SUPPORTING INFORMATION for:

DNA damage and genome instability by G-quadruplex ligands are mediated by R-loops in human cancer cells.

Alessio De Magis[a,1,2], Stefano G. Manzo[a,1,3], Marco Russo[a], Jessica Marinello[a], Rita Morigi[a], Olivier Sordet[b] and Giovanni Capranico[a,4]

[a] Department of Pharmacy and Biotechnology, Alma Mater Studiorum University of Bologna, via Selmi 3, 40126 Bologna, Italy, and [b]Cancer Research Center of Toulouse (CRCT), INSERM, Université de Toulouse, Université Toulouse III Paul Sabatier, CNRS, Toulouse, France.

[1] A.D.M. and S.G.M. contributed equally to this work.

[2] Present address: Universitätsklinikum Bonn, Sigmund-Freud-Straße 25, 53127 Bonn, Germany

[3] Present address: Division of Gene Regulation, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

[4] To whom correspondence may be addressed: Phone: +39-0512091209. Email: giovanni.capranico@unibo.it

SI METHODS

Compound synthesis, analysis and preparation

FG and FA (SI Appendix Fig. 1 A) were synthesized as described previously (1). The melting points are uncorrected. Elemental analysis data (C, H, N) were within ±0.4% of the theoretical values. ^{1}H and ^{13}C NMR spectra were determined with a Varian MR 400 MHz (ATB PFG probe). Abbreviations are: ar = aromatic, im = imidazole, ph = phenyl, py = pyridine, pym = pyrimidine, th = thiazole, imi = imidazoline. Chemical shifts (referenced to solvent signal) are in δ (ppm) and J in Hz. IR spectra were recorded in Nujol on a Nicolet Avatar 320 ESP (ν_{max} is expressed in cm^{-1}). High resolution mass spectral (HRMS) data were recorded on a Waters Xevo Q-Tof spectrometer. For 2,8-diphenyldiimidazo[1,2-a:1',2'-c]pyrimidine-3,9-dicarbaldehyde (FA): mp: 246-247°C; ^{1}H NMR ($CDCl_3$), 7.36 (1H, d, py, J=7.2), 7.50 (3H, m, ph), 7.58 (3H, m, ph), 7.88 (2H, m, ph), 8.24 (2H, m, ph), 9.34 (1H, d, py, J=7.2), 10.16 (1H, s, CHO), 11, 51 (1H, s, CHO); ^{13}C NMR ($CDCl_3$), 105.34, 120.94, 124.54, 128.14, 128.58, 129.55, 130.08, 130.28, 130.78, 131.07, 131.14, 132.01, 140.75, 144.01, 153.28, 155.57, 180.63, 181.68; IR (Nujol): 1670, 1629, 1506, 764, 687 cm^{-1}; HRMS (AP-ESI) m/z: calcd for $C_{22}H_{14}N_4O_2Na_1$ $[M+Na]^+$ 389.1015, found 389.1014; combustion analysis (calcd., found for $C_{22}H_{14}N_4O_2$): C (72.12, 72.22), H (3.85, 3.86), N (15.29, 15.25). For (2E,2'E)-2,2'-((2,8-diphenyldiimidazo[1,2-a:1',2'-c]pyrimidine-3,9-diyl)bis(methanylylidene))bis(hydrazine-1-carboximidamide) (FG): mp: 268-270°C; ^{1}H NMR (DMSO-d_6), 7.29 (1H, d, py, J=8.0), 7.53 (6H, m, ph), 7.84 (8H, broad, NH), 7.88 (2H, d, ph, J=7.4), 8.01 (2H, d, ph, J=7.4), 8.64 (1H, s, CH), 9.28 (1H, d, py, J=8.0), 9.56 (1H, s, CH), 12.19 (1H, s, NNH), 12.53 (1H, s, NNH); ^{13}C NMR (DMSO-d_6), 103.59, 115.31, 118.12, 127.93, 128.43, 128.84, 128.94, 129.09, 129.41, 131.74, 133.21, 138.39, 138.60, 139.58, 142.16, 145.79, 146.08, 154.77, 155.19; HRMS (AP-ESI) m/z: calcd for $C_{24}H_{24}N_{12}$ $[M+H]^{2+}$ 240.1123, found 240.1124; IR (Nujol): 1681, 1614, 1152, 777, 722 cm^{-1}; combustion analysis (calcd., found for $C_{24}H_{22}N_{12}\cdot 2HCl$): C (52.27, 52.32), H (4.39, 4.38), N (30.48, 30.45). Thus,

IR, ^{1}H NMR, ^{13}C NMR and HRMS data of FG and FA are in agreement with assigned structures (SI Appendix Fig. 1 A). Pyridostatin and Braco-19 (SI Appendix Fig. 1 A) were purchased from Merck.

Stock solutions of FG, FA and Braco-19 were in 100% DMSO at 10 mM (FG and Braco-19) or 2 mM (FA). Pyridostatin stock solution was in 100% ddH_2O at 5 mM. To avoid repeating freeze-thawing, compound stock solutions were aliquoted in small vials of 30 µL each and stored at -20°C. Compounds were diluted to the final concentration in cell culture medium immediately before use. NU7441 (a DNA-PK inhibitor) was from Tocris Bioscience (Bristol UK), stocked in DMSO at 10 mM and diluted in culture medium to a final concentration of 10 µM immediately before use. Other chemical reagents were from Merck, if not otherwise indicated.

Cell culture conditions.

U2OS cells were grown in Dulbecco's modified Eagle's medium (DMEM, Carlo Erba Reagents) supplemented with 10% fetal bovine serum (FBS, Carlo Erba Reagents) and 2 mM L-glutamine (Carlo Erba Reagents). U2OS_T-Rex and U2OS_T-Rex_RH cells were grown in DMEM supplemented with 10% tetracycline-free FBS (Takara ref #631106), 2 mM L-glutamine. 500 µg/mL hygromycin B (Introvigen ref #10687010) and 1.5 µg/mL puromycin (Sigma-Aldrich ref #P8833). WI38 cells were grown in modified Eagle's medium (MEM) supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids (Gibco, Life Technologies ref #11140-035), and 1 mM sodium pyruvate (Gibco, Life Technologies ref #11360-039). All cell lines were maintained by trypsinization and splitting 2-3 times a week, and incubated at 37°C in 5% CO2.

Antibody purification and storage

BG4 antibody. The plasmid expressing an engineered antibody specific to G4 structures (BG4) (2) was kindly obtained by S. Balasubramanian (University of Cambridge, UK), and transfected into BL21 (DE3) *E. coli* cells. BG4 protein expression was activated with the autoinduction protocol

as described already (3). BG4 was purified with Protino® Ni-IDA (Macherey-Nagel ref #745250.10) pre-charged with Ni_{2+} ions and eluted with 250 mM Imidazole (Merck) in PBS (137 NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4), pH 8.0. The eluted antibody solution was concentrated with Amicon Ultra-15 Centrifugal Filter Units (Millipore ref #UFC903024) and imidazole was removed by several washes with PBS, pH 8.0. BG4 antibody was conserved at 4°C, in presence of 0.02% sodium azide, for few months. Purity of each preparation was checked by SDS-PAGE.

S9.6 antibody. The murine HB-8730 hybridoma cell line was kindly obtained by S. Gabor (Drebecen, Hungary) (4) and regularly checked for human cell contamination. HB-8730 cells were cultured in IMDM medium (Gibco, Thermo Fisher Scientific ref #42200-014) adjusted with 1.5 g/L sodium bicarbonate supplemented with 4 mM L-glutamine and 0.1 mM hypoxanthine, 0.016 mM thymidine and 20% horse serum (Carlo Erba Reagents). Culture density was maintained between 10^5 and 10^6 cells/ml and fluid renewal was performed every 2-3 days. For antibody purification, a column filled with 2 ml of Sepharose CL-4B (protecting column, Merck ref #CL4B200) was assembled over another column filled with 2 ml of Protein A Sepharose Cl-4B (binding column, GE Healthcare ref#17-0780-01), and then whased with 200 ml of phosphate buffer (5 mM NaH_2PO_4, 94.7 mM Na_2HPO_4, pH 8.0). 800 ml of cellular supernatant was then loaded into the columns and let flow through them by gravity (from 4 to 6 hours). The columns were then washed with 200 ml phosphate buffer. Protein A-Sepharose® CL-4B protecting column was removed, and the binding column was washed with additional 300 ml phosphate buffer. Eppendorf tubes were then filled with 250 µl of Tris 1M pH 8.5 and the column washed over with C-buffer pH 3.7 (68.8 mM Citric Acid, 35.9 mM Tris-Na-citrate), collecting the drops into the eppendorf tubes. Tubes were mixed well and fractions observed at Thermo Scientific™ NanoDrop™ spectrophotometers to select those containing the protein. Positive fractions were mixed together and centrifuged in Amicon Ultra-15 Centrifugal Filter Units. Buffer exchange was performed resuspending the filtrate in 1X PBS and centrifuging the solution again in the same centrifugal unit. Protein concentration was determined by Bradford Assay

and S9.6 antibody was conserved at -80° C in aliquots (after thawing, each aliquot was immediately used or maintained at 4 °C for few days). Each purified S9.6 stock was checked for purity by SDS-PAGE, and binding specificity was checked in cells expressing exogenous RNaseH1 (U2OS_RH cell line) and by using DRIP (SI Appendix Fig. 1). S9.6 stocks were also titrated with immunofluorescence microscopy to determine the optimal concentration to use in IF experiments.

Immunofluorescence microscopy

Detection of G4 with BG4 antibody.

Cells were pre-fixed with a solution 50% DMEM and 50% methanol/acetic acid (3:1) at RT for 10 minutes. After a brief wash with methanol/acetic acid (3:1), cells were fixed with methanol/acetic acid (3:1) at RT for 10 min. Cells were then permeabilized with 0.1% Triton™ X-100 (Merck) in PBS at RT for 3 minutes. Cells were exposed to blocking solution (2% milk in PBS, pH 7.4) for 1 hour at RT and then incubated with 2 µg per slide of BG4 antibody in blocking solution (2 hours at RT). Cells were then incubated with 1:800 of a rabbit antibody against the DYKDDDDK epitope (Cell Signaling ref #2368) in blocking solution for 1 hour. Next, cells were incubated at RT with 1:1000 Alexa Fluor 488 goat anti-rabbit IgG (Life technologies ref #A11008) in blocking solution for 1 hour. After each step, cells were washed three times for 5 minutes with 0.1% Tween-20 in PBS under gentle rocking. For nuclear staining, cells were incubated with 2 µg/µL of DAPI (Merck ref #D9542) or 1µg/ml of Hoechst 33342 for 30 minutes. The cover glasses were mounted with Mowiol® 4-88 (Merck ref #81381).

Detection of DNA:RNA hybrids with S9.6 antibody.

Cells were fixed with ice-cold methanol at room temperature (RT) for 10 minutes. After a brief wash with PBS, cells were permeabilized with acetone (Merck) for 1 minute on ice. After 3

washes with PBS, cells were blocked with 3% bovine serum albumine (BSA, Merck)/0.1% Tween-20/SSC 4X for 1 hour and then incubated with S9.6 and 1:1000 anti-Nucleolin (Abcam ref #ab22758) or 1:500 anti-mCherry (GeneTex ref #GTX128509) antibodies diluted in 3% BSA/0.1% Tween-20/SSC 4X for 1 hour at RT. Cells were then incubated at RT with 1:1000 Alexa Fluor 594 goat Anti-Mouse IgG (Life technologies ref #A11032) and 1:1000 Alexa Fluor 488 goat anti-rabbit IgG (Life technologies ref #A11008) in 3% BSA/0.1% Tween-20/SSC 4X for 1 hour. After each step, cells were washed with 4X SSC. For nuclear staining, cells were then incubated with 2 µg/µL of DAPI or 1µg/ml of Hoechst 33342. Finally, cover glasses were mounted with Mowiol® 4-88.

Co-staining of cells with BG4 and S9.6 antibodies.

Cell were fixed with ice-cold methanol at room temperature (RT) for 10 minutes. After a brief wash with PBS, cells were permeabilized with 0.5% Triton™ X-100 in PBS at RT for 15 minutes. Cells were blocked with 8% BSA/PBS and then incubated with 2 µg per slide each of BG4 and S9.6 antibodies diluted in 2% BSA/PBS for 2 hours. Cells were then incubated with 1:800 of DYKDDDDK Tag antibody (Cell Signaling ref #2368) in 2% BSA/PBS for 1 hour. Next, cells were incubated at RT with 1:1000 Alexa Fluor 594 goat Anti-Mouse IgG (Life technologies ref #A11032) and Alexa Fluor 488 goat anti-rabbit IgG (Life technologies ref #A11008) in 2% BSA/PBS for 1 hour. After each step, cells were washed with PBS. For nuclear staining, cells were incubated with 2 µg/µL of DAPI or 1µg/ml of Hoechst 33342 for 15 minutes. Finally, cover glasses were mounted with Mowiol® 4-88.

Detection of γH2AX.

After wash with 1X PBS cells were fixed with 4% paraformaldehyde (PFA)/PBS 1X at RT 10 minutes. After 2 washes with PBS, cells were permeabilized with 0.5% Triton™ X-100/PBS at RT for 15 minutes. Cells were blocked with 8% BSA/PBS and then incubated at RT with 1:500 anti-

γH2AX antibody (Millipore ref #05-636) diluted in 1% BSA/PBS for 2 hours. Next, cells were incubated at RT with 1:1000 Alexa Fluor 488 goat Anti-Mouse IgG (Life technologies ref #A11011) in 1% BSA/PBS for 1 hour. After each step, cells were washed with PBS. For nuclear staining, cells were incubated with 2 µg/µL of DAPI or 1µg/ml of Hoechst 33342 for 15 minutes. The cover glasses were mounted with Mowiol® 4-88.

Co-staining of cells with γH2AX and S9.6 antibodies.

Cells were fixed with 4% PFA/PBS for 15 min, washed 3 times with 1X PBS and blocked for 1 hr at RT with 2% BSA/PBS. Cells were then incubated with S9.6 and anti-H2AX (Abcam ref #ab11175) overnight at 4°C. Cells were washed 3 times in 1X PBS and incubated at RT for 1 hour with 1:1000 Alexa Fluor 488 goat Anti-Mouse IgG (Life technologies ref #A11011) and Alexa Fluor 594 goat Anti-Rabbit IgG (Life technologies ref #A11037) diluted in 2% BSA/PBS.

Detection of DNA repair factors

For ATM and 53BP1, cells were pre-extracted with CSK buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM Sucrose, 3 mM $MgCl_2$, 0.5% Triton™ X-100) with Halt™ Protease Inhibitor Cocktail (Thermo Fisher ref #87785). The buffer was added drop by drop and incubated for 3 min at RT. After one wash with PBS, cells were fixed with 2% PFA/PBS at RT for 15 minutes. After 3 washes with PBS, cells were incubated with 1:500 anti-γH2AX antibody (Millipore ref #05-636) or anti-H2AX (Abcam ref #ab11175) and 1:500 anti-p53BP1 (Cell signalling ref #S1778) or anti-pATM (Cell signalling ref #S1981) or anti-53BP1 (Novus ref #NB-100-305) antibodies diluted in 5% BSA/PBS for 1 hour under gentle agitation. Next, cells were incubated at RT for 1 hour with 1:1000 Alexa Fluor 488 goat Anti-Mouse IgG (Life technologies ref #A11011) or Alexa Fluor 488 goat anti-rabbit IgG (Life technologies ref #A11008) and Alexa Fluor 594 goat Anti-Rabbit IgG (Life technologies ref #A11037) or Alexa Fluor 594 goat Anti-Mouse IgG (Life technologies ref #A11032) diluited in 5%

BSA/PBS. After each step, cells were washed with PBS. For nuclear staining, cells were incubated with 2 µg/µL of DAPI or 1µg/ml of Hoechst 33342 for 15 minutes. The cover glasses were mounted with Mowiol® 4-88.

For RAD51, after 2 washes with PBS, cells were fixed with ice-cold methanol at 4°C for 15 minutes. After 3 washes with PBS, cells were permeabilized with 0.5% Triton X-100/PBS at RT for 5 minutes. After 3 washes with PBS, cells were blocked with 10% BSA/0.3% Triton X-100/PBS for 1 hour under agitation. Then, cells were incubated overnight at 4°C with 1:500 anti-RAD51 antibody (Millipore ref #PC130) diluted in 1% BSA/0.3% Triton X-100/PBS. Cells were then incubated with 1:1000 Alexa Fluor 488 goat anti-rabbit IgG (Life technologies ref #A11008) in 1% BSA/0.3% Triton X-100/PBS for 1 hour. After each step, cells were washed with PBS. For nuclear staining, cells were incubated with 2 µg/µL of DAPI or 1µg/ml of Hoechst 33342 for 15 minutes. The cover glasses were mounted with Mowiol® 4-88.

Cell image analysis

Fluorescence signal was determined using ImageJ software with the following formula: Corrected Total Cell Fluorescence (CTCF) = Integrate Density - (Area of selected cell x Mean Fluorescence of Background Readings). Nucleoplasmic compartment was calculated by subtracting the nucleolar signal, obtained by anti-Nucleolin staining, from the total nuclear fluorescence. For graphical representation of signal distribution, we used box-and-whisker plots using GraphPad Prism 6 software with the following settings: boxes: 25–75 percentile range; whiskers: 10–90 percentile range. Statistical significance was determined by “Kolmogorov-Smirnov” non-parametric test. Data analyses were performed with Excel and GraphPad, and all figures were prepared with Adobe Illlustrator.

Exogenous RNaseH1 gene expression in cells

Transient human RNaseH1 expression was performed in U2OS cells. 24 hours after seeding, cells were transfected with 2.5 µg of pCAG-RNAseH1-GFP (green fluorescent protein) or Turbo-GFP plasmids (negative control) using Lipofectamine™ 2000 Transfection Reagent (Thermo Fisher ref #11668027). pCAG-RNAseH1-GFP plasmid was a kind gift from Frederic Chedin (Davis, CA, USA). Exogenous enzyme expression was verified with fluorescence microscopy.

Exogenous human RNaseH1 expression was also performed in U2OS_T-Rex_RH and U2OS_RH cells. Before activation of plasmid-borne RNaseH1, antibiotics (hygromycin and puromycin) were removed from culture medium for 24 hours, and then RNaseH1 expression was induced with 2 µg/mL of doxycycline for 48 hours. Exogenous enzyme expression was verified by western blot and/or fluorescence microscopy of mCherry tag for U2OS_T-Rex_RH cells.

***BRCA2* gene silencing**

24 hours after seeding, U2OS or U2OS_RH cells were forward transfected with 20 nM siRNA specific for *BRCA2* gene (Hs_BRCA2_7 FlexiTube siRNA ref#S102653595, Qiagen) or 20 nM scramble siRNA (LifeTechnlogies, Negative Control #2 ref#4390847) using Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher ref #13778075). Protein knockdown was assessed by western blot of nuclear proteins extracted from cells 72 hours post transfection.

Genomic mapping of R loops by DRIP-seq

We used DNA:RNA immunoprecipitation (DRIP) methodologies to immunoprecipitate and isolate DNA:RNA duplexes from genomic DNA preparations by using S9.6 antibody and to map genome-wide R loop structures, as described previously (5, 6)□. Our DRIP protocol follows in details.

Cellular DNA purification and fragmentation.

Cells were resuspended in Lysis Buffer (30 mM Tris-HCL pH 8.0, 1 mM EDTA, 1% SDS), scraped and collected in a 1.5 ml tube. 100 µg of Proteinase K were added to the tubes and the samples were incubated overnight at 37°C. The day after, DNA was precipitated adding 2.5 volume of 100% Ethanol, 1/10 volume of 3M NaOAc and 1 ul of glycogen (the latter is optional). After gently inversion, the DNA was precipitated. The DNA was washed 5 times with 70% ethanol and then allowed to air dry. Pellet was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA) pH 8.0, and incubated on ice until the DNA is completely resuspended. Genomic DNA was then digested ON at 37°C using a restriction enzyme cocktail (80 U HindIII, 40 U XbaI, 40 U BsrgI, 40 U SSpI) in Tango Buffer 1X (Thermo Fisher Scientific). The day after, cells were incubated for additional 2 hours with 80 U EcoR1 in 2X Tango Buffer. DNA samples were checked for equal rates of fragmentation by agarose gels, and DNA quantification was performed with a Thermo Scientific™ NanoDrop™ spectrophotometers.

DNA-RNA hybrid immunoprecipitation.

4 µg of collected gDNA and an input equivalent to 10% were resuspended in TE (10 mM Tris-HCl, 1 mM EDTA), pH 8.0. In order to have a control for a quantitative PCR (qPCR) the input was conserved at 4°C. Samples were pretreated, or not, with 25 U of *E. coli* RNaseH (Ambion-Life Technologies ref #AM2293) for 2 hours at 37°C. 10 µg of S9.6 antibody were added to the samples in binding buffer (100 mM Na_3PO_4, pH 7.0, 1.4 mM NaCl, 0.5% Triton™ X-100) and incubated overnight at 4°C under rotation (20 rpm). The day after, the complex DNA/S9.6 was incubated with a mixtures of Protein G Sepharose® 4 Fast Flow (GE Healthcare ref #17-0618-01) and Protein A-Sepharose® CL-4B (GE Healthcare ref #17-0780-01) beads that were previously washed and equilibrated with binding buffer. After 2 hours in rotation at 4°C, the DNA/S9.6/beads complex was washed 3 time at 4°C in Binding buffer 1X (diluted in TE buffer) and centrifugated at 4°C 4 minutes at 4000 G. After last centrifugation, the binding buffer was removed accurately and the pellet was

resuspended in elution buffer (50 mM Tris-HCl, 10 mM EDTA, 0.5% SDS). Samples were incubated with 100 µg of proteinase K at 55°C for 45 minutes or more. The supernatant was collected in a new tube and DNA was extracted using phenol-chloroform extraction protocol followed by DNA precipitation overnight at -20°C. The DNA was then precipitated by centrifugation, washed with 70% ethanol and then resuspend in 50 mM Tris-HCl, pH 8, 10 mM EDTA, 0.5% SDS.

Preparation of DNA libraries and sequencing.

DRIP immunoprecipitates obtained from 40 micrograms of digested genomic DNA were pooled together and sonicated. Ligation of Illumina Truseq adapters was performed according to manufacturer's instructions. NGS was performed by Biodiversa S.r.l. (Rovereto,TN, Italy) using Illumina platform Hiseq4000.

RNA-seq

Total cellular RNA was purified with the acid phenol method, quantified by UV absorbance and quality controlled by electrophoresis. RNA was then depleted of rRNA by Ribo-zero rRNA Removal Kit (Illumina) and libraries prepared with NEBNext Ultra Directional RNA library prep Kit for Illumina (NEB #E7420S) following manufacturer instructions. RNA sequencing was performed on Illumina HiSeq4000 platform (pair-end 2X150 bp) at Biodiversa S.r.l. (Rovereto,TN, Italy).

Quantitative PCR (qPCR)

Real-time PCR were performed using Applied Biosystems StepOne and SYBR Select Master Mix for CFX (Applied Biosystems). Quantification and melting curve analyses were performed using StepOne Software v2.2.3 as indicated by the supplier. Specificity of PCR products was routinely controlled by melting curve analysis.

Bioinformatic analyses of genomic R loop maps.

Mapping.

Raw FASTQ reads were trimmed to remove adaptor contamination using Trimmomatic (7) and aligned to the human reference genome version hg19 with bwa (8) using aln and sampe commands. Reads with a mapping quality below ten, marked as positional duplicates and mapped in ENCODE blacklist regions (9) were excluded from further analysis. Reads filtering and downsampling normalization were performed with Samtools suite (10) using view and rmdup commands.

The calculation of fragment sizes for read pairs, the analysis of correlation between replicates and the Spearman correlation coefficient calculation was performed with Deeptools suite (11) using bamPEFragmentSize, multiBamSummary and plotCorrelation commands. Genomic signal tracks were built with Deeptools suite using bamCoverage command. Integrative Genomics Viewer (12) was used for genomic track visualization.

Peak calling and peak processing.

DRIP–seq peaks were called using MACS2.0 (13) using default parameters and FDR<0.05. Peak consensus between replicates was assessed using Bedtools suite (14). Peak annotation on gene (RefSeq database) was performed using custom python and R script. To establish DNA:RNA hybrid to one of the two DNA strands for further analysis, we assigned each R-loop peak to the most expressed gene, using custom python script, and the hybrid was then assigned to the template strand. “Shuffled” peak set was calculated randomly shuffling each peak set over the genome using Bedtools shuffleBed command. Every peak set was shuffled 1000 times.

Signal plot over TSS were calculated using Bedtools coverage command. TSS region were divided in four categories on the base of the gene expression level (top 10%, high expression; mid 57%, intermediate expression; low 33%, low expression; silent, no expression) as established by

RNA-seq experiments (high expression, FPKM>57.5; intermediate expression, 5.5<FPKM<57.5; low expression, 1<FPKM<5.5; no expression, FPKM<1). In addition, TSS categories were also defined based on the presence of a CpG island over a 4kb window and on the presence of a GC skew region in CpG island promoters. GC skew region were determined using SkewR tool with High threshold model set (5).

Differential analysis.

Differential analysis of peak signal level was performed using Bedtools coverage command and DeSeq2 (15). We called differential expressed peak with a *p*-value < 0.001 and a treated/control fold change > 1.5.

Differential analysis of peak length was performed using only peaks that were present both in control and treatment samples. We performed a t-test and robust moderated t-test from limma R package (16) and then select only peaks with a treated/control size fold-change > 1.5 and a *p*-value < 0.05. Randomization was performed using bedtools shuffle, and shuffling each peak set only on genomic regions containing expressed genes with a CpG island extended by 5 kb upstream and 5 kb downstream. Matched unchanged peak sets were prepared choosing, for every extended peak, an unchanged peak with the same dimension and the same sub-genic localization (Upstream, Genebody, Downstream). Randomization was performed in the same way of the extended peak sets. Intersection with G4 set were performed using bedtools suite. Statistical significance was determined with the Kolmogorov-Smirnov test.

Detection of proteins by western blottings

Before performing Western Blotting analysis, cells were lysed and proteins extracted with the following protocols:

Whole Cell Extract (WCE).

Whole-cell lysates were prepared by lysing the cells in boiling buffer (1% SDS, 10 mM Tris pH 7.4) in the presence of Halt™ Protease Inhibitor Cocktail, EDTA-Free -100X- (Thermo Fisher ref #87785) and Halt™ Phosphatase Inhibitor Single-Use Cocktail (Thermo Fisher ref #78428). The viscosity of the samples is reduced by brief sonications.

Cellular extracts for ATM and pATM.

After washing with cold 1X PBS, cells were scraped in PBS, collected in a 1.5 ml tube, and lysed with cold Lysis Buffer (50mM Tris-HCl pH 8.0, 300 mM NaCl, 0.4% NP-40, 10 mM MgCl2, 5mM DTT, Halt™ Protease Inhibitor Cocktail, EDTA-Free -100X- (Thermo Fisher ref #87785) and Halt™ Phosphatase Inhibitor Single-Use Cocktail (Thermo Fisher ref #78428). Cells were then centrifuged at 13000 rpm, and supernatants were transferred to fresh tubes containing dilution buffer (50 mM Tris-HCl, pH 8.0, 0.4% NP-40, 5 mM DTT).

Immunoblotting.

Proteins were separated by SDS-PAGE (polyacrilammide percentage: 6% for ATM, pATM; 10% for mCherry-RNaseH1) and immunoblotted with the following antibodies: anti-ATM (Santa-Cruz ref # sc23921), anti-ATM-pS1981 (Cell Signalling ref #S1981), anti-mCherry (Genetex ref #GTX128509). Immunoblotting was revealed by chemiluminescence using ChemiDoc MP System (Bio-Rad).

Cytofluorimetry

Cytofluorimetry was performed as previously described in (Manuscript & Magnitude, 2013). Briefly, cells were detached using 0.2X Trypsin-EDTA, and resuspended in cell culture medium. Cells were counted and collected (usually >150,000). After centrifugation, cells were resuspended in ice-

cold PBS containing 1% FBS. Cells were then fixed in ice-cold 100% ethanol and stained with 50 µg/mL of propidium iodide.

Cell survival MTT assay

Cell killing activity of tested compounds was determined with the MTT assay in U2OS and U2OS_RH cell lines. After 24 hours of treatment, compounds were removed from culture medium and cells were further cultured in fresh medium for two days. Then, 500 µg/ml of Thiazolyl Blue Tetrazolium Bromide (MTT) solution (5 mg/ml in PBS, Merck ref #2128) was added to each well and incubated for 90 minutes at 37°C. Medium was subsequently removed from wells and precipitated formazan crystals were solubilized in 300 µl of DMSO. 100 µl of the solution was transferred in 96-well and absorbance at 595 nm was measured using a multiplate reader. Linear regression parameters were determined to calculate the IC_{50} (GraphPad Prism 4.0, Graph Pad Software Inc.).

List of primers used to determine R-loop levels by DRIP-qPCR

Gene name and sequence name are followed by primer sequence in the 5'>3' direction:

PLEKHG6_2	Peak_7494_Fw1	CTCTGTGACTTGCTTATCA
	Peak_7494_Rv2	GACACACTAAGAAAGAGACC
COPS2	Peak_11435_Fw1	AAGTAACTCAACTGCATAGC
	Peak_11435_Rv1	GGGAACTTAACAAGATGTAA
MEGF11	Peak_11727_Fw1	GAATGGATATTATGGGAAG
	Peak_11727_Rv1	AAGAAGTGAGGAAGAACAG
TLE3	Peak_11834_Fw1	CAGTCTCTCCTCAAAGAGTA
	Peak_11834_Rv1	ATAAGATTGTGTGTTTGTCC
ACSF3	Peak_14157_Fw1	ATGTAGATCTATGAGCAAGC

	Peak_14157_Rv1	AATTTAGACTCAGGACAGC
SEPT9	Peak_16078_Fw1	GGTAGTGTCTTCTTCCAGTA
	Peak_16078_Rv1	CTCTGTAGAAGGGTGTAATG
LRRC8D	Peak_1746_Fw2	GAGATGTTTAGTCTGGTGTC
	Peak_1746_Rv2	AATGGAATTAATAAAGATGC
SPTBN4	Peak_18077_Fw1	TTCCATCCATCCTCTATC
	Peak_18077_Rv1	GTGGAAGGAATAGGAAAG
TSGA10	Peak_20255_Fw1	AATTAAACTTTCTTCCTCCT
	Peak_20255_Rv1	CTTTCCTCTAGTGATATTGC
VSIG8	Peak_2618_Fw1	GTCTTCTGGAGTCACAGCCC
	Peak_2618_Rv1	TGCCGATTTGTTTGCCTGTG
TBC1D7	Peak_29811_Fw2	CCCTCATGTTGCAGTCCACT
	Peak_29811_Rv2	CCCCTCAGTCTGAGCAAGTG
GBGT1	Peak_36062_Fw1	CAGGTCTTTGGGTCCCCATC
	Peak_36062_Rv1	CAAACAAGCCGTCCAAGGTG
PLEKHG6_1	Peak_7276_Fw2	CGCATCACCAAGTACCCACT
	Peak_7276_Rv2	GATTCACCAGAGTCCCAGCC
DDX24	Peak_10329_Fw1	TACTTGGGGCAGACACAAGC
	Peak_10329_Rv1	CCAGCAAGAAGAACGTCGGA
BAHD1	Peak_10792_Fw1	GCCTACCACTTTGCTGGGAT
	Peak_10792_Rv1	TGCAACTTTTTCCCTGGGGT
RPL13A	Fw	AATGTGGCATTTCCTTCTCG
	Rv	CCAATTCGGCCAAGACTCTA
EIF5A	Fw	GGTCGAGTCAGTGCGTTC
	Rv	GCAGTTCCATCTTCTCCC

17

SNRPN	Fw	GCCAAATGAGTGAGGATGGT
	Rv	TCCTCTCTGCCTGACTCCAT

SI RESULTS AND FIGURES

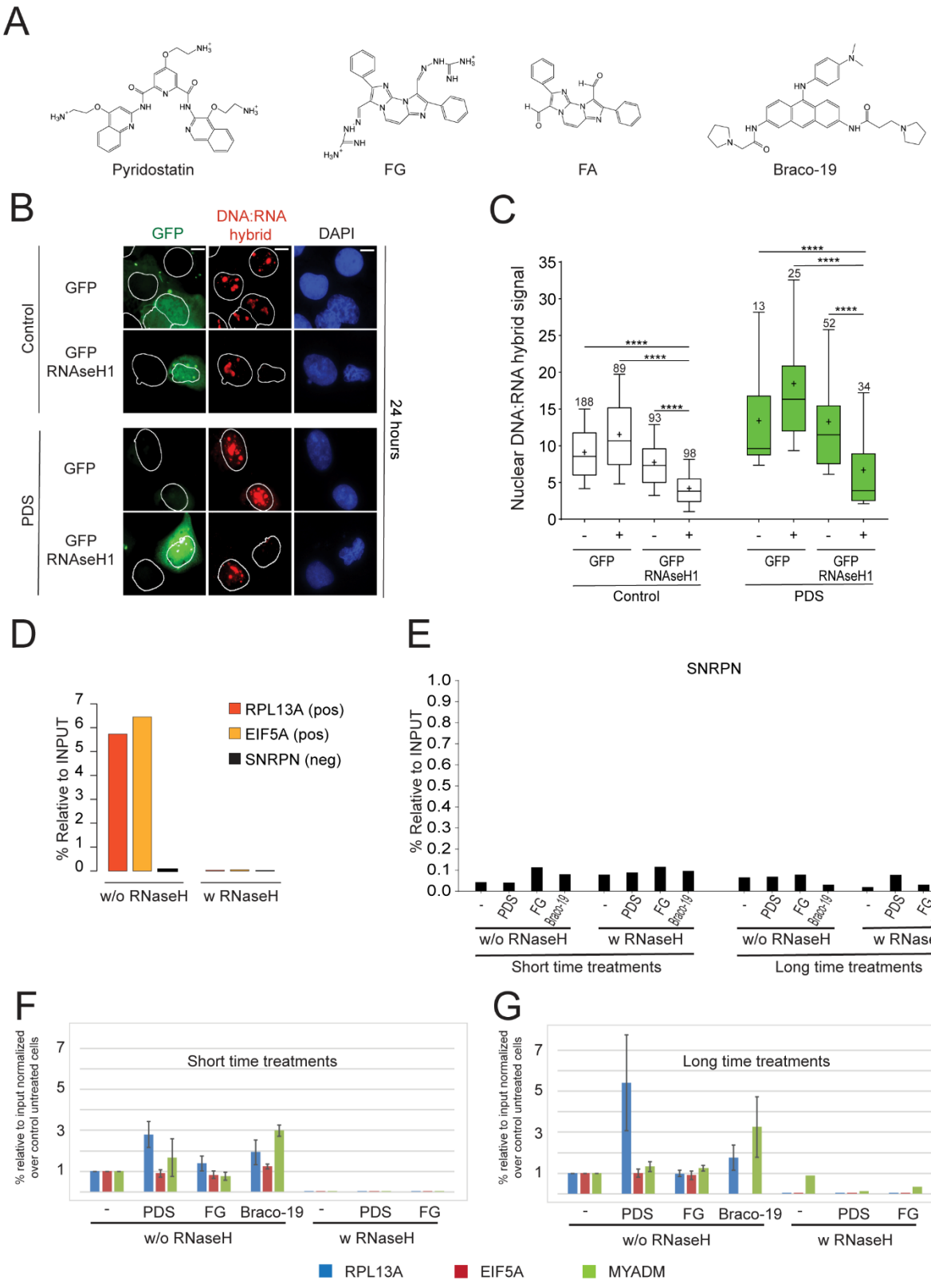


Figure S1. Compound structures and validation of S9.6 antibody. (**A**) Chemical structures of the studied compounds. (**B**) Effects of exogenous human RNaseH1 expression in U2OS cells on

fluorescence signals detected with S9.6 antibody. To assess the specificity of S9.6 Ab, we transiently overexpressed human RNaseH1, which specifically degrades RNAs annealed to DNA strands, in U2OS cells. Marked R-loop accumulation at highly transcribed genomic regions has been reported in the model organism *S. cerevisiae* when RNaseH1 gene is deleted (17, 18). U2OS cells were transiently transfected with GFP- and GFP-RNaseH1-expressing vector DNA, and then treated/untreated with 10 μM of PDS for 24 hours. Cells were then fixed and stained with S9.6 antibody (red) under high-stringency buffer conditions as described in SI Materials and Methods. In green, fluorescence signals of GFP or GFP-RNaseH1 as indicated. Scale bars: 10 μm. The results showed that human RNaseH1 overexpression abolished S9.6 signals in U2OS cells untreated or treated with PDS for 24 hours, supporting a specific recognition of DNA:RNA hybrids by S9.6 under our high-stringency conditions. (**C**) Quantification of S9.6 signals in cells expressing RNaseH1 and treated with PDS in comparison with cells not expressing RNaseH1 or expressing GFP alone (panel B). Total nuclear fluorescence is shown, and data were from two biological replicates. RNaseH1 expression abolished or strongly reduced the hybrid signal in cells untreated or treated with PDS. Thus, the results showed that the nuclear S9.6 signal was largely due to DNA:RNA hybrids. Asterisk numbers indicate the level of statistical significance: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$, as determined with the Kolmogorov/Smirnov parametric test. (**D**) In addition, we set up DRIP experiments to assess S9.6 specificity. DRIP-qPCR analysis of R-loop positive (pos) and negative (neg) loci (5, 6) with the purified S9.6 antibody. DRIP and qPCR were performed in untreated U2OS cells as described in Online Method. Treatments of cellular DNA with *E. coli* RNaseH (an enzyme that degrades RNA only when annealed to DNA) was performed after restriction enzyme digestion and prior to S9.6 addition and immunoprecipitation of DNA. The results are reported as percentage of recovered DNA relative to input DNA. Thus, pre-treatment of DNA with RNaseH fully abolishes DNA recovery supporting the specificity of S9.6 antibody under our experimental conditions. It must be noted that the negative genomic region (SNRPN), which does not

form R loops (5, 6), showed an enrichment at least 100-fold less than positive loci. (**E**) G4 binders do not increase of R-loops at the negative genomic locus SNRPN by DRIP-qrtPCR. The results of two biological replicates are reported as percentage of recovered DNA relative to input DNA. After DNA immunoprecipitated with S9.6, DNA enrichment was determined by qrtPCR as described in SI Materials and Methods. Short and long treatments are 5 minutes and 24 hours, respectively. Compound concentrations are: 10 μM for PDS and FG, and 15 μM for Braco-19. w/o and w indicate without and with RNaseH pre-treatment, respectively, of cellular DNA prior immunoprecipitation. (**F, G**) G4 binders induce some increase of R-loops at positive genomic loci (RPL13A, EIF5A and MYADM, (5, 6)) by DRIP-qrtPCR that are lost upon pre-treatment of DNA with RNaseH. Experiments were performed as described and graph details are as in (E). The bars show the fold-increase of treated samples over control untreated samples. Thus, the *E. coli* RNaseH fully abolished the DNA recovery at specific gene loci known to have R-loops (5, 6, 19) with DRIP experiments performed with U2OS cells treated with G4 ligands.

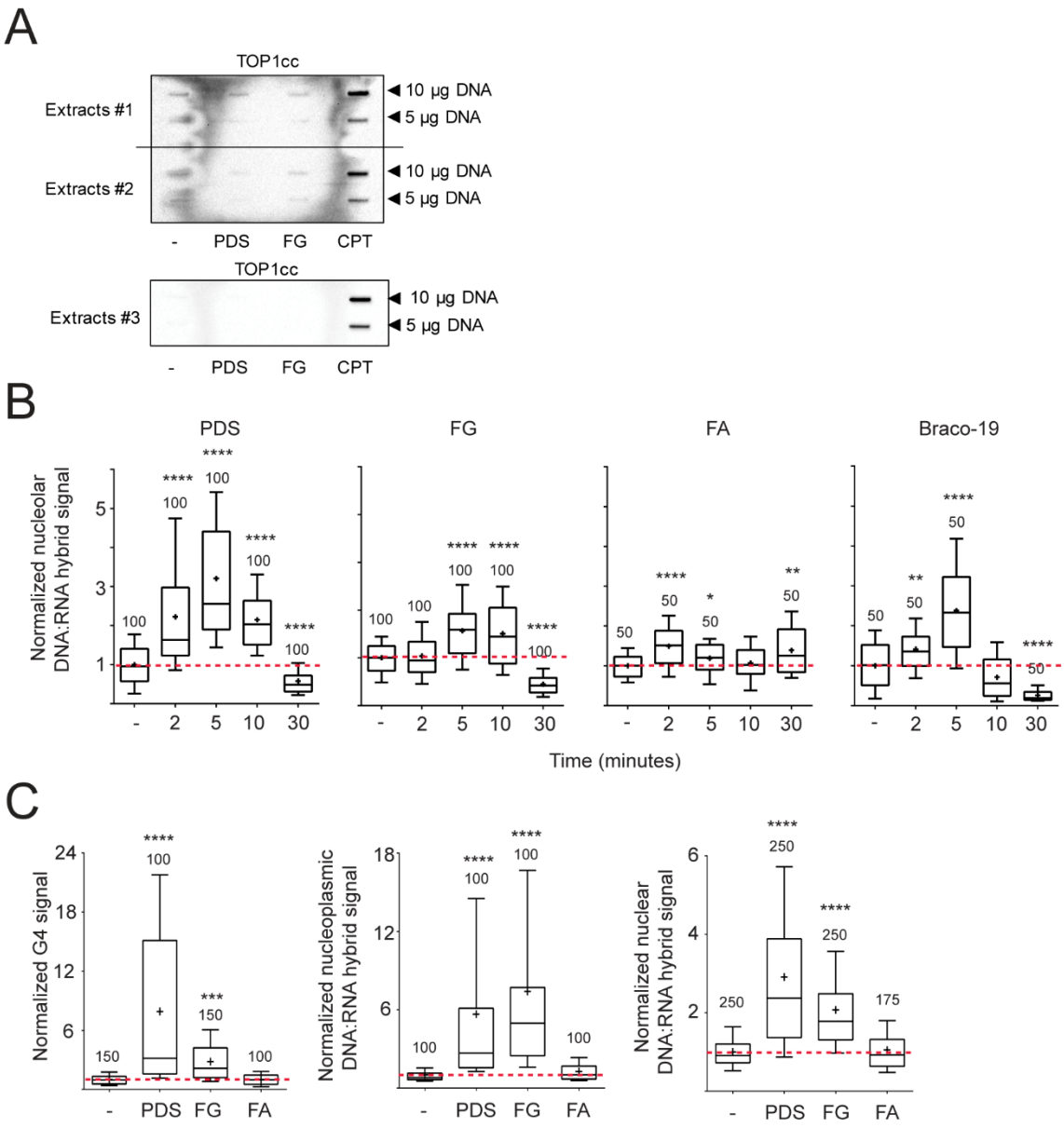


Figure S2. PDS and FG are not topoisomerase I poisons, and G4 binders increase DNA:RNA hybrid levels in the nucleolus. (**A**) FG and PDS are not topoisomerase I poisons as they do not increase Top1-DNA cleavage complexes (Top1cc) (20, 21)□. U2OS cells were left untreated or treated for 1 hour with PDS (10 µM), FG (10 µM) or camptothecin (CPT, 1 µM). Two concentrations of genomic DNA (10 and 5 µg) were probed with an anti-Top1cc antibody (Millipore, MABE1084). Data show the results of 3 biological replicates as described already (22). (**B**) Nucleolar levels of hybrids in U2OS cells treated with the indicated compound for different time periods. Cells were stained with S9.6 and anti-nucleolin antibodies as shown in Figure 1 A. Boxplots are reported as

detailed in legend of Figure 1 B. (**C**) Hybrid and G4 levels after 24 hour treatment with the indicated compounds as in Figure 1 C. Boxplots are shown as detailed in the legend to Figure 1 B. Data for all graphs are from at least two biological biological replicates.

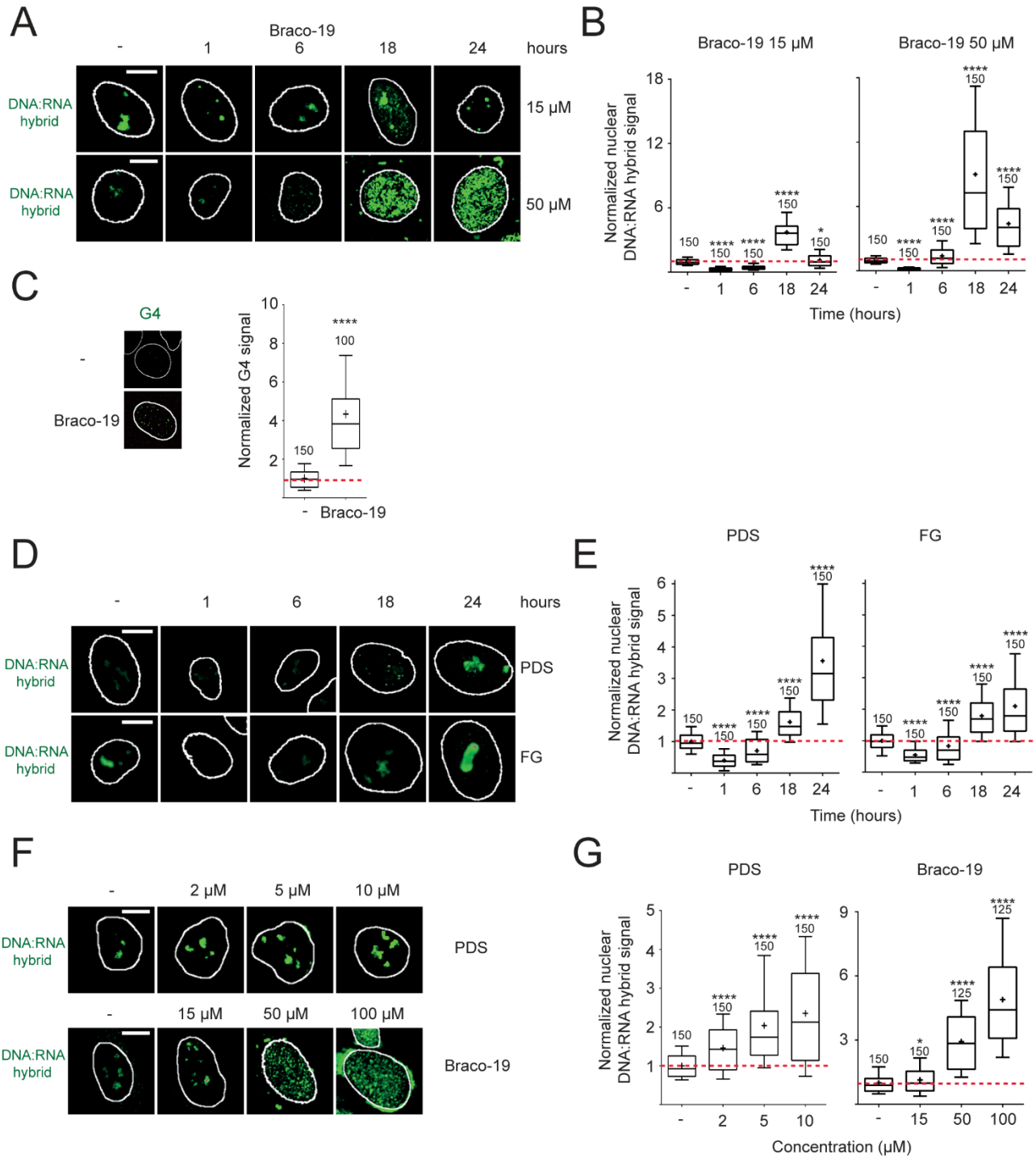


Figure S3. Kinetics and dose-dependance of hybrid induction by G4 ligands in U2OS cells. (**A**) Kinetics of hybrid increase in U2OS cells treated with Braco-19 at the indicated concentrations. (**B**) Nuclear hybrid levels were determined by fluorescence intensity of S9.6 signals in cells shown in panel A). Results are from two biological replicates, and normalized values over controls are shown as reported in legend of Figure 1 B. (**C**) Increase of G4 foci in U2OS cells treated with Braco-19 (15

μM) for 24 hours. Cell images and quantitative analyses are reported on the left and right sides, respectively. Y-axis reports G4 foci normalized to untreated cells and results are from two biological replicates. (**D**) Kinetics of hybrid formation in U2OS cells treated with 10 μM of PDS or FG. (**E**) Nuclear hybrid levels were determined by fluorescence intensity of S9.6 signals in cells shown in panel D). Results are from two biological replicates, and normalized values over controls are shown as reported in legend of Figure 1 B. (**F**) The indicated G4 binders increase hybrid signals in a dose dependent manner. Cells were treated for 24 hours with 2, 5, 10 μM of PDS or 15, 50, 100 μM of Braco-19. (**G**) Nuclear hybrid levels were determined by fluorescence intensity of S9.6 signals in cells shown in panel F). Results are from two biological replicates, and normalized values over controls are shown as reported in legend of Figure 1 B. Scale bars: 10 μm.

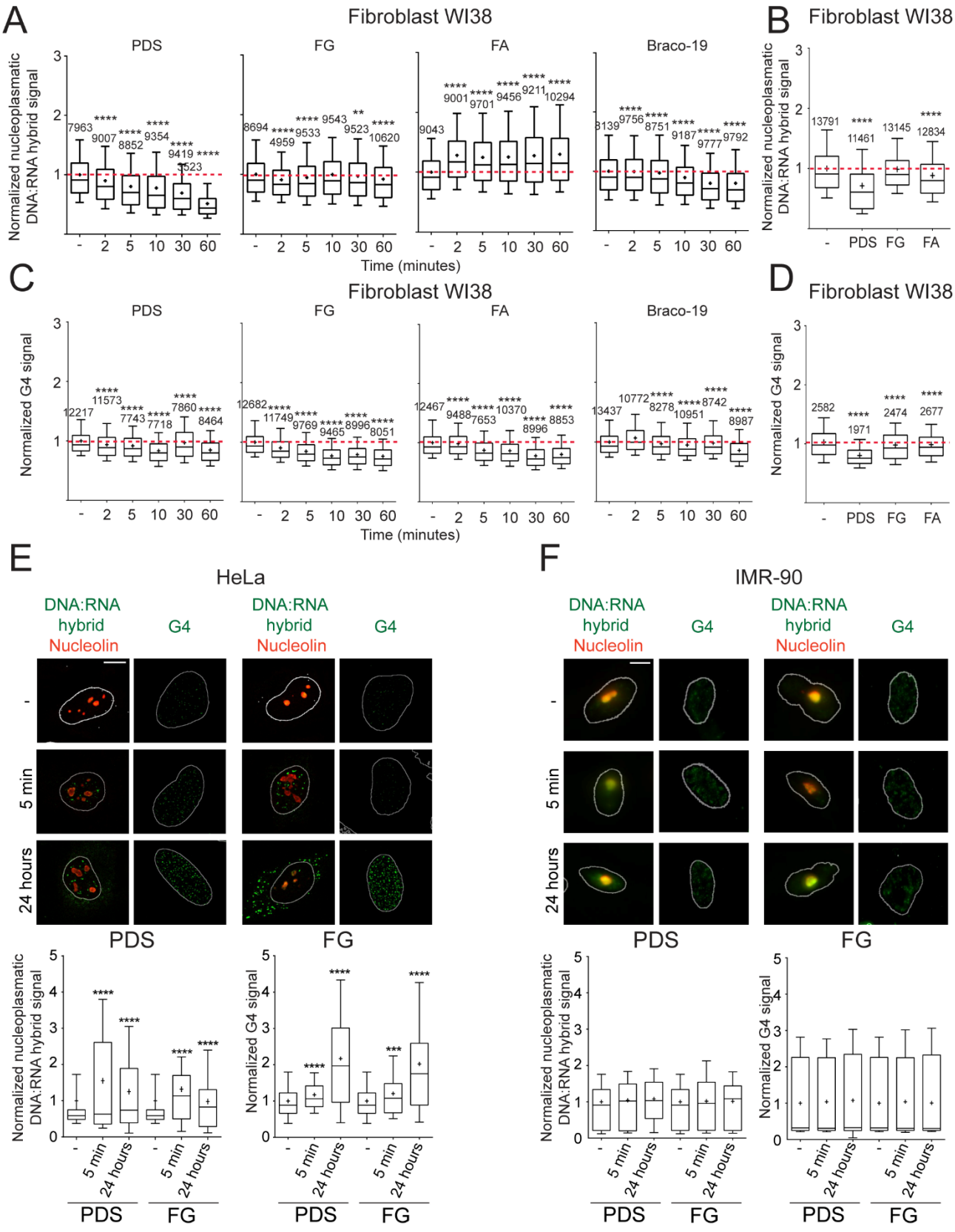
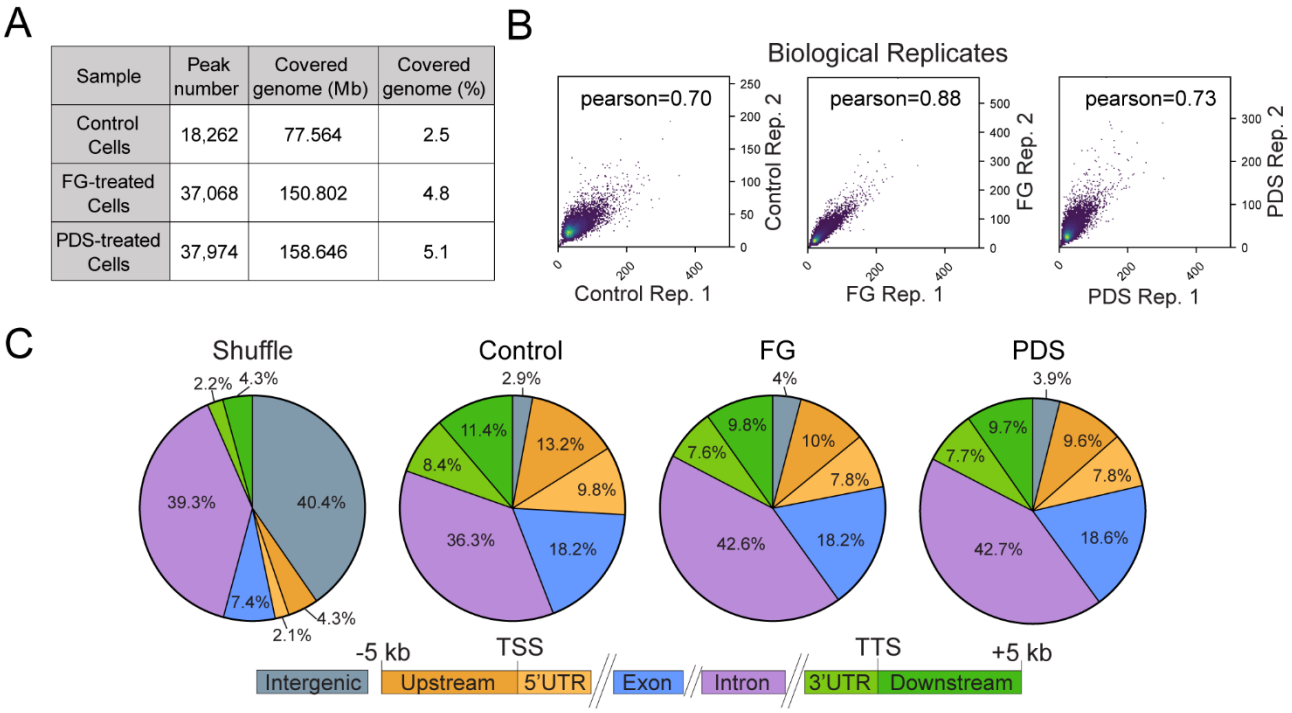


Figure S4. G4 binders induce nuclear hybrids and G4s in HeLa cancer cells but not in normal IMR-90 and WI-38 fibroblasts (**A**) Nucleoplasmatic hybrid levels in WI38 fibroblasts treated with

10 μM of PDS, FG, FA or 15 μM of Braco-19 for the indicated time periods, and then stained with S9.6 and Nucleolin antibodies. (**B**) Nucleoplasmatic hybrid levels in WI38 fibroblasts treated with 10 μM of PDS, FG or FA for 24 hours. (**C**) Levels of nuclear G4s in WI38 fibroblasts treated for the indicated times with 10 μM of PDS, FG, FA or 15 μM of Braco-19. (**D**) Nuclear G4 levels in WI38 fibroblasts treated with 10 μM of PDS, FG or FA for 24 hours. (**E**) Levels of nucleoplasmatic hybrids and nuclear G4 in HeLa cancer cells. Cells were treated for 5 minutes or 24 hours with FG or PDS, then fixed and stained with S9.6, nucleolin or BG4, as indicated. (**F**) Levels of nucleoplasmatic hybrids and nuclear G4 in normal human IMR-90 fibroblasts. Experiments were performed as in panel (E). In all the above panels, results are from two biological replicates, and graphs are as described in previous SI Appendix Figures. Asterisks indicate the level of statistical significance: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$, as determined with the Kolmogorov/Smirnov parametric test.

A

Sample	Peak number	Covered genome (Mb)	Covered genome (%)
Control Cells	18,262	77.564	2.5
FG-treated Cells	37,068	150.802	4.8
PDS-treated Cells	37,974	158.646	5.1



E

Sample	Control	FG-treated cells	PDS-treated cells	G4 motifs
Control Cells	1	-	-	-
FG-treated cells	0.81	1	-	-
PDS-treated cells	0.78	0.92	1	-
G4 motifs	0.57	0.60	0.53	1

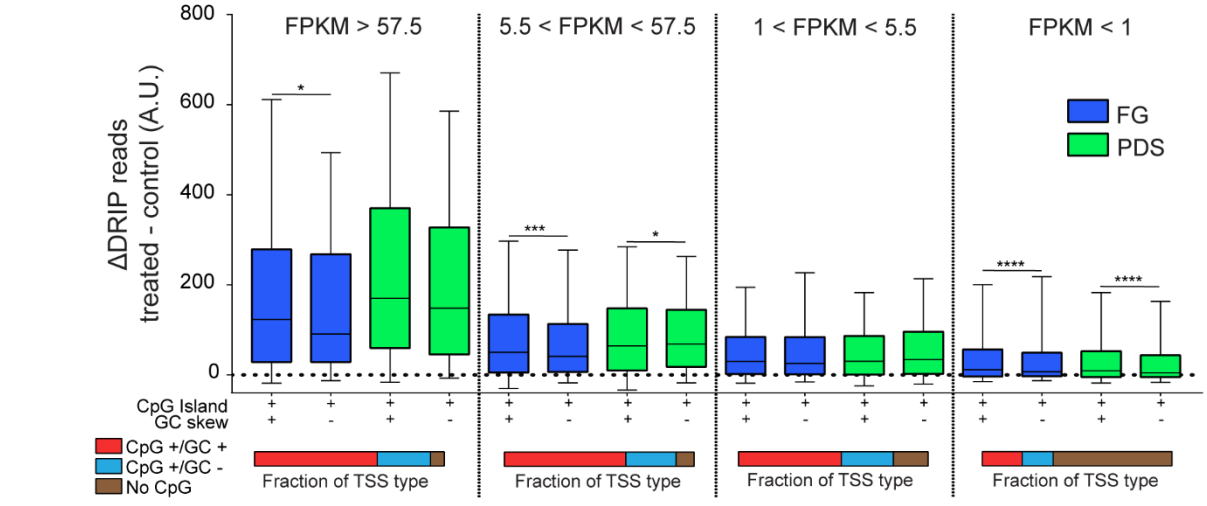


Figure S5. Detection, distribution and pattern correlation of R-loop peaks in U2OS cells treated with PDS or FG. (**A**) Total R loop peaks identified in control and G4 binder-treated cells and corresponding covered genome. (**B**) Scatterplots showing correlation between DRIP-seq peak biological replicates of Control, FG and PDS treated cells. Each dot represents a DRIP-seq peak. (**C**)

Distribution of DRIP peaks for Control, FG and PDS across the genomic compartments depicted below. Numbers indicate the percentage occupied by each compartment. On the left, the graph shows the compartment distribution of randomly shuffled peaks over the full genome. (TSS) transcription start site; (TTS) transcription termination site. (**D**) Distribution of R-loop peaks in control (blue) and treated (red) cells in comparison with the distribution of Observed Quadruplex (G4 motifs established experimentally using PDS in 22. Only regions overlapping expressed genes in U2OS cells are considered. Chromosome 11 is shown as a representative example. (**E**) Pearson correlation coefficients among R loop peak distributions and Observed Quadruplex - PDS (G4 motifs) in the whole genome. Only regions overlapping expressed genes in U2OS cells are considered. (**F**) Box plots showing ΔDRIP-seq read counts between treatment and control condition at promoter TSS divided on the base of transcription level, the presence of CG islands and the presence of GC Skew at CG island level. Analyzed regions are from 2000 bp upstream to 2000 bp downstream the TSS. Genes are split into four categories based on transcript levels as established by RNA-seq data and indicated at the top of graphs (FPKM is Fragments Per Kilobase of gene exon model per Million reads mapped). As indicated below, TSS promoters with CG islands and GC skew constitute 64.7%, 64%, 54.3% and 21.1 % for the four gene sets from left to right, respectively. Statistical significance was determined with the Kolmogorov-Smirnov test. Asterisks indicate p-values: **, $p<0.01$; ***, $p<0.001$; ****,$p<0.0001$.

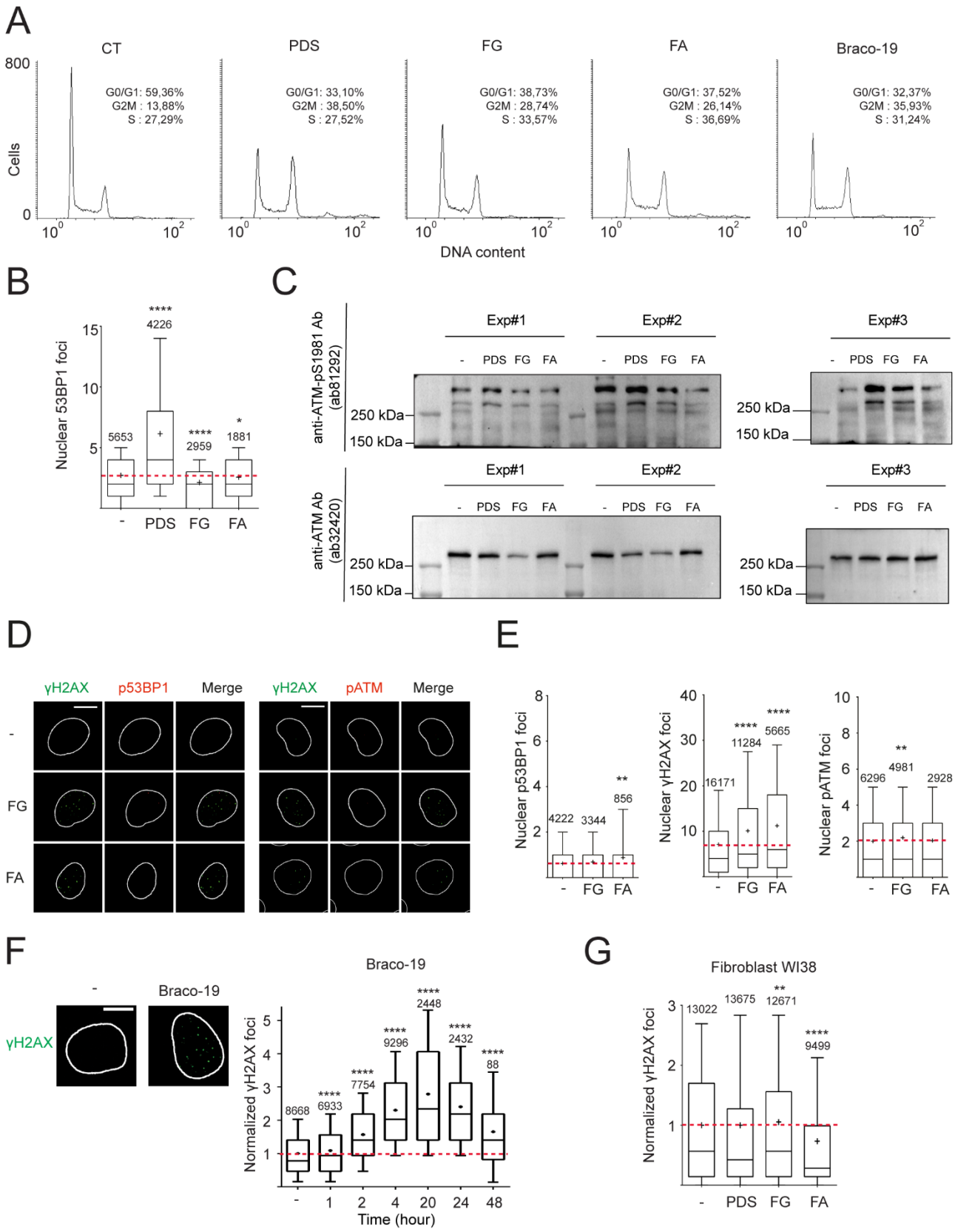


Figure S6. G4 binders induce cell cycle block at G2/M and DDR in human cancer U2OS cells.

(**A**) Human U2OS cells were treated with 10 µM of PDS, FG, FA or 15 µM of Braco-19 for 24 hours.

Then, cells were stained with propidium iodide (PI) and analyzed by flow cytometry. (**B**) Levels of 53BP1 foci in U2OS cells treated for 24 hours with the indicated ligands. (**C**) Full gel images of pATM and ATM western blots of the three biological replicates in U2OS cells treated for 24 hours with the indicated compounds. (**D**) γH2AX (green) and p53BP1 (red), on the left panel, and γH2AX (green) and pATM (red), on the right panel, induced by 10 μM of FG or FA for 24 hours in U2OS cells. After treatment, cells were co-stained with the corresponding specific antibodies. (**E**) Levels of p53BP1, γH2AX and pATM foci in cells treated as in panel (D). (**F**) γH2AX foci induced by 24 hours of treatment with 15 μM of Braco-19. Levels of Braco-19-induced γH2AX foci are shown on the right. The data are from two biological replicates. (**G**) Levels of γH2AX foci in WI38 fibroblast treated with 10 μM of PDS, FG or FA for 24 hours. Data are from two biological replicates. All boxplots show the results of two biological replicates and are reported as detailed in legend of Figure 1 B. Scale bars: 10 μm.

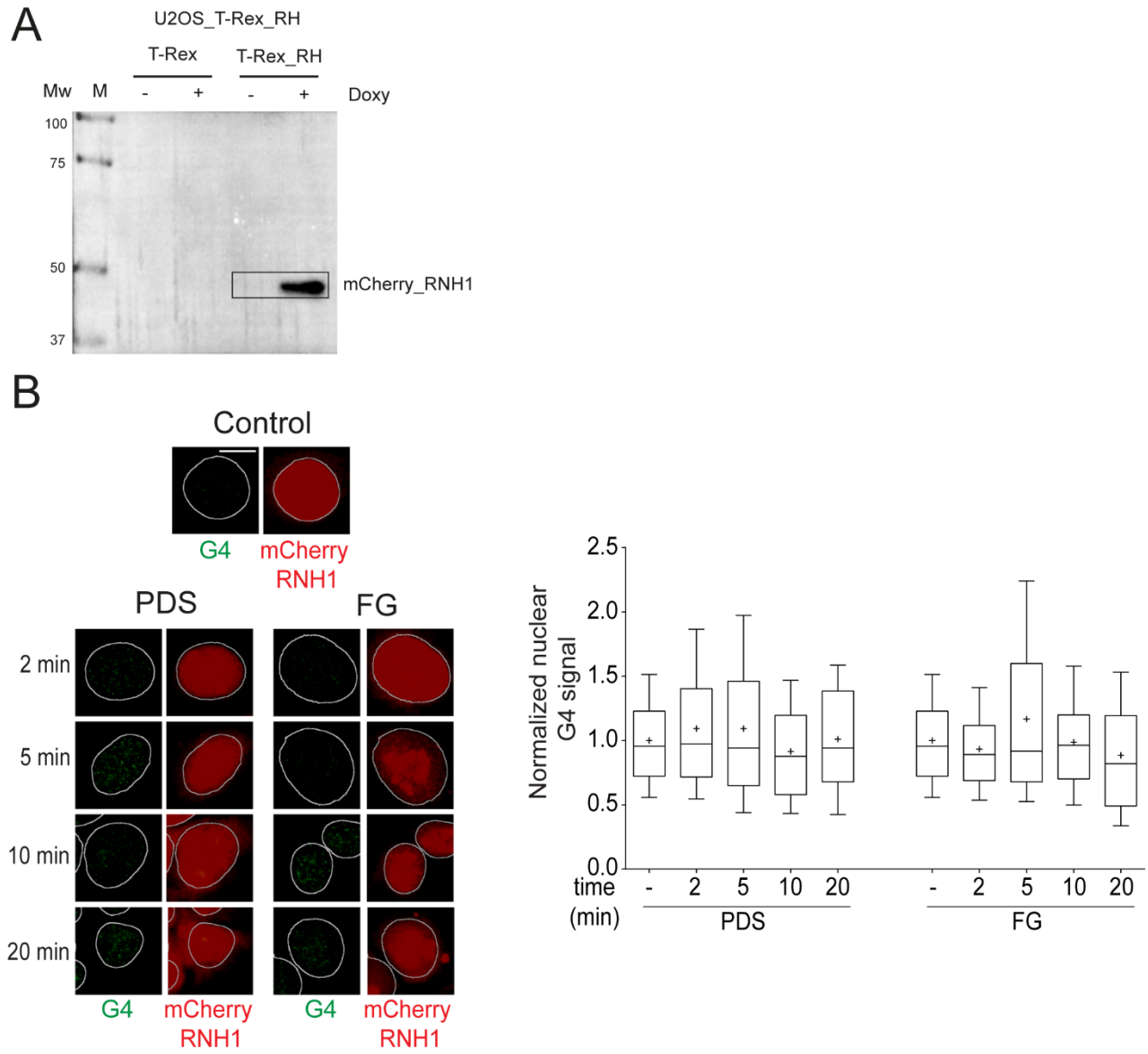


Figure S7. Expression of mCherry-RNaseH1 in the studied cells and G4 foci formation in cells overexpressing mcherry-RNaseH1. (**A**) Western blot analysis of mCherry-RNaseH1 expression in U2OS_TREX_RH cells treated with Doxycycline as detected with an mCherry-specific antibody. M, marker of molecular weight (Mw); numbers are in kDa. (**B**) G4 foci in U2OS_T-Rex_RH cells treated with PDS or FG (10 µM). U2OS_T-Rex_RH cells were exposed to Doxycycline for 24 hours, to overexpress mCherry-RNaseH1, and then incubated with the G4 ligand for the indicated times. On the left, cell images showing the G4 foci or mCherry-RNaseH1

expression as indicated. On the right, G4 foci levels in the same cells treated as indicated; G4 foci values are normalized to that of control, untreated cells.

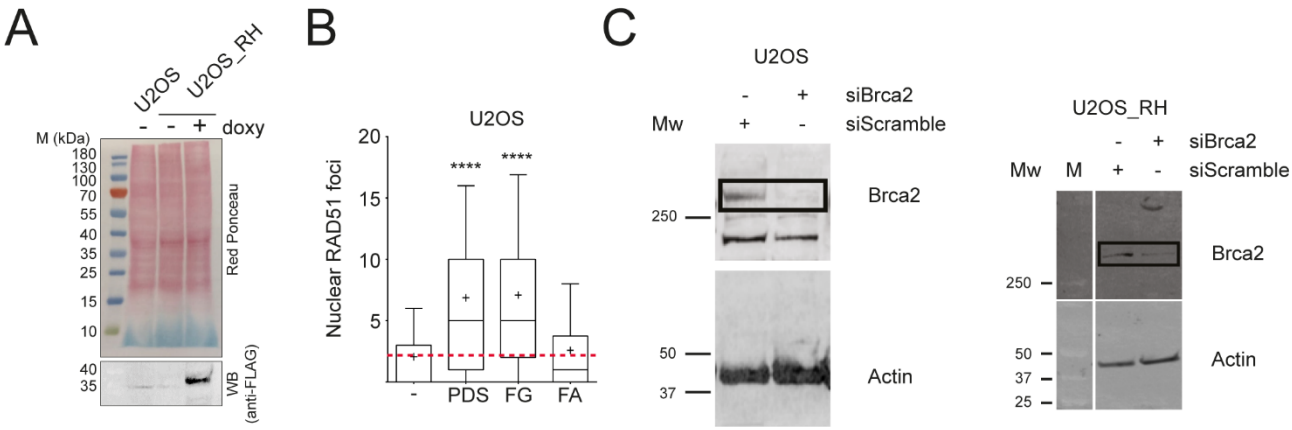


Figure S8. Western blotting analyses and quantification of Rad51 foci. (**A**) Red ponceau staining (top) and Western blot analysis (below) of RNaseH1 expression in U2OS_RH cells treated with Doxycycline; RNaseH1 is detected with an antiFLAG-specific antibody. M, marker of molecular weight in kDa. (**B**) Levels of RAD51 foci in U2OS cells treated with 10 μM of PDS, FG, FA for 24 hours. The data are from at two biological replicates and cell images are shown in Figure 4 H. (C) Western blot analysis of BRCA2 silencing in U2OS cells (left) and RNaseH1-expressing vector stably-transfected U2OS_RH cells (right). M, marker of molecular weight (Mw); numbers are kDa.

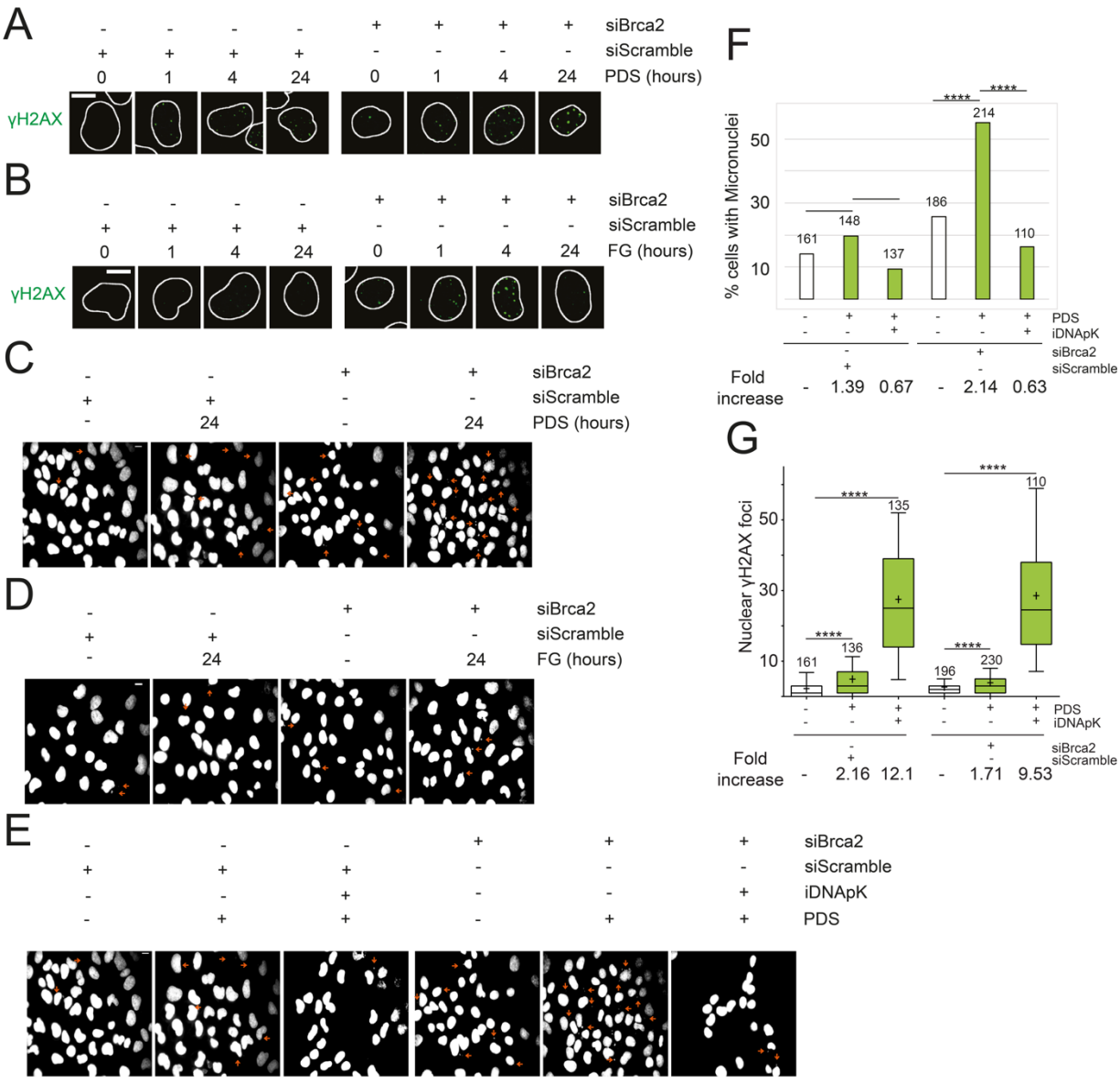


Figure S9. PDS-induced micronuclei in *BRCA2*-silenced U2OS cells and the effects of a DNA-PK inhibitor. (**A**) PDS-induced γH2AX foci in U2OS cell. U2OS cells have been silenced with siBRCA2 and siScramble, then treated with 10 μM of PDS for 1, 4, 24 hours. Cells were then stained with γH2AX antibody. (**B**) FG-induced γH2AX foci in U2OS cell. U2OS cell have been silenced with siBRCA2 and siScramble, then treated with 10 μM of FG for 1, 4, 24 hours. Cells were then stained with γH2AX antibody. (**C**) Micronuclei induced in scramble cells and cells lacking BRCA2 by 24 hours treatment with PDS. (**D**) Micronuclei induced in scramble cells and cells lacking BRCA2 by 24 hours treatment with FG. (**E**) Micronuclei induced in scramble cells and cells lacking BRCA2 by

24 hours treatment with PDS and DNA-PK inhibitor. (**F**) Micronuclei induced in U2OS cells silenced with a siBRCA2 and siScramble, then treated with 10 μM of PDS and DNA-PK inhibitor for 24 hours. The data show a representative experiment and cell images are shown in the panel (E). (**G**) PDS-induced γH2AX foci in U2OS cells of experiments shown in (E and F). Scale bars: 10 μm.

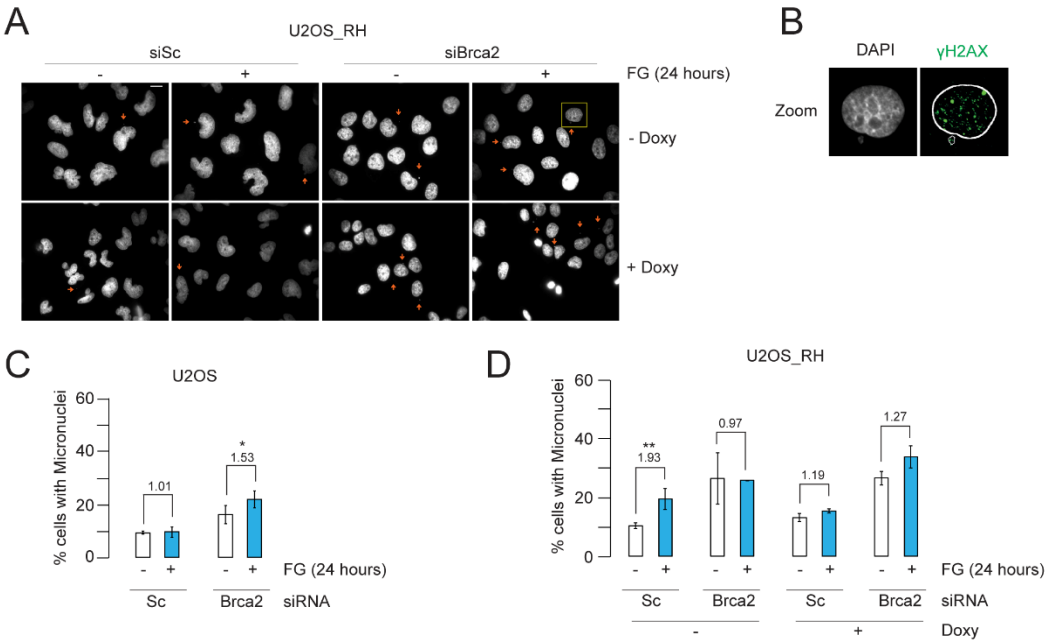


Figure S10. FG effects on micronuclei generation in U2OS cell lines. (**A**) Micronuclei induced in U2OS_RH cells transfected with scrambled siRNA (siSc) or siRNA against *BRCA2* (siBRCA2), and then treated with FG for 24 hours. Small red arrows indicate single micronuclei. Doxy, doxycycline treatment of cells to activate expression of exogenous RNaseH1. (**B**) Enlargement of the cell in the yellow square in panel (a). The image shows a micronucleus positive for γH2AX labelling. (**C**) Fractions of U2OS cells with micronuclei with and without *BRCA2* silencing and 24-hour treatments with FG. The data are from at least two biological replicates. Bars show mean values +/- SEM. (**D**) Fractions of U2OS_RH cells with micronuclei with and without *BRCA2* silencing, doxycycline and FG treatments as indicated. The data are from at least two biological replicates. Bars show mean values +/- SEM. For all graphs, fold-increase values are shown above the bars and are treated/control ratios of mean values. Statistical significance was determined with the Kolmogorov-Smirnov tests considering full cell populations. Asterisks show p-value as follows: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. Scale bars: 10 µm.

Supplemental References

1. Andreani A, et al. (2004) Potential Antitumor Agents. 34.(1) Synthesis and Antitumor Activity of Guanylhydrazones from Imidazo[2,1-b]thiazoles and from Diimidazo[1,2-a:1,2-c]pyrimidine. *Anticancer Res* 24:203–212.
2. Biffi G, Tannahill D, McCafferty J, Balasubramanian S (2013) Quantitative visualization of DNA G-quadruplex structures in human cells. *Nat Chem* 5(3):182–6.
3. Hänsel-Hertsch R, Spiegel J, Marsico G, Tannahill D, Balasubramanian S (2018) Genome-wide mapping of endogenous G-quadruplex DNA structures by chromatin immunoprecipitation and high-throughput sequencing. *Nat Protoc* 13(3):551–564.
4. Székvölgyi L, et al. (2007) Ribonucleoprotein-masked nicks at 50-kbp intervals in the eukaryotic genomic DNA. *Proc Natl Acad Sci U S A* 104(38):14964–9.
5. Ginno PA, Lim YW, Lott PL, Korf I, Chédin F (2013) GC skew at the 59 and 39 ends of human genes links R-loop formation to epigenetic regulation and transcription termination. *Genome Res* 23(10):1590–1600.
6. Manzo SG, et al. (2018) DNA Topoisomerase I differentially modulates R-loops across the human genome. *Genome Biol* 19(1):100.
7. Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30(15):2114–2120.
8. Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25(14):1754–1760.
9. Consortium TEP (2012) An integrated encyclopedia of DNA elements in the human genome. *Nature* 489(7414):57–74.
10. Li H, et al. (2009) The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25(16):2078–2079.
11. Ramírez F, et al. (2016) deepTools2: a next generation web server for deep-sequencing data analysis. *Nucleic Acids Res* 44(W1):W160–W165.
12. Thorvaldsdottir H, Robinson JT, Mesirov JP (2013) Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief Bioinform* 14(2):178–192.
13. Zhang Y, et al. (2008) Model-based Analysis of ChIP-Seq (MACS). *Genome Biol* 9(9):R137.
14. Quinlan AR, Hall IM (2010) BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 26(6):841–842.
15. Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15(12):550.

16. Smyth GK (2004) Linear Models and Empirical Bayes Methods for Assessing Differential Expression in Microarray Experiments. *Stat Appl Genet Mol Biol* 3(1):1–25.

17. Chan YA, et al. (2014) Genome-Wide Profiling of Yeast DNA:RNA Hybrid Prone Sites with DRIP-Chip. *PLoS Genet* 10(4):e1004288.

18. El Hage A, Webb S, Kerr A, Tollervey D (2014) Genome-Wide Distribution of RNA-DNA Hybrids Identifies RNase H Targets in tRNA Genes, Retrotransposons and Mitochondria. *PLoS Genet* 10(10):e1004716.

19. Sanz LA, et al. (2016) Prevalent, Dynamic, and Conserved R-Loop Structures Associate with Specific Epigenomic Signatures in Mammals. *Mol Cell* 63(1):167–178.

20. Pommier Y, Marchand C (2011) Interfacial inhibitors: targeting macromolecular complexes. *Nat Rev Drug Discov* 11(1):25–36.

21. Capranico G, Marinello J, Chillemi G (2017) Type I DNA Topoisomerases. *J Med Chem* 60(6). doi:10.1021/acs.jmedchem.6b00966.

22. Mouly L, et al. (2018) PARP-1-dependent RND1 transcription induced by topoisomerase I cleavage complexes confers cellular resistance to camptothecin. *Cell Death Dis* 9(9):931.