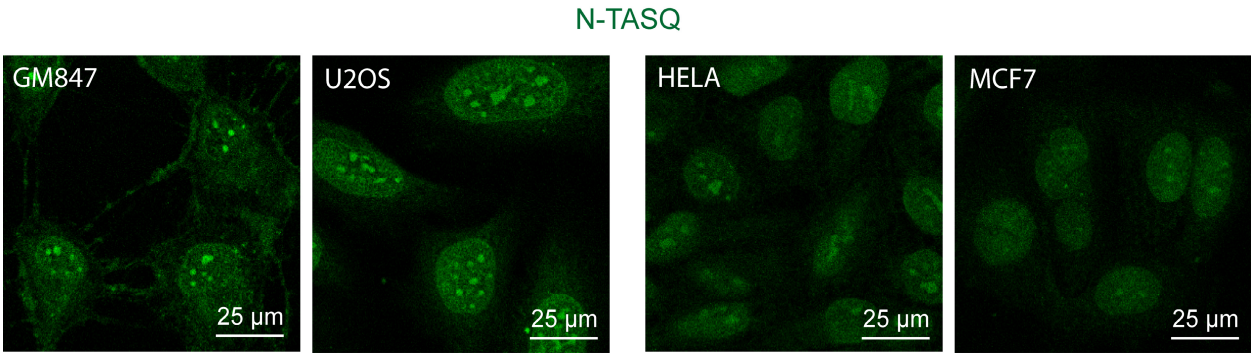
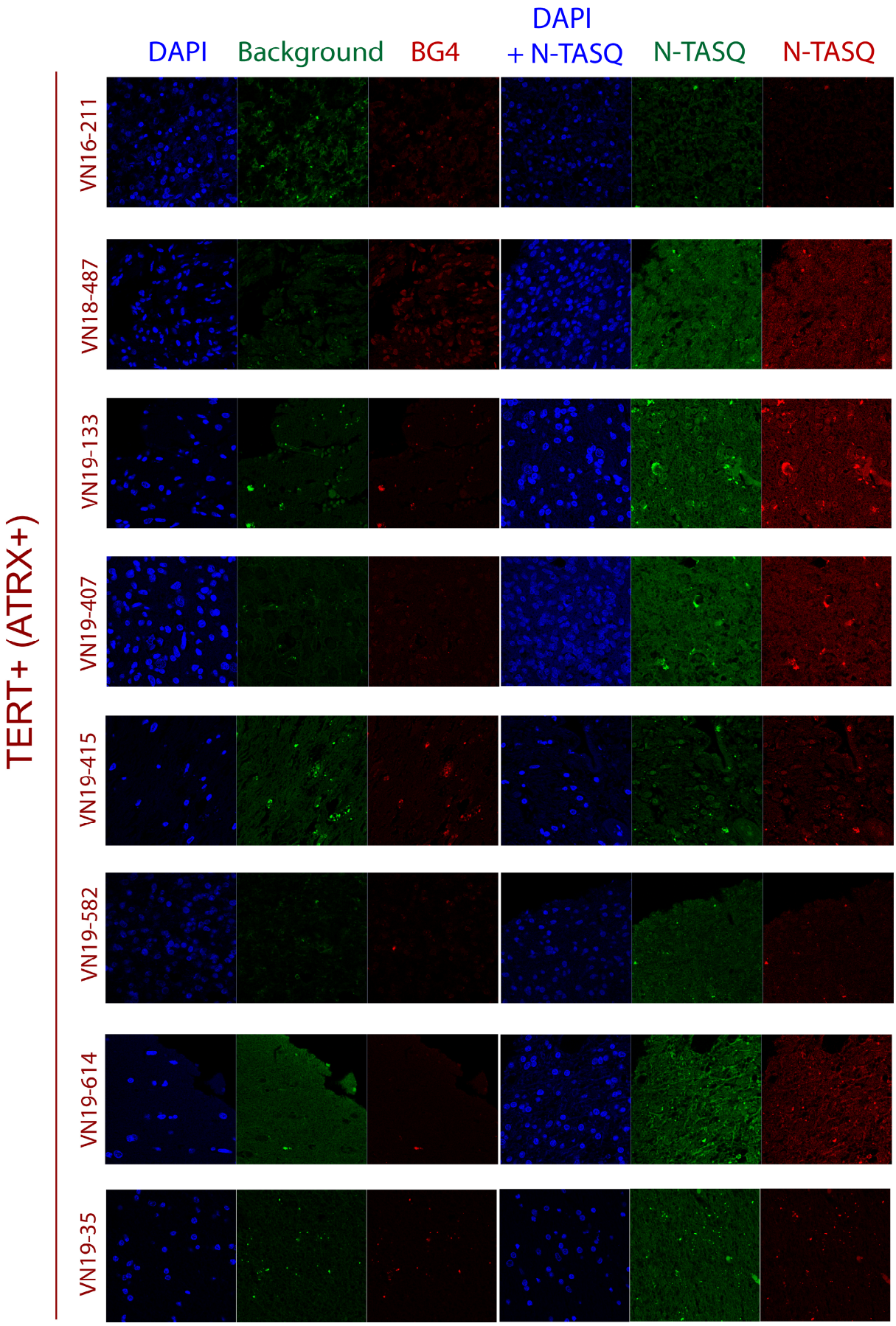
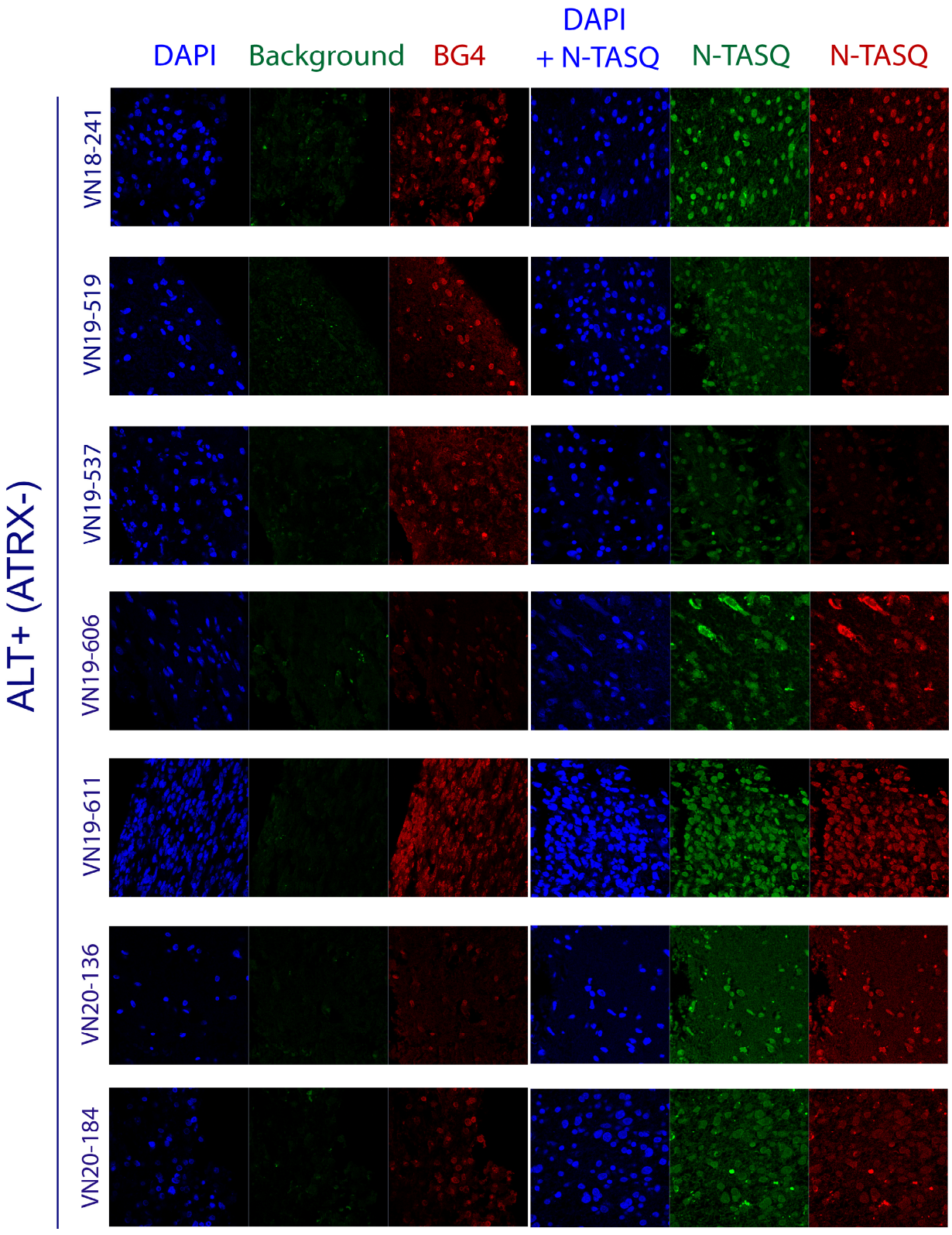
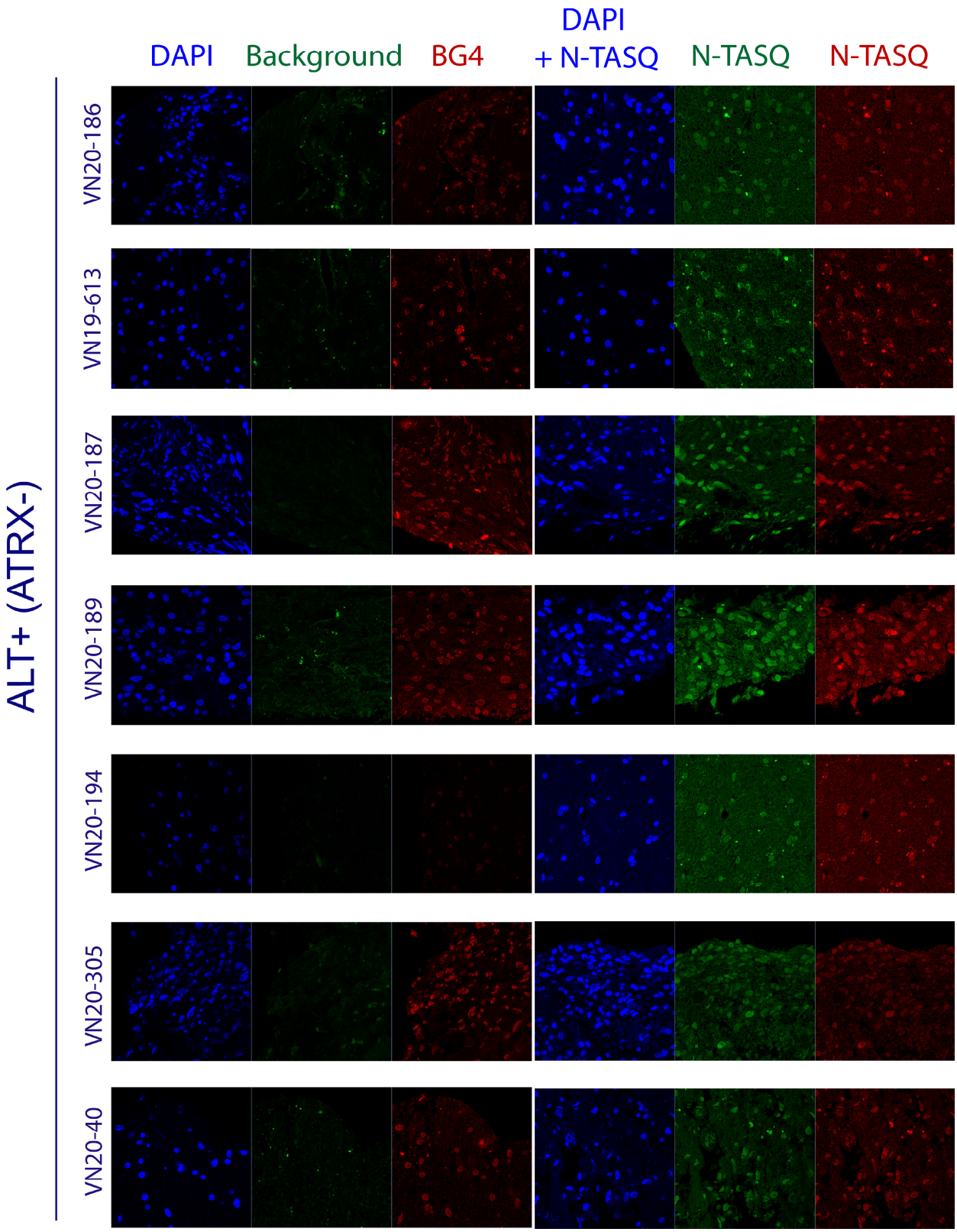


Figure 11. N-TASQ staining images in ALT+ cells (GM847 and U2OS) and TERT+ cells (HELA and MCF7)


DAPI
Background
BG4
DAPI
+ N-TASQ
N-TASQ
N-TASQ
TERT+ (ATRX+)
VN16-211
VN18-487
VN19-133
VN19-407
VN19-415
VN19-582
VN19-614
VN19-35


DAPI
Background
BG4
DAPI
+ N-TASQ
N-TASQ
N-TASQ
ALT+ (ATRX-)
VN18-241
VN19-519
VN19-537
VN19-606
VN19-611
VN20-136
VN20-184


DAPI
Background
BG4
DAPI
+ N-TASQ
N-TASQ
N-TASQ
ALT+ (ATRX-)
VN20-186
VN19-613
VN20-187
VN20-189
VN20-194
VN20-305
VN20-40

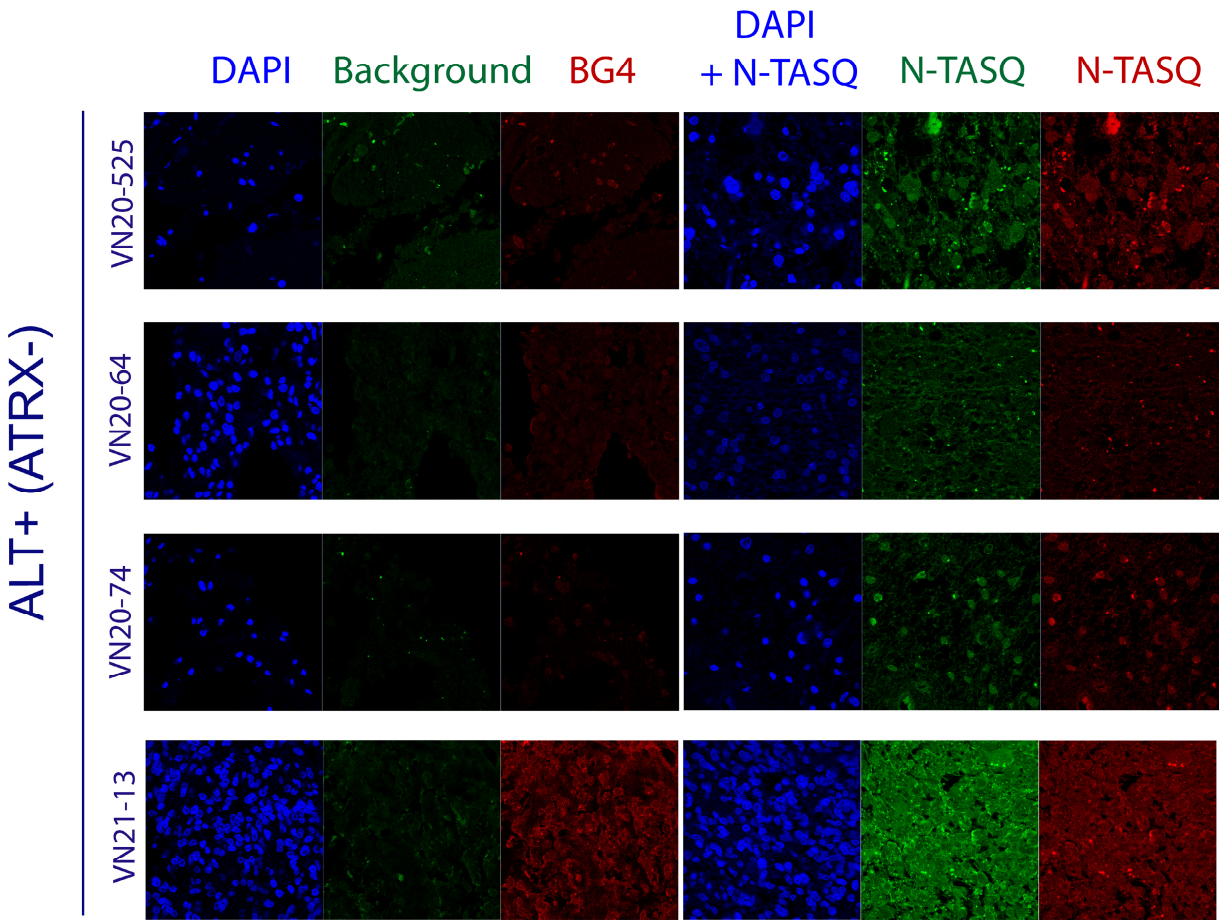


Figure 12. Examples of raw images of BG4 and N-TASQ IHC staining in low-grade glioma tissue. Blue patient IDs indicates ATRX- status while red patient IDs indicates ATRX+ status.

---Other Tables---

Table 1. Specifications of cell lines used					
Cell line name	**Source or Vendor**	**Tissue**	**Cell type**	**Disease/ Transformation method**	**Telomere maintenance status [ref]**
GM847 (GM00847)	Coriell Institute	Skin	Fibroblast	SV40-transformed	ALT+ [3]
SKLU1 (SK-LU-1)	ATCC HTB-57	Lung	Epithelial	Adenocarcinoma	ALT+ [4]
U2OS (U-2 OS)	ATCC HTB-96	Bone	Epithelial	Osteosarcoma	ALT+ [4,5]
SAOS-2	ATCC HTB-85	Bone	Epithelial	Osteosarcoma	ALT+ [4,5]
HELA (HeLa)	ATCC CCL-2	Cervix	Epithelial	Adenocarcinoma	TERT+ [3,4]
MCF7 (MCF-7)	ATCC HTB-22	Breast	Epithelial	Adenocarcinoma	TERT+ [6]
HT1080 (HT-1080)	ATCC CCL-121	Connective tissue	Epithelial	Fibrosarcoma	TERT+ [7]
SUSM1 (SUSM-1)	Gift from Kathy Collins' Lab	Liver	Fibroblast	Gammy ray- and 4NQO-transformed	ALT+ [3]
HELA 1.3	Gift from Kathy Collins' Lab	Cervix	epithelial	HELA subclone with longer telomere length	TERT+ [8]

Table 2. Specifications of antibodies used in ICC, CHIP and PLA				
Antibody Name	**Vendor (Catalog #)**	**Type**	**Target of antibody**	**Application in this study**
BG4	Homemade (purified from BL21 DE3 expressing pSANG10-3F-BG4 plasmid)	Single-chain fragment with FLAG tag	G-quadruplex	ICC, ChIP, ChIP-ReChIP, PLA
Anti-FLAG M2	Sigma (F3165)	Mouse monoclonal primary antibody	FLAG tag	ICC
Anti-FLAG M2	Sigma (F1804)	Mouse monoclonal primary antibody	FLAG tag	PLA
DYKDDDK Tag Rabbit mAB	Cell Signaling (14793)	Rabbit monoclonal primary antibody	FLAG tag	ICC
Anti-DNA-RNA Hybrid, clone S9.6	Millipore (MABE1095)	Mouse monoclonal primary antibody	DNA/RNA- hybrid (R-loop)	ICC, ChIP, ChIP-ReChIP
Anti-S9.6 antibody	Absolute Antibody (Ab01137-23.0)	Rabbit monoclonal primary antibody	DNA/RNA- hybrid (R-loop)	PLA
TRF2 Antibody	Novus Biologicals (NB110-57130)	Rabbit polyclonal primary antibody	TRF2	ICC
Anti-phospho-H2A.X, clone JBW301	Millipore-Sigma (05-636)	Mouse monoclonal primary antibody	yH2AX	ICC, ChIP, ChIP-ReChIP
PML antibody	Santa Cruz (sc-966)	Mouse monoclonal primary antibody	PML	ICC
Goat anti-mouse IgG, Alexa Fluor 488	Invitrogen (A11001)	Goat polyclonal secondary antibody	Mouse IgG	ICC
Goat anti-mouse IgG, Alexa Fluor 594	Invitrogen (A11032)	Goat polyclonal secondary antibody	Mouse IgG	ICC
Goat anti-rabbit IgG, Texas Red	Invitrogen (T2767)	Goat polyclonal secondary antibody	Rabbit IgG	ICC

REFERENCES

1) Chung,C.T., Niemela,S.L. and Miller,R.H. (1989) One-step preparation of competent Escherichia coli: transformation and storage of bacterial cells in the same solution. Proceedings of the National Academy of Sciences, **86**, 2172–2175.
2) Zeng,X.-L., Thumati,N.R., Fleisig,H.B., Hukezalie,K.R., Savage,S.A., Giri,N., Alter,B.P. and Wong,J.M.Y. (2011) The accumulation and not the specific activity of telomerase ribonucleoprotein determines telomere maintenance deficiency in X-linked dyskeratosis congenita. Human Molecular Genetics, **21**, 721–729.
3) TM;Englezou,B. (2021) Telomere elongation in immortal human cells without detectable telomerase activity. The EMBO journal, **14**.
4) Bryan,T.M., Englezou,A., Dalla-Pozza,L., Dunham,M.A. and Reddel,R.R. (1997) Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nature Medicine, **3**, 1271–1274.
5) Scheel,C., Schaefer,K.-L., Jauch,A., Keller,M., Wai,D., Brinkschmidt,C., van Valen,F., Boecker,W., Dockhorn-Dworniczak,B. and Poremba,C. (2001) Alternative lengthening of telomeres is associated with chromosomal instability in osteosarcomas. Oncogene, **20**, 3835–3844.
6) Kyo S;Takakura M;Kanaya T;Zhuo W;Fujimoto K;Nishio Y;Orimo A;Inoue M (2021) Estrogen activates telomerase. Cancer research, **59**.
7) Holt,S.E., Aisner,D.L., Shay,J.W. and Wright,W.E. (1997) Lack of cell cycle regulation of telomerase activity in human cells. Proceedings of the National Academy of Sciences, **94**, 10687–10692.
8) Takai,K.K., Hooper,S., Blackwood,S., Gandhi,R. and de Lange,T. (2010) In Vivo Stoichiometry of Shelterin Components*. Journal of Biological Chemistry, **285**, 1457–1467.