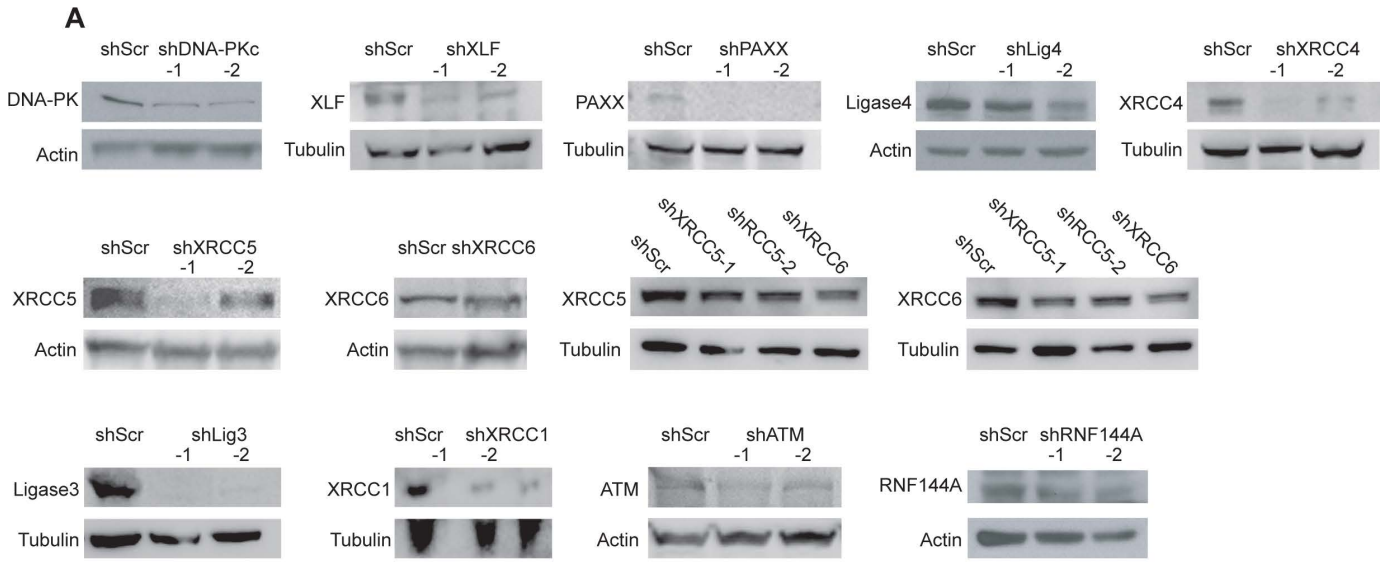


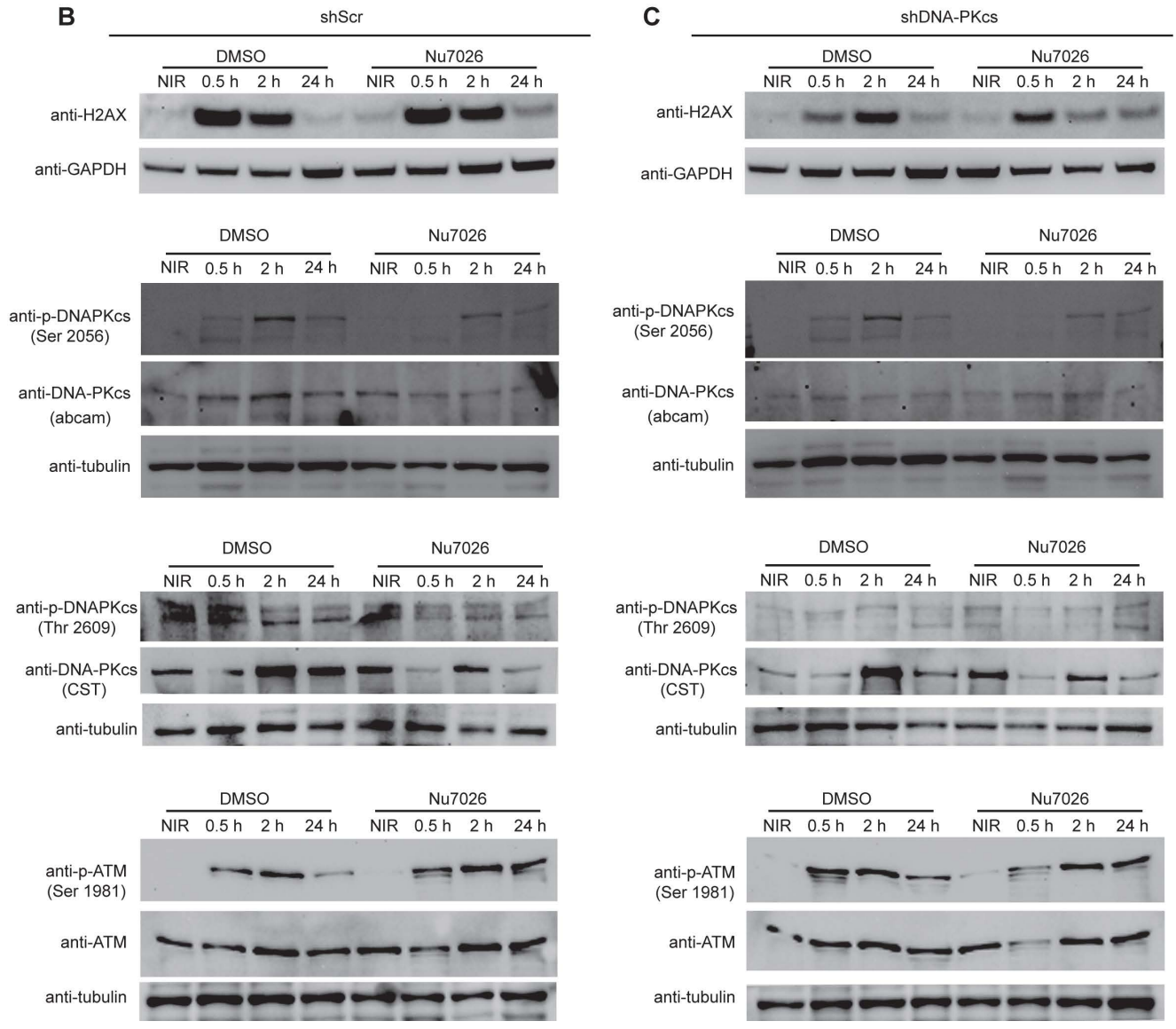


Fig. S1: Western blot analysis of protein expression.

A. Verification of the efficiency of corresponding shRNAs by Western blot. Cells were collected from passage 2 or 3 after selection with puromycin and whole cell lysates were extracted. **B, C.** Western blot for corresponding proteins in the presence or absence of Nu7026 (10 μM) in response to irradiation. shScr (**B**) and shDNA-PKcs (**C**) were treated with DMSO or Nu7026 (10 μM) 1 h before 6 Gy. Cell lysate was collected at the indicated time points. Approximately 70 μg total protein was loaded into each lane.

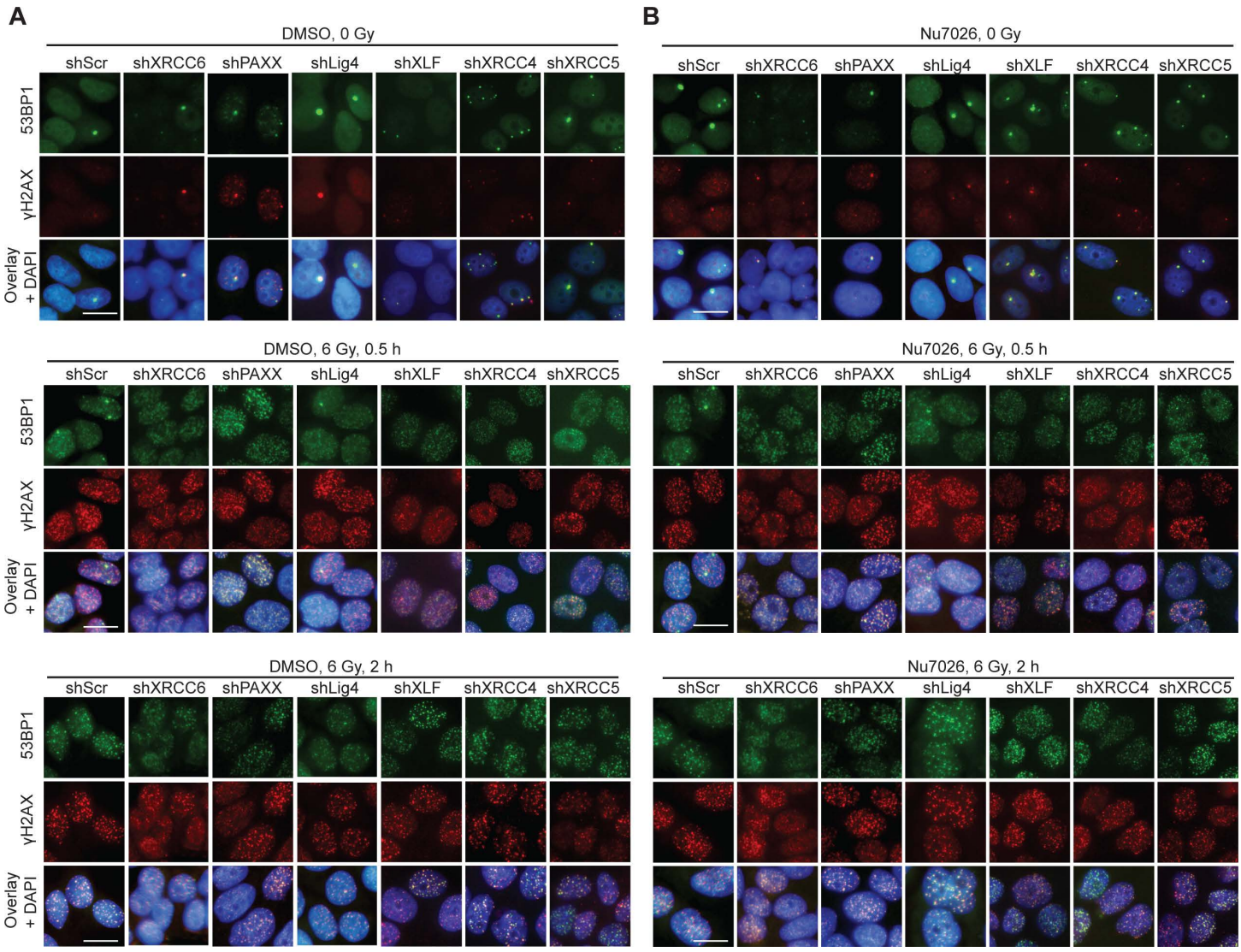


Fig. S2: DNA-PKcs inhibitor Nu7026 does not alter foci formation in cells expressing shRNAs targeting NHEJ repair factors.

A, B. Immunofluorescence analysis of γH2AX and 53BP1. shScr and shNHEJ were treated with DMSO (**A**) or Nu7026 (10 μM) (**B**)1 h before 6 Gy. Cells were stained at indicated time points after radiation with anti-53BP1 (green) or anti-γH2AX (red). Three color overlay with DAPI (blue) shown for representative examples. Scale bar = 20 μm.

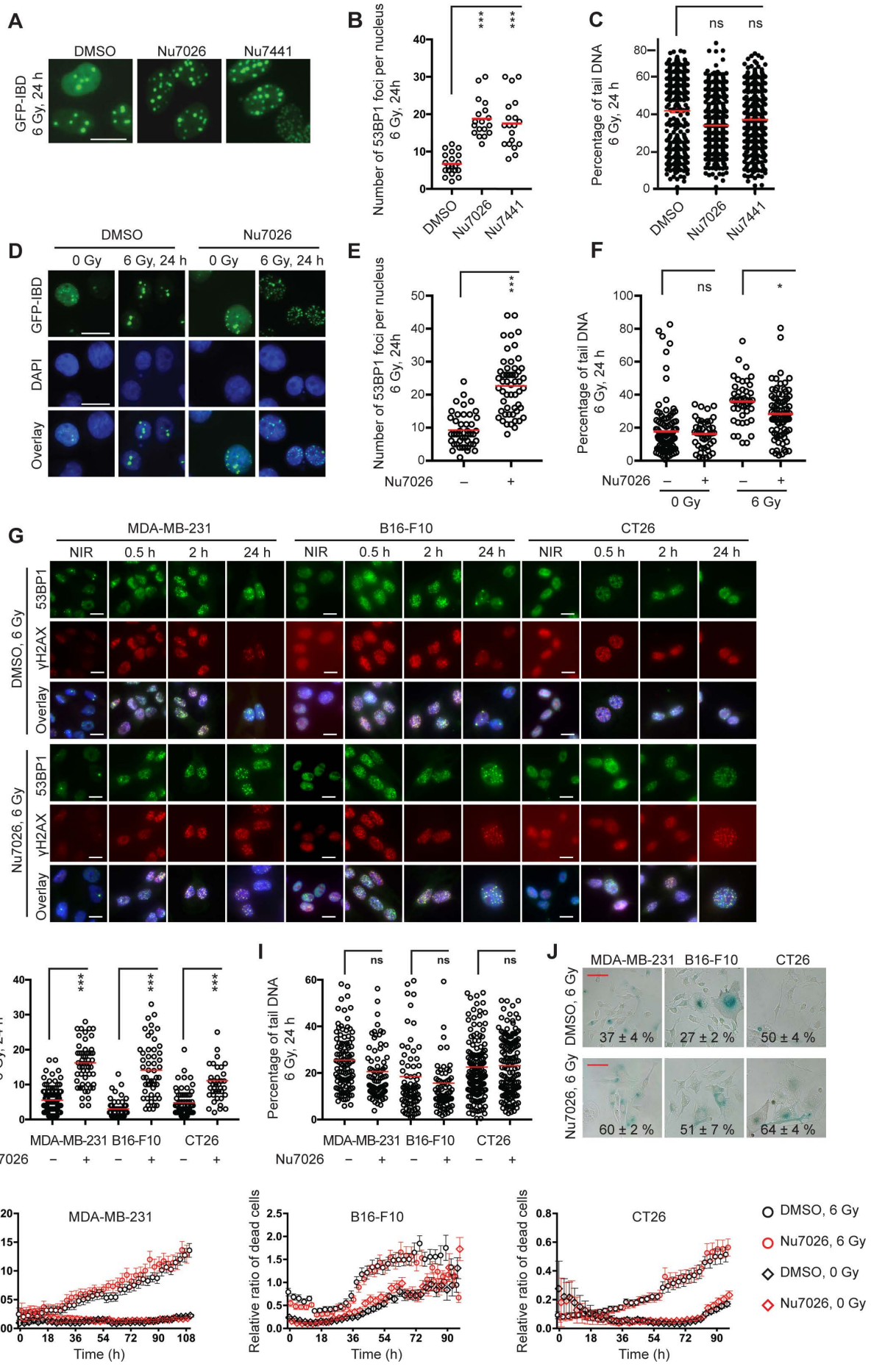


Fig. S3: The effect of DNA-PKcs deficiency on foci resolution is not a cell-type- or inhibitor-specific phenotype.

A. Fluorescence imaging of GFP-IBD foci in MCF7 cells. MCF7 cells were treated with DMSO, Nu7026 (10 μM) or Nu7441 (1 μM) 1 h before 6 Gy. Representative images are shown at 24 h after irradiation. Scale bar = 20 μm. **B.** Quantification of GFP-IBD foci as in **A**. N > 20 cells, mean ± s.d. (red bar). **C.** Quantitative analysis of DSBs in MCF7 cells detected by neutral comet assay after treatment as in **A.** Cells were collected 24 h after 6 Gy. N > 100. **D**. Live-cell fluorescence imaging of GFP-IBD reporter for 53BP1 in MDA-MB-435 cells. MDA-MB-435$^{GFP\text{-}IBD}$ cells were treated with DNA-PKcs inhibitor Nu7026 (10 μM) 1 h before IR and images captured 24 h after 0 or 6 Gy. Representative images are shown. Scale bar = 20 μm. **E.** Quantification of GFP-IBD foci as in **D** at 24 h. N > 50 cells, mean ± s.d. (red bar). **F.** Quantitative analysis of DSBs in MDA-MB-435 cells by neutral comet assay after treatment with DMSO or Nu7026 (10 μM)1 h before 0 or 6 Gy. Cells were collected 24 h after 6 Gy. **G.** Immunofluorescence analysis of γH2AX and 53BP1. MDA-MB-231, B16-F10, and CT26 cells were treated with DMSO or Nu7026 (10 μM) 1 h before 6 Gy. Cells were stained at the indicated time points after radiation with anti-53BP1 (green) or anti-γH2AX (red). Three color overlay with DAPI (blue) shown for representative examples. Scale bar = 20 μm. **H.** Quantification of 53BP1 foci as in **G** at 24 h. N > 100 cells, mean ± s.d. (red bar). **I**. Quantitative analysis of DSBs in MDA-MB-231, B16-F10, and CT26 cells by neutral comet assay after treatment with DMSO or Nu7026 (10 μM)1 h before 6 Gy. Cells were collected 24 h after 6 Gy. N > 100 cells, mean ± s.d. (red bar). **J.** SA-βGal staining in indicated cells. Cells were treated as in **I** and stained 5 d after 6 Gy. At least 5 images were captured with randomly selected, non-overlapping fields. Representative images are shown. Mean percentage of SA-βGal^{+} cells ± s.d. from five 20X fields indicated. Scale bar = 200 μm. **K.** Growth curve of indicated cells. Cell were treated as in **I**. Cell growth was recorded by IncuCyte S3 immediately after 6 Gy for 4 d. Unpaired t-test compared to DMSO controls, ***, $p < 0.001$; *, $p < 0.05$; ns, $p > 0.05$.

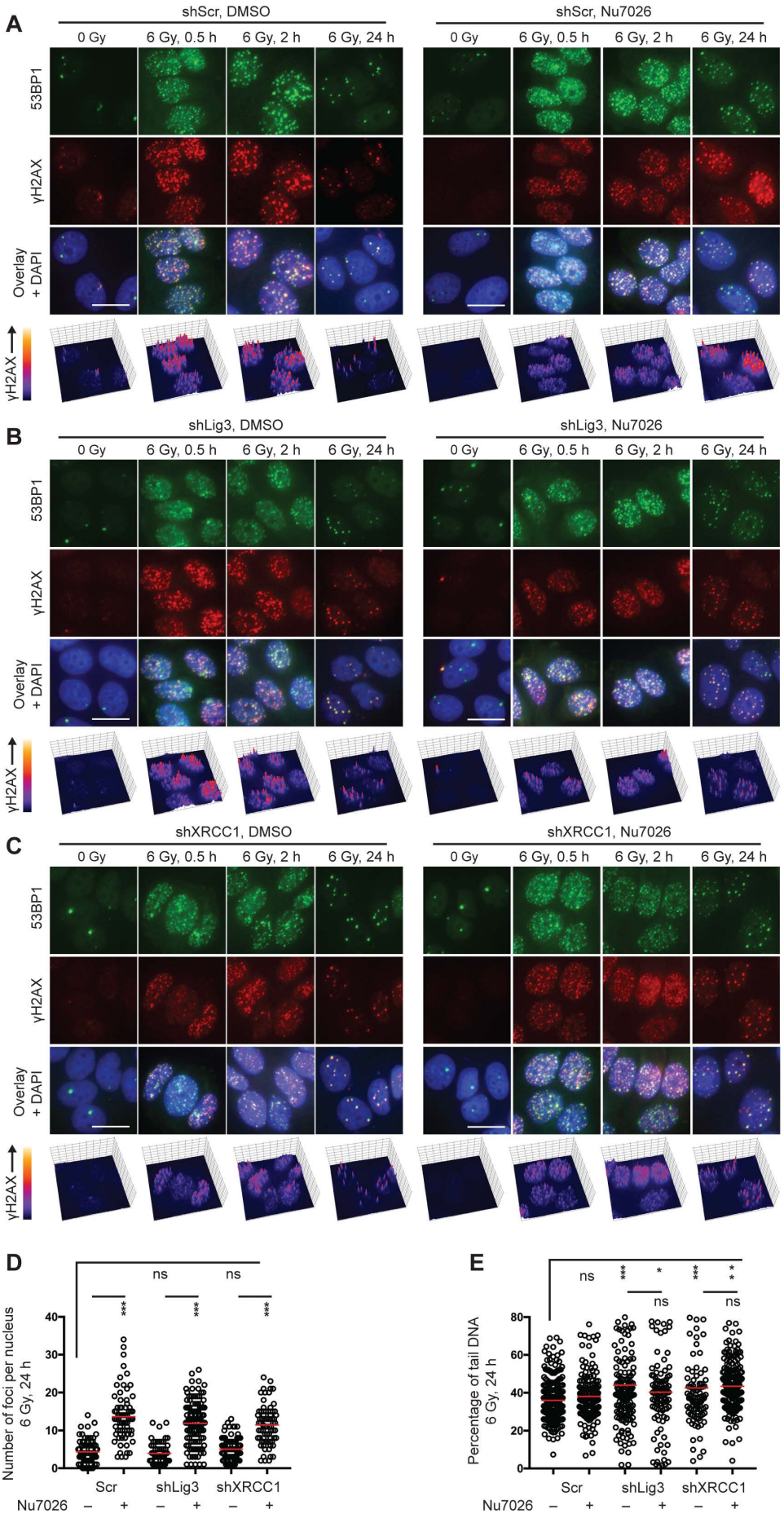


Fig. S4: Inhibition of DNA-PKcs induces persistent foci without further delaying DSB repair in cells with alt-EJ defects.

A, B and **C**, Immunofluorescence analysis of γH2AX and 53BP1. shScr (**A**) control and shLig3 (**B**) and shXRCC1 (**C**) targeting alt-EJ were treated with Nu7026 (10 μM) 1 h before 6 Gy. At indicated time points after irradiation, cells were analyzed for foci with anti-53BP1 (green) or anti-γH2AX (red), shown as three color overlay with DAPI (blue) and perspective plots of γH2AX staining intensity for representative examples from each condition. Scale bar = 20 μm. **D.** Quantification of γH2AX foci as in **A, B** and **C** at 24 h after 6 Gy. N > 100 cells, mean ± s.d. (red bar). Unpaired t-tests compared to non-Nu7026 controls, ***, $p < 0.001$. **E.** Quantitative analysis of DSBs in MCF7 cells by neutral comet assay after treatment as in **A.** Cells were collected 24 h after 6 Gy. N > 100 cells, mean ± s.d. (red bar). Unpaired t-tests compared to DMSO controls, ***, $p < 0.001$; **, $p < 0.01$.

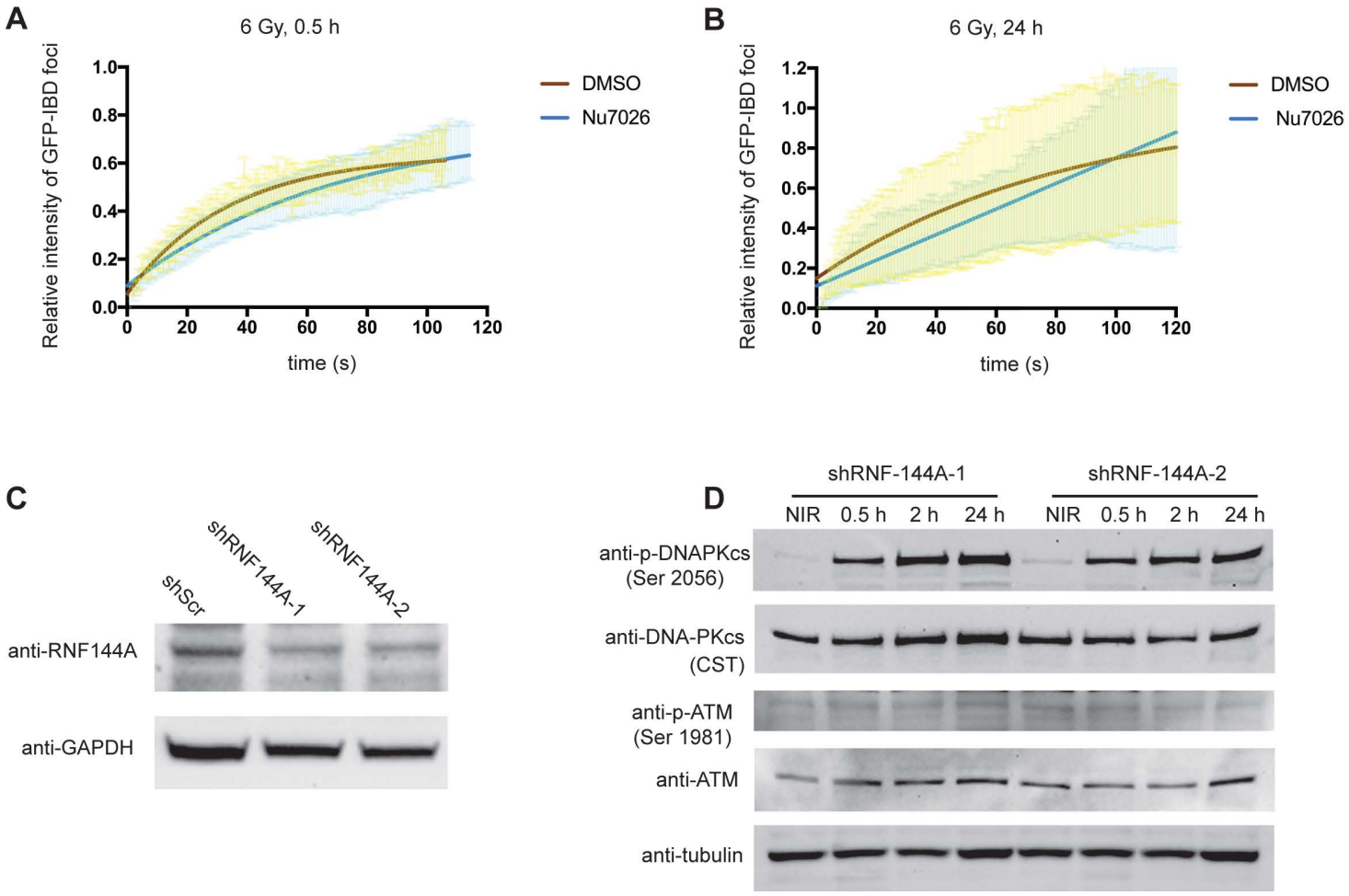


Fig. S5: Fluorescence recovery after photobleach (FRAP) analysis of molecular exchange in GFP-IBD foci and the effect of shRNF-144A on DNA-PKcs and ATM in response to irradiation.

A, B. Fluorescence recovery after photobleach (FRAP) analysis of molecular exchange of the GFP-IBD reporter in foci formed in MCF7$^{GFP\text{-}IBD}$ cells treated with Nu7026 (10 μM) or DMSO 1 h before 6 Gy and examined at 0.5 h (**A**) and 24 h (**B**) after irradiation, representing newly formed and persistent foci respectively. Data were fitted with easyFRAP. N > 10 foci, mean (solid line) ± s.d. **C.** Western blot validation of shRNF-144A. Two shRNA-144A constructs were used. **D.** Western blot analysis of the indicated proteins in shRNF-144A cells after 6 Gy. Cell lysate was collected at the indicated time points. Approximately 70 μg total protein was loaded for each sample.

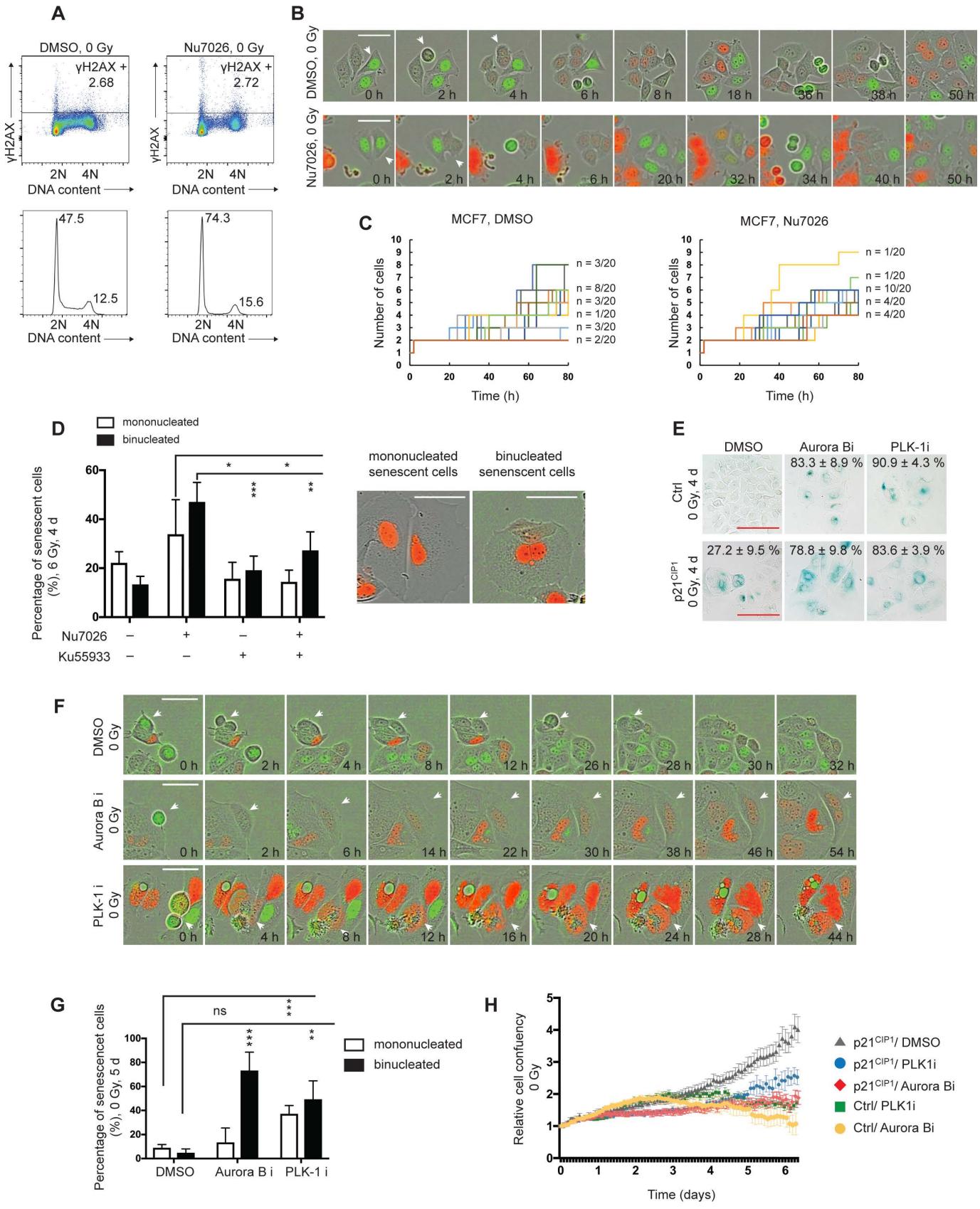
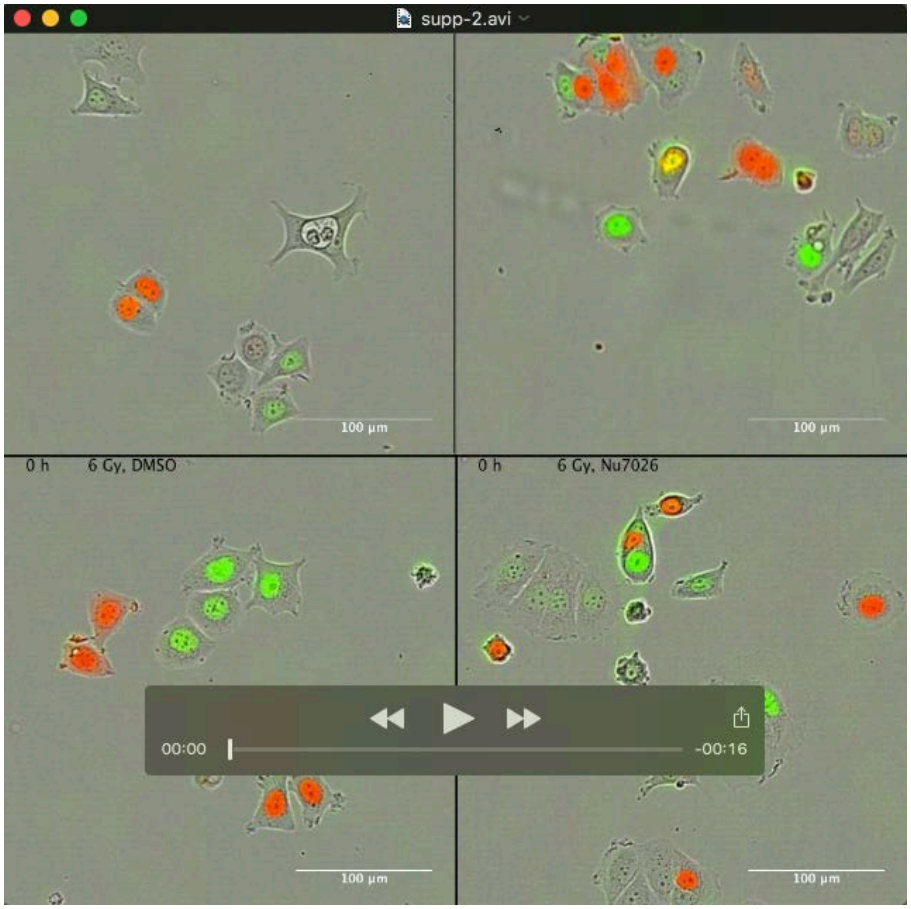


Fig. S6: Tracking of cell behaviors after treatments.

A. Flow cytometry analysis of DNA content (DAPI) and γH2AX in cells treated with DMSO or Nu7026 (10 μM) and then collected after 24 h. The 2D dot plots (upper) show γH2AX staining across the cell cycle. The gate for γH2AX$^+$ was set based these unirradiated cell sample. The histograms (lower) show relative abundance at each DNA content. Data acquired from 50,000 cells per sample. **B.** Time-lapse analysis of MCF7-FUCCI cells treated with DMSO or Nu7026 (3 μM) for 24 h before imaging. Representative cells shown at indicated times. Carets indicate cells that perform mitosis and cytokinesis during time course. Scale bar = 50 μm. **C.** Tracking cell division of single cells. MCF7 cells were treated as in **B**. The time at which rounding up for mitosis was first observed was set as 0 h. Then, 20 cells were tracked for each condition, revealing distinct trajectories of completed cell division and/or mitotic slippage. **D.** ATM inhibitor Ku55933 partially rescues mitotic slippage caused by Nu7026 after 6 Gy. (Left) Analysis of senescent morphology in MFC7 cells treated with DMSO, Nu7026 (3 μM), Ku55933 (0.3 μM) or the combination for 1 h before 6 Gy and tracked by time-lapse recording. More than 50 cells from 10 randomly captured, non-overlapping images, were tracked for 4 d after irradiation. Cells with senescent morphology were classified as mono- or binucleated. Histogram shows mean ± s.d. **E.** SA-βGal staining in MCF7 cells. MCF7 control (Ctrl) or p21^{CIPI} overexpression (p21) cells were treated with DMSO, Aurora B inhibitor or PLK-1 inhibitor and stained after 4 d. At least 5 images were captured from randomly selected, non-overlapping fields. Representative images are shown. Mean percentage of SA-βGal$^+$ cells ± s.d. from five 20x fields indicated. Scale bar = 200 μm. **F.** Time-lapse analysis of MCF7-FUCCI cells treated with DMSO, Aurora B inhibitor (Aurora B i) or PLK-1 inhibitor (PLK-1 i). Representative cells shown at indicated times. Carets indicate cells that performed mitosis and cytokinesis during the time course. Scale bar = 50 μm. **G.** Analysis of senescent morphology in MCF7 cells treated as in **F**. More than 50 cells from 10 randomly captured, non-overlapping images, were tracked to 5 d after irradiation. Cells with senescent morphology were classified as mono- or binucleated. Histogram shows mean ± s.d. for each condition. **H**, Automated proliferation analysis from time-lapse imaging over 6 d comparing control (Ctrl) or p21^{CIPI} overexpression (p21) cells treated with DMSO, Aurora B inhibitor or PLK-1 inhibitor. For statistical analysis, unpaired t-test, **, $p < 0.01$; ***, $p < 0.001$. (Right) Diagram represents mononucleated or binucleated senescent cells. Scale bar = 50 μm.



Movie 1. DNA-PKcs inhibitor Nu7026 induces mitotic slippage after irradiation, related to Figure 7 and Figure S5.

Time-lapse imaging of MCF7-FUCCI cells treated with DMSO or Nu7026 (3 µM) following 6 Gy irradiation or not. Cells were treated with DMSO or Nu7026 (3 µM) for 1 h, irradiated with 0 Gy or 6 Gy and then images were taken at 2 h intervals. Following 0 Gy, most of the cells underwent normal mitosis in which parent cells divided into two separated daughter cells approximately every 24 h in the presence or absence of Nu7026. Following 6 Gy, though some of the surviving cells treated with DMSO recovered from irradiation after a few cell cycles, others lost the capability for cell division and accumulate as flattened and enlarged cells displaying characteristic senescent morphology. However, many surviving cells with Nu7026 treatment entered the cell cycle but failed to complete cytokinesis and yielded binucleated cells, which adopted a senescent phenotype over time. Most cells with senescent morphology terminally arrested while expressing mCherry-hCdt1 (red), representing G1 cell cycle stage. Scale bar = 100 µm.

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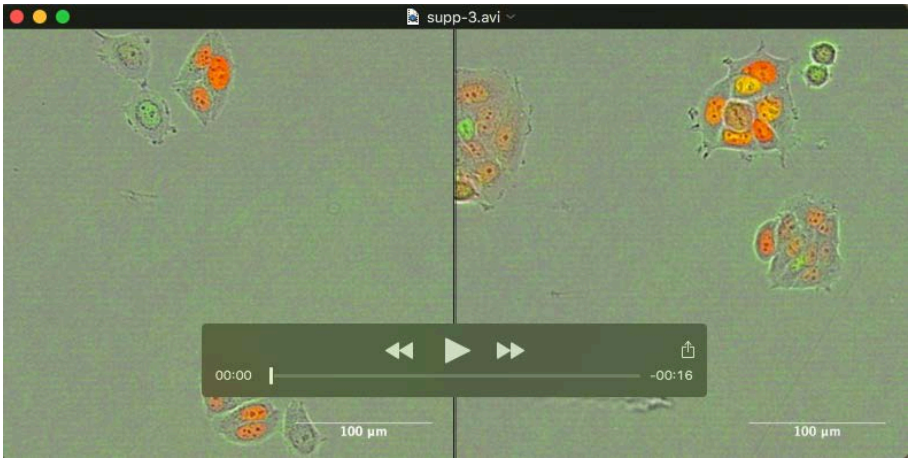
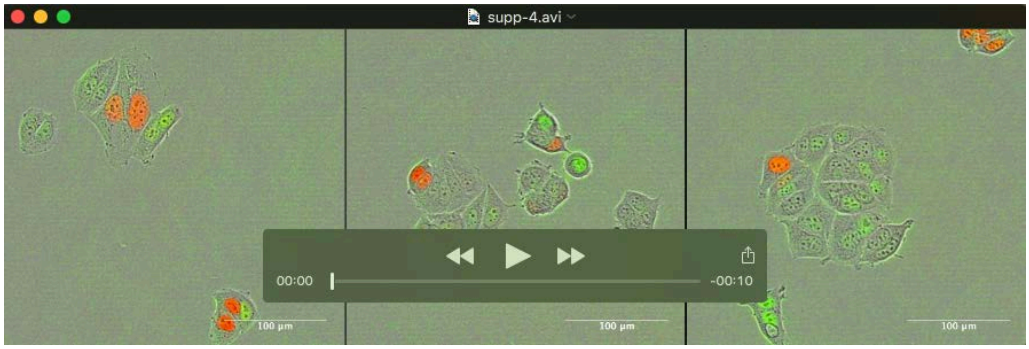


Movie 2. ATM inhibitor Ku55933 partially rescues mitotic slippage caused by Nu7026 after 6 Gy, related to Figure 7 and Figure S6.

Time-lapse imaging of MCF7-FUCCI cells treated with Ku55933 (0.3 µM) or the combination of Ku55933 and Nu7026 (3 µM) following 6 Gy. Cells were treated with inhibitors for 1 h, irradiated with 6 Gy and then images were taken at 2 h intervals. In both cases, most surviving cells entered the cell cycle and completed cytokinesis, but many did not progress further. A high proportion of these daughters contained micronuclei and died instead of continuing on to senescence. Scale bar = 100 µm.



Movie 3. Mitosis and/or cytokinesis defects in unirradiated cells is sufficient to promote accelerated senescence, related to Figure 7 and Figure S7.

Time-lapse imaging of MCF7-FUCCI cells treated with DMSO, Aurora B kinase inhibitor, or PLK-1 inhibitor. Images were taken at 2 h intervals beginning 1 h after inhibitors treatment. With DMSO treatment, most of the cells underwent normal mitosis in which cell divided approximately every 24 h. With Aurora B kinase or PLK-1 inhibitor treatment, cells underwent mitotic slippage and/or catastrophe. Though many cells died, surviving cells eventually displayed senescent phenotype with mCherry-hCdt1 (red, G1) expression. Scale bar = 100 µm.

Movie 4. Overexpression of p21^{CIP1}rescues cell death induced by Aurora B or PLK1 inhibitors and yields homogeneous senescence, related to Figure 7 and Figure S7.

Time-lapse imaging of MCF7-FUCCI or p21^{CIPI} overexpression (p21) cells treated with DMSO, Aurora B or PLK1 inhibitors. Images were taken at 2 h intervals beginning 1 h after inhibitors treatment. In MCF7 cells with DMSO treatment, most of cells underwent cell cycle every 24 h. In MCF7 cells treated with Aurora B or PLK1 inhibitors, cells displayed mitotic slippage and some cells developed senescent morphology while some binucleate cells died rather than entering senescence. P21 overexpression can induce senescence on its own but when combined with the Aurora B or PLK1 inhibitors, the binucleate cells were rescued from death, yielding nearly homogeneous senescence.

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Table S1. List of chemical probes and their working concentrations.

Chemical probe	Targeted protein	Company	Catalog #	Working concentration
Nu7026	DNA-PKcs	Selleckchem	S2893	10 μM
Ku55933	ATM	Selleckchem	S1092	5 μM
CHIR124	Chk1	Cayman	16553	0.5 μM
Chk2 inhibitor	Chk2	Cayman	17552	5 μM
MK-8745	Aurora A	MedChem Express	HY-13819	1 μM
AZD1152-HQPA	Aurora B	MedChem Express	10126	0.5 μM
GSK461364	PLK-1	MedChem Express	50877	0.5 μM

Table S2. List of shRNAs.

Targeted protein	Sigma MISSION shRNA Catalog #	shRNA sequence
DNA-PKcs (PRKDC)(a)	TRCN0000195491	CCGGCCTCCAGGTTAGGATTAATTGCTCGAGCAATTAATCCTAACC TGGAGGTTTTTTG
DNA-PKcs (PRKDC)(b)	TRCN0000194719	CCGGCCTGAAGTCTTTACAACATATCTCGAGATATGTTGTAAAGAC TTCAGGTTTTTTG
ATM(a)	TRCN0000039948	CCGGCCTTTCATTCAGCCTTTAGAACTCGAGTTCTAAAGGCTGAAT GAAAGGTTTTTG
ATM (b)	TRCN0000039951	CCGGGCCTCCAATTCTTCACAGTAACTCGAGTTACTGGAAGAATTG GAGGTTTTTG
XRCC4 (a)	TRCN0000040117	CCGGCCTCAGGAGAATCAGCTTCAACTCGAGTTGAAGCTGATTCT C CTGAGGTTTTTG
XRCC4 (b)	TRCN0000009875	CCGGTGTGTGAGTGCTAAGGAAGCTCTCGAGAGCTTCCTTAGCAC T CACACATTTTTG
XRCC5(a)	TRCN0000295856	CCGGAGAGGAAGCCTCTGGAAGTTCCTCGAGGAACTTCCAGAGG C TTCCTCTTTTTTG
XRCC5(b)	TRCN0000307986	CCGGAATCTAAGAGAGCTGCCATCGCTCGAGCGATGGCAGCTCT C TTAGATTTTTTTG
XRCC6 (a)	TRCN0000039608	CCGGCGACATAAGTCGAGGGACTTTCTCGAGAAAGTCCCTCGAC T TATGTCGTTTTTG
XLF (a)	TRCN0000275632	CCGGTACCATGGACTTTAGGTATATCTCGAGATATACCTAAAGTCC ATGGTATTTTTG
XLF (b)	TRCN0000275628	CCGGGCTAGCAACGTTACTTCATATCTCGAGATATGAAGTAACGTT GCTAGCTTTTTG
PAXX(C9orf142)(a)	TRCN0000263653	CCGGCTCTTCTTACCAGACCCAGATCTCGAGATCTGGGTCTGGTA A GAAGAGTTTTTG
PAXX(C9orf142)(b)	TRCN0000263654	CCGGACAGAGCATCCCTGACGCTTTCTCGAGAAAGCGTCAGGGA T GCTCTGTTTTTTG
Ligase4(a)	TRCN0000040004	CCGGGCCCGTGAATATGATTGCTATCTCGAGATAGCAATCATATTC ACGGGCTTTTTG
Ligase4(b)	TRCN0000040005	CCGGGCTCGCATCTAAACACCTTTACTCGAGTAAAGGTGTTTAGAT GCGAGCTTTTTG
RNF144A(a)	TRCN0000004413	CCGGGAACGAGATTGAGTGCATGGTCTCGAGACCATGCACTCAA T CTCGTTCTTTTT
RNF144A(b)	TRCN0000421486	CCGGATGTTGAGCTCTTGATCAAAGCTCGAGCTTTGATCAAGAGC T CAACATTTTTTTG
XRCC1(a)	TRCN0000007912	CCGGCCTTCTGGTCACCTCATCTTTCTCGAGAAAGATGAGGTGACC A GAAGGTTTTT
XRCC1(b)	TRCN0000007913	CCGGCCAGTGCTCCAGGAAGATATACTCGAGTATATCTTCCTGGAG C ACTGGTTTTT
Ligase3(a)	TRCN0000048499	CCGGCCGGATCATGTTCTCAGAAATCTCGAGATTTCTGAGAACATGA TCCGGTTTTTG
Ligase3(b)	TRCN0000048500	CCGGGCTGAGTAACTCCAACAGCAACTCGAGTTGCTGTTGGAGTT A CTCAGCTTTTTG

Table S3. List of antibodies.

Antibody/protein	Company	Catalog #	Dilution
γ-H2AX, clone JBW301	EMD Millipore	05-636	1:1000
γ-H2AX(Alexa 647 conjugate)	Cell Signaling Technology	9720	1:50
γ-H2AX	Cell Signaling Technology	9718	1:1000
53BP1	Novus	NB100-304	1:1000
DNA-PKcs	Abcam	ab32566	1:500
DNA-PKcs	Cell Signaling Technology	12311	1:1000
DNA-PKcs (Phospho T2609)	Abcam	ab97611	1:500
DNA-PKcs (Phospho S2056)	Abcam	ab124918	1:1000
ATM	Abcam	ab78	1:200
ATM	Cell Signaling Technology	2873	1:1000
ATM (Phospho S1981)	Cell Signaling Technology	4526	1:1000
XRCC5	Abcam	ab80592	1:1000
Ku80	Cell Signaling Technology	2180	1:1000
XRCC6	Abcam	ab202022	1:1000
XRCC4	Abcam	ab97351	1:1000
PAXX	Abcam	ab126353	1:1000
XLF	Abcam	ab33499	1:1000
Ligase4	Abcam	ab26039	1:800
RNF144A	Abcam	ab89260	1:100
Actin (HRP conjugate)	Proteintech	HRP-60008	1:10000
Tubulin (HRP conjugate)	Proteintech	HRP-66031	1:10000