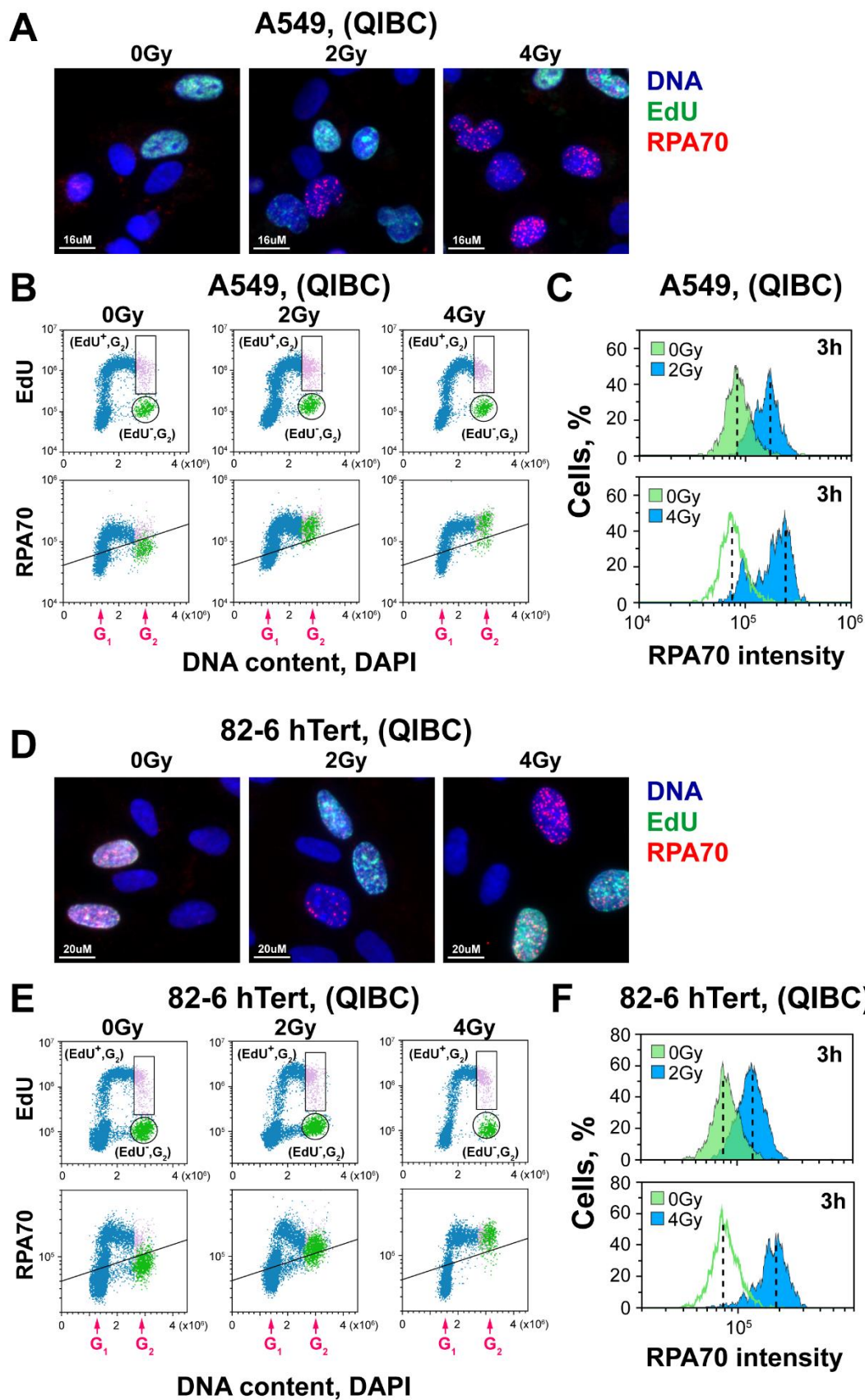


Supplementary Information

Increased resection at DSBs in G₂-phase is a unique phenotype associated with DNA-PKcs defects that is not shared by other factors of c-NHEJ

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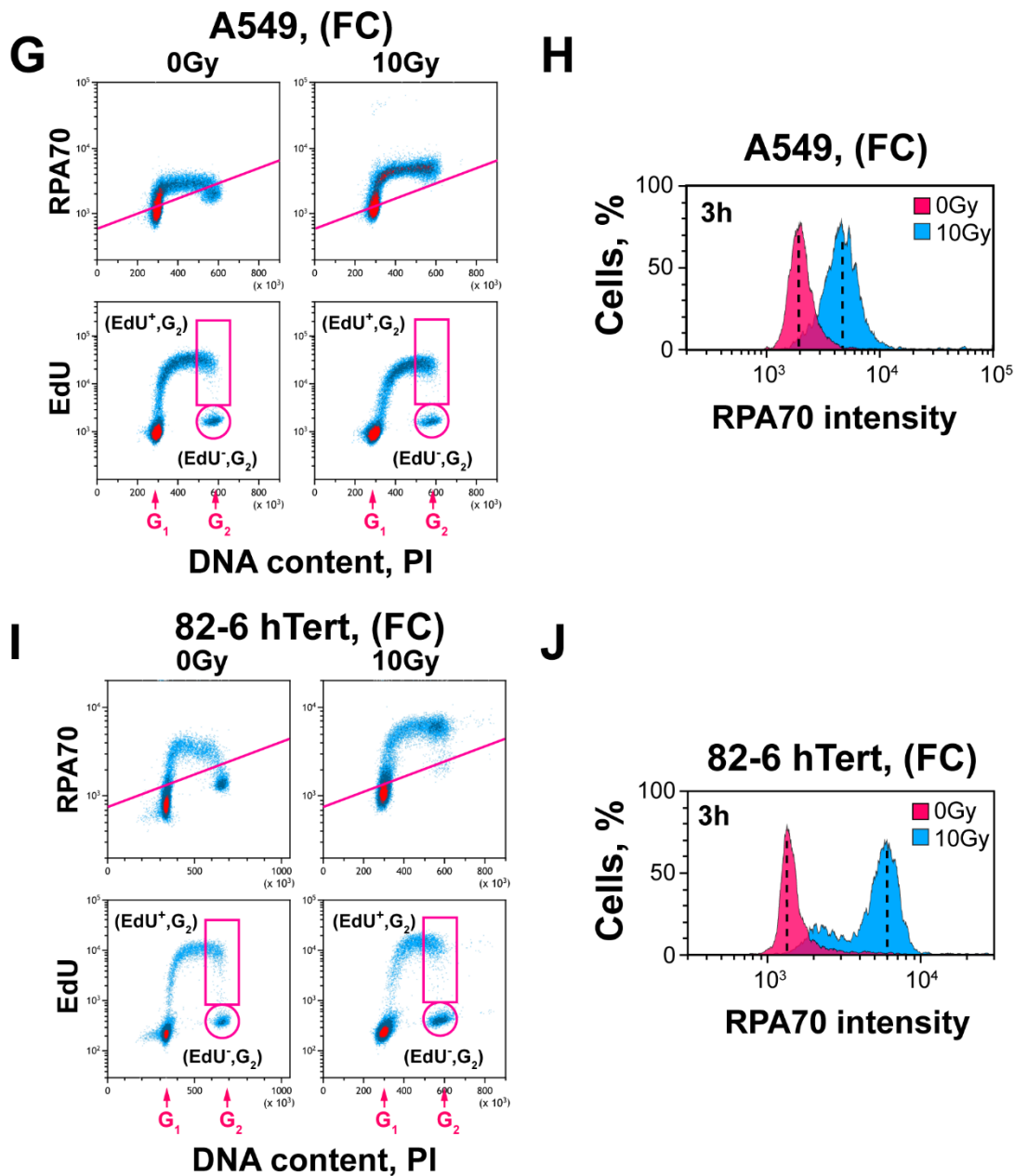


Figure S1. Outline of methodology to measure resection in G_2 -phase. A) Representative AxioScan Z1 immunofluorescent images, obtained during QIBC analysis of A549 cells irradiated with 2 and 4 Gy X-rays. DNA was counterstained with DAPI and DAPI intensity (blue) was assessed to determine the progression of cells through the cell cycle. EdU intensity signal (green) was evaluated to determine whether cells were irradiated in S-phase, while the accumulation of RPA70 (red) onto chromatin was an indication for ongoing DNA end resection. B) Dot plots of RPA70, EdU and DAPI intensities obtained by QIBC analysis. The gates utilized to specifically select cells in different cell cycle phases

are also depicted. EdU positive (EdU⁺, violet) or EdU negative (EdU⁻, green) cells, represent the cells irradiated, respectively, in S- or G₂-phase of the cell cycle. Counterstaining with DAPI allows the determination of DNA content, which reflects the cell cycle phase during analysis. For our experiments, we have selected cells irradiated in G₂-phase and analyzed in the G₂-phase (EdU⁻, G₂-cells, green). C) The RPA70 intensity signals in the selected gates (EdU⁻, G₂-cells) are shown as histogram plots. DNA end-resection after IR is visualized by the increase in the signal intensity between irradiated and non-irradiated cells. The ratio of the means between the two signal intensities is used as a parameter to quantitate resection in the experiments shown here. D, E and F) As in panels A, B and C for 82-6 hTert cells. G) Three parametric flow cytometry analysis of RPA70 signal intensity in A549 cells, showing the gates utilized to select cells irradiated in the G₂-phase and analyzed in G₂-phase of the cell cycle (EdU⁻, G₂-cells). H) The RPA70 intensity signals in the selected gates (EdU⁻, G₂-cells) are plotted as histogram plots. Other details as in Fig. S1A-C. I) As in panel G, for 82-6 hTert cells. J) As in panel H, for 82-6 hTert cells.

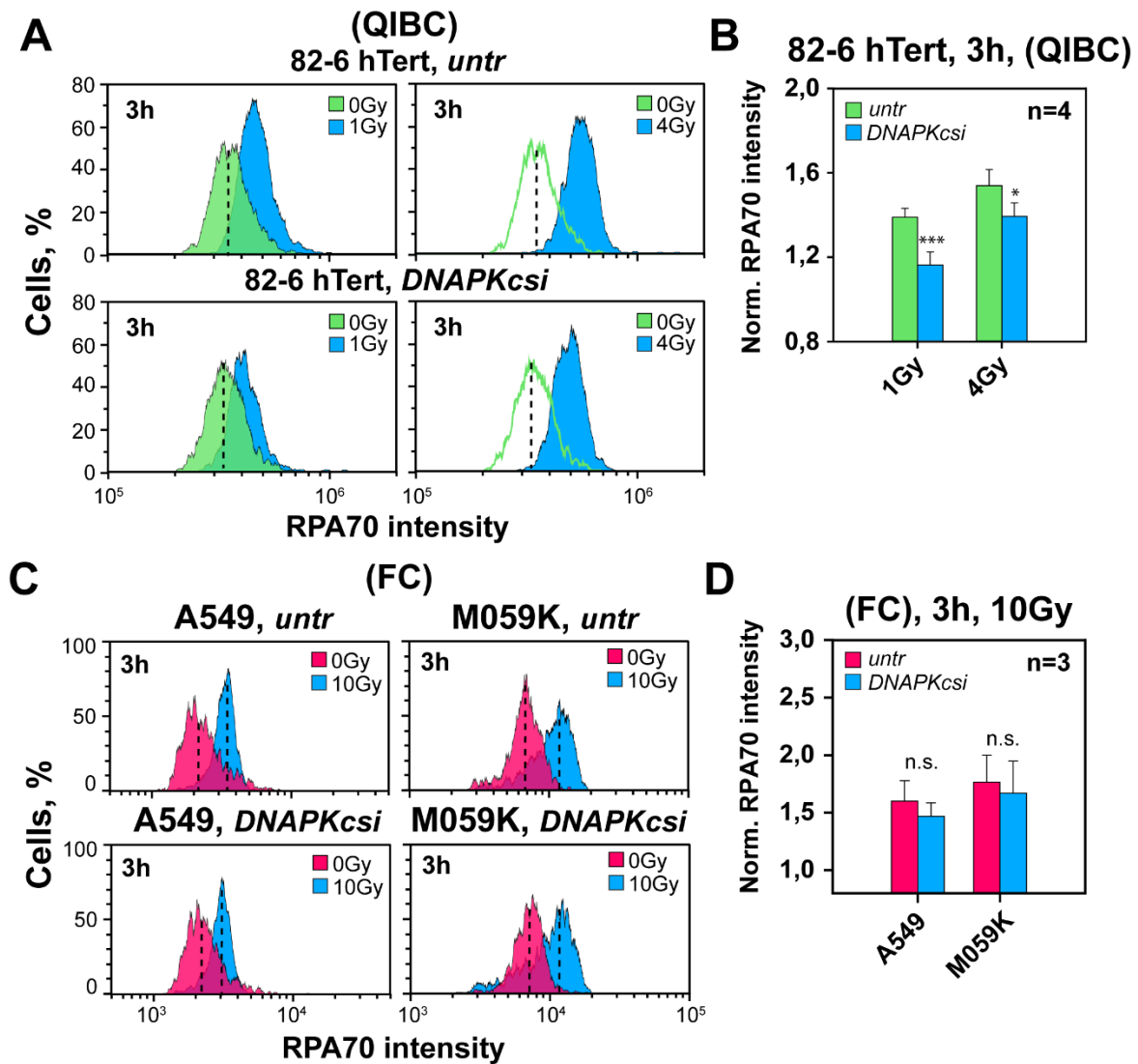


Figure S2. Resection analysis in DNA-PKcs deficient cells. A) QIBC analysis of RPA70 signal at DSBs in 82-6 hTert cells treated or not with *DNAPKcsi* (*NU7441*), 3 h after exposure to 1 Gy or 4 Gy. Other details as in Fig. 1B. B) Bar plot showing the normalized RPA70 signal intensity from three experiments, obtained as shown in panel A. C) FC-based analysis of resection in wild-type A549 and M059K cells, treated or not with *DNAPKcsi*. DNA end-resection is measured 3 h after exposure to 10 Gy (other details as in Fig. 1D). D) Bar plot showing the normalized RPA70 signal intensity from three experiments, obtained as shown in panel C.

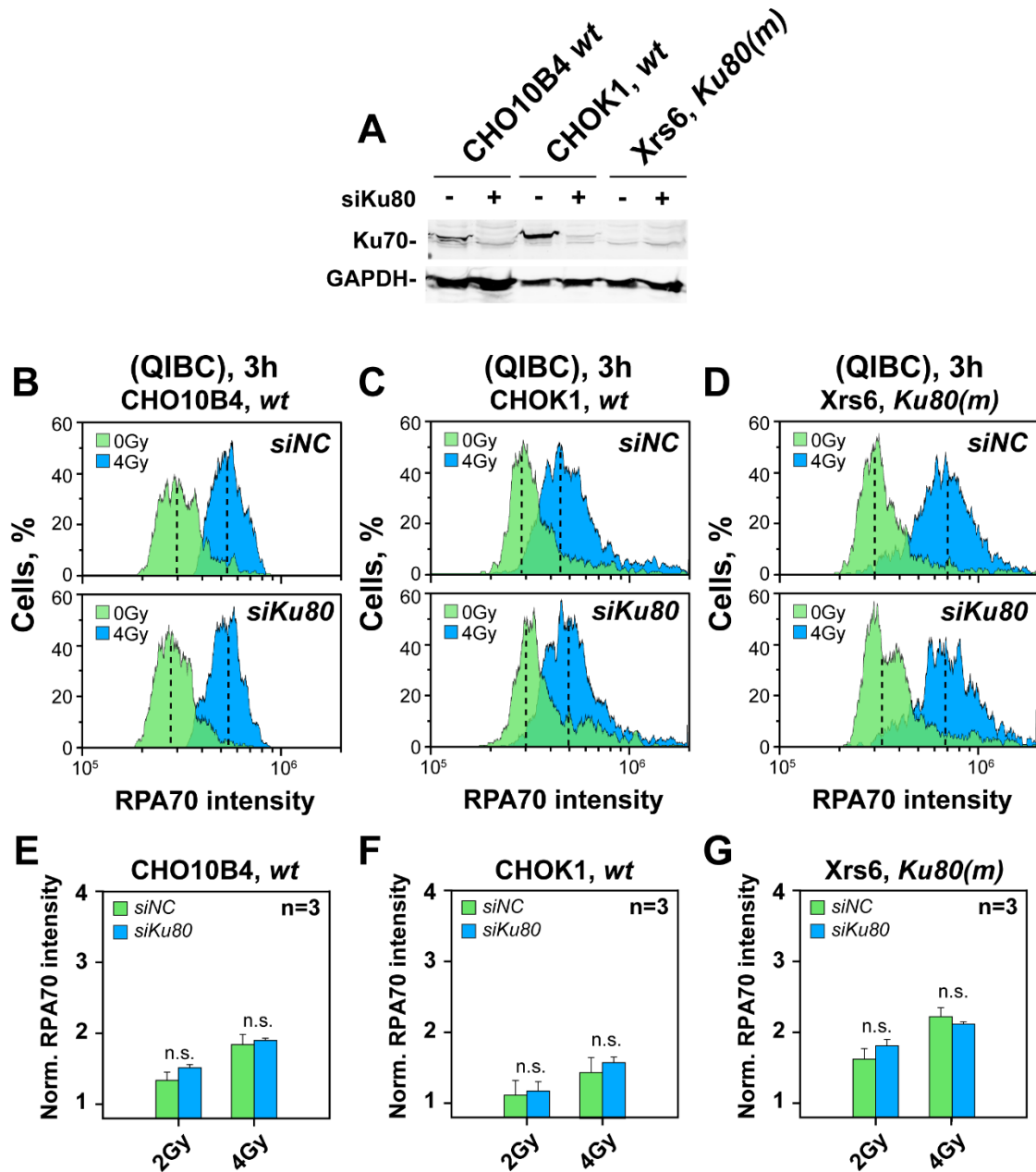


Figure S3. Resection analysis in CHO cells after knockdown of Ku. A) Western blot analysis showing the level of Ku70 in CHO10B4, CHOK1 and Xrs6 cells 24 h after transfection with a siRNA against Ku80, or with a non-specific control siRNA. KU70 serves here as a proxy for KU80, as the anti-KU80 antibody we used failed to recognize the hamster protein. B) Representative histograms of RPA70 intensity in mock transfected and Ku depleted CHO10B4(wt) cells, obtained by QIBC analysis 3 h after exposure to 2 or 4 Gy. C) As in panel B, for CHOK1(wt) cells. D) As in panel B, or the Ku80 mutant. E) Quantitative analysis of RPA70 signal in CHO10B4(wt) cells. F) As in panel E, but for CHOK1(wt) cells. G) As in panel E, for Xrs6 cells. The results represent the mean and SE from 3 independent determinations.

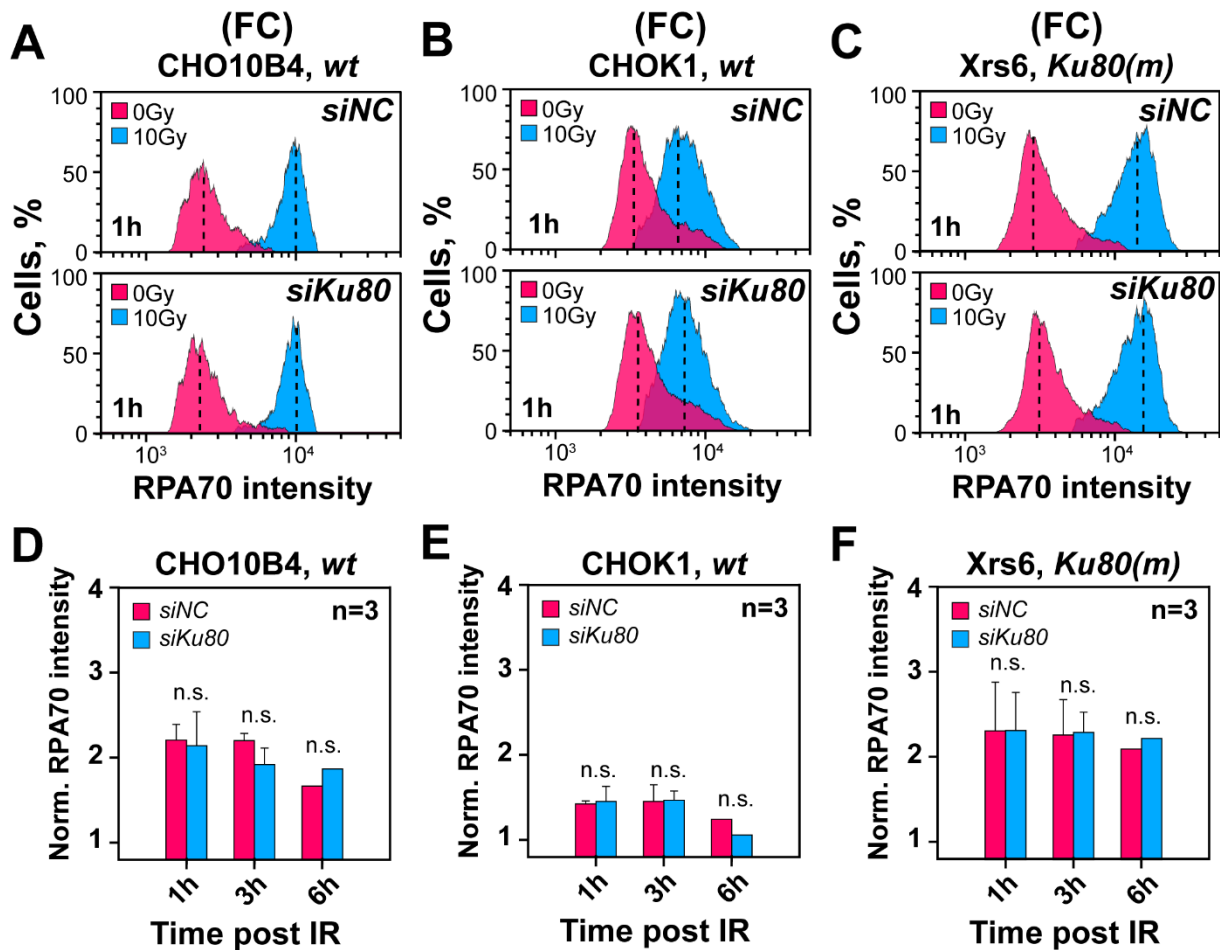


Figure S4. Resection after Ku knockdown in CHO cell lines. A) Representative histograms of RPA70 intensity in mock transfected and Ku depleted CHO10B4(wt) cells, obtained by FC analysis 1 h after exposure to 10 Gy. B) As in panel A, for CHOK1 cells. C) As in panel A, for Xrs6 cells. D) Quantitative analysis of RPA70 signal in CHO10B4 cells. E) As in panel D, for CHOK1 cells. F) As in panel D, for Xrs6 cells. The results represent the mean and SE from 3 independent determinations.

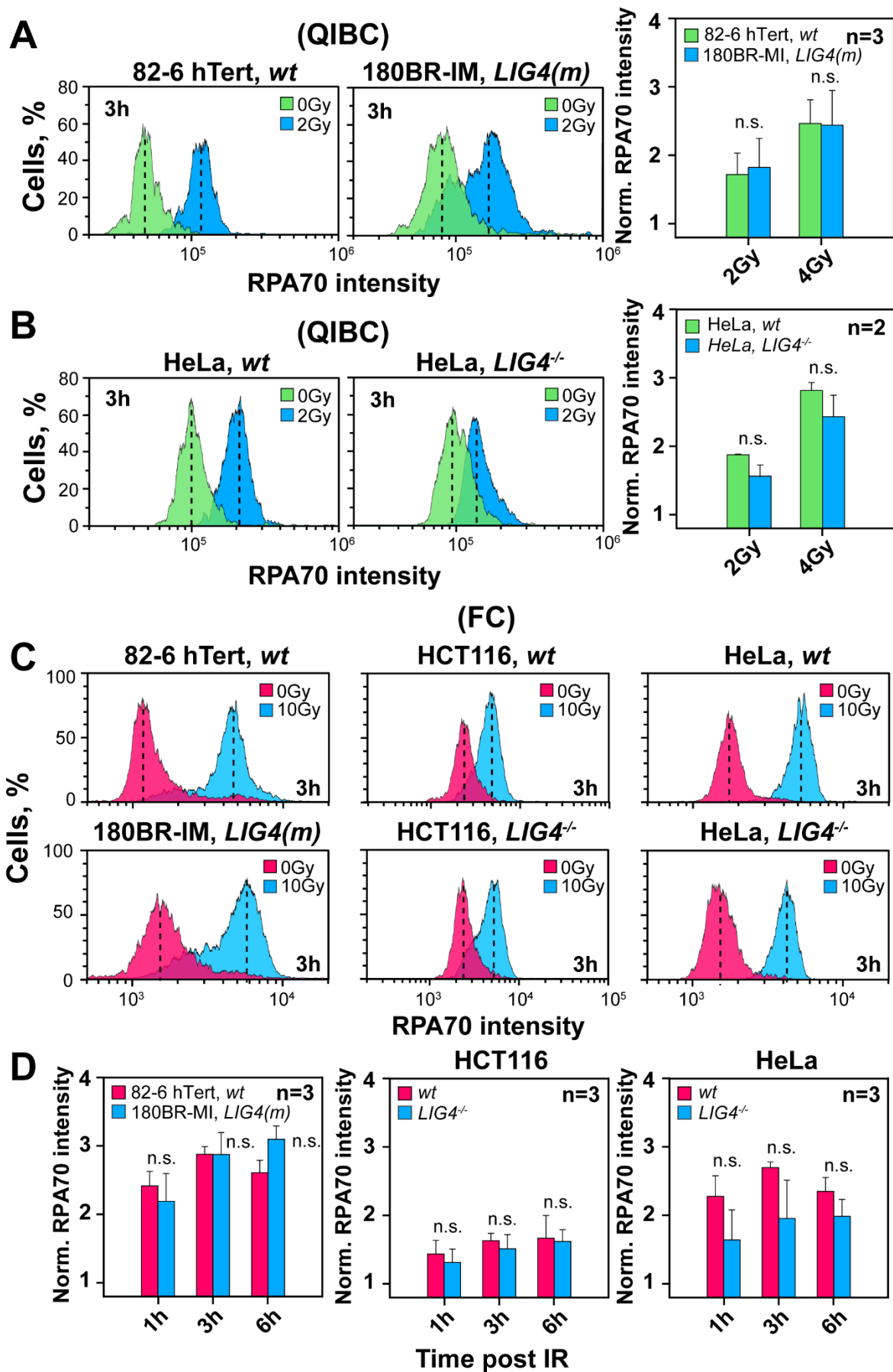


Figure S5. *Resection analysis in *LIG4* deficient cells.* A) QIBC analysis of RPA70 in wt, 82-6 hTert and *LIG4* deficient 180BR-M human cell lines. The bar plot is showing the normalized RPA70 signal intensity from three independent determinations. B) As in panel A for *LIG4* proficient and deficient HeLa cells. C) FC-based analysis of RPA70 signal intensity in human cell lines deficient in *LIG4*. Other details as in Figure 2B. D) Quantification of RPA70 signal from three experiments such as those shown in panel A.

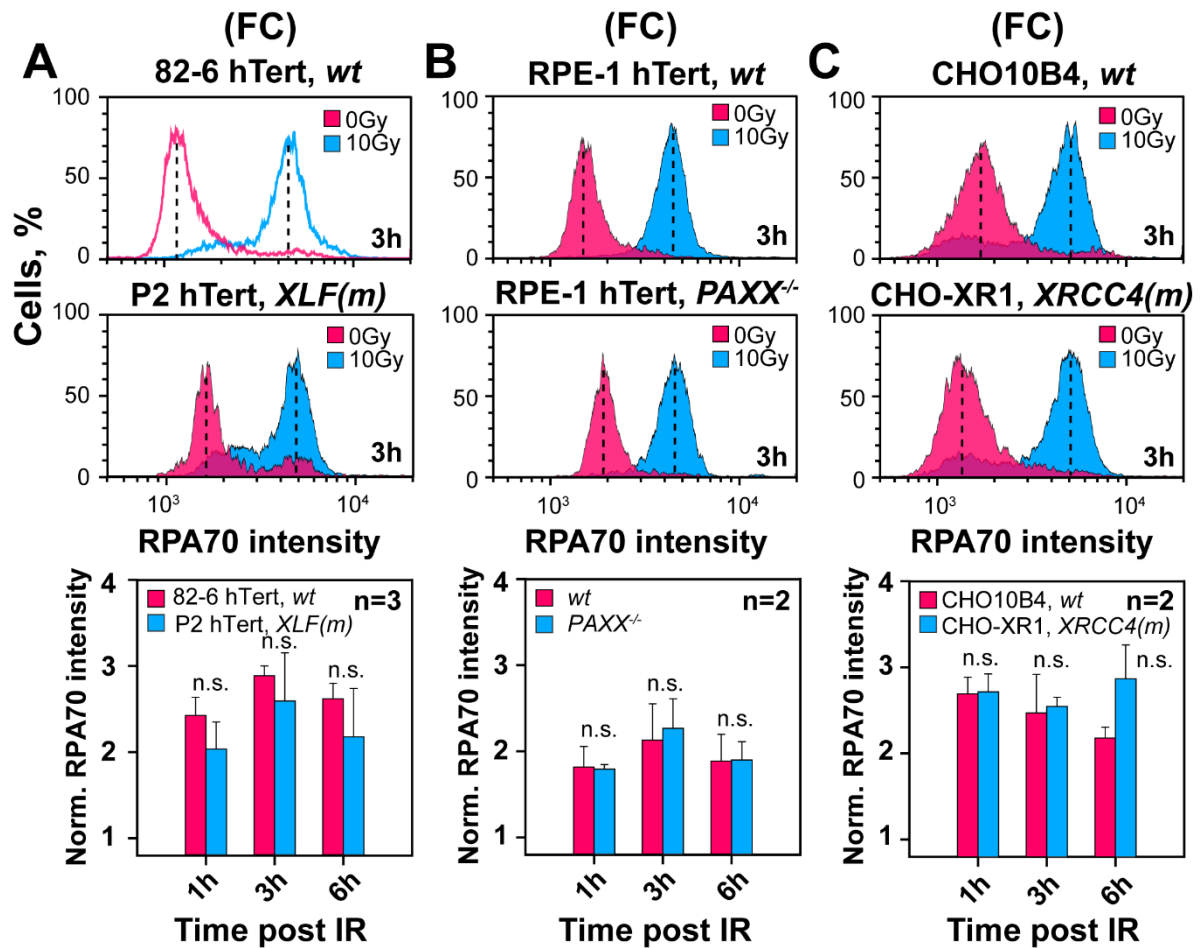


Figure S6. FC-based analysis of resection in *c-NHEJ* deficient cells. A) Resection in *XLF*^{-/-} cells. B) Resection in *PAXX*^{-/-} cells. C) Resection in *XRCC4*^{-/-} cells. All cell lines were exposed to 10 Gy and analyzed at 1 h, 3 h, and 6 h after irradiation. The experiments are repeated thrice and the bar graphs at the bottom show the combined results. Plotted are the mean and SE from three independent experiments.

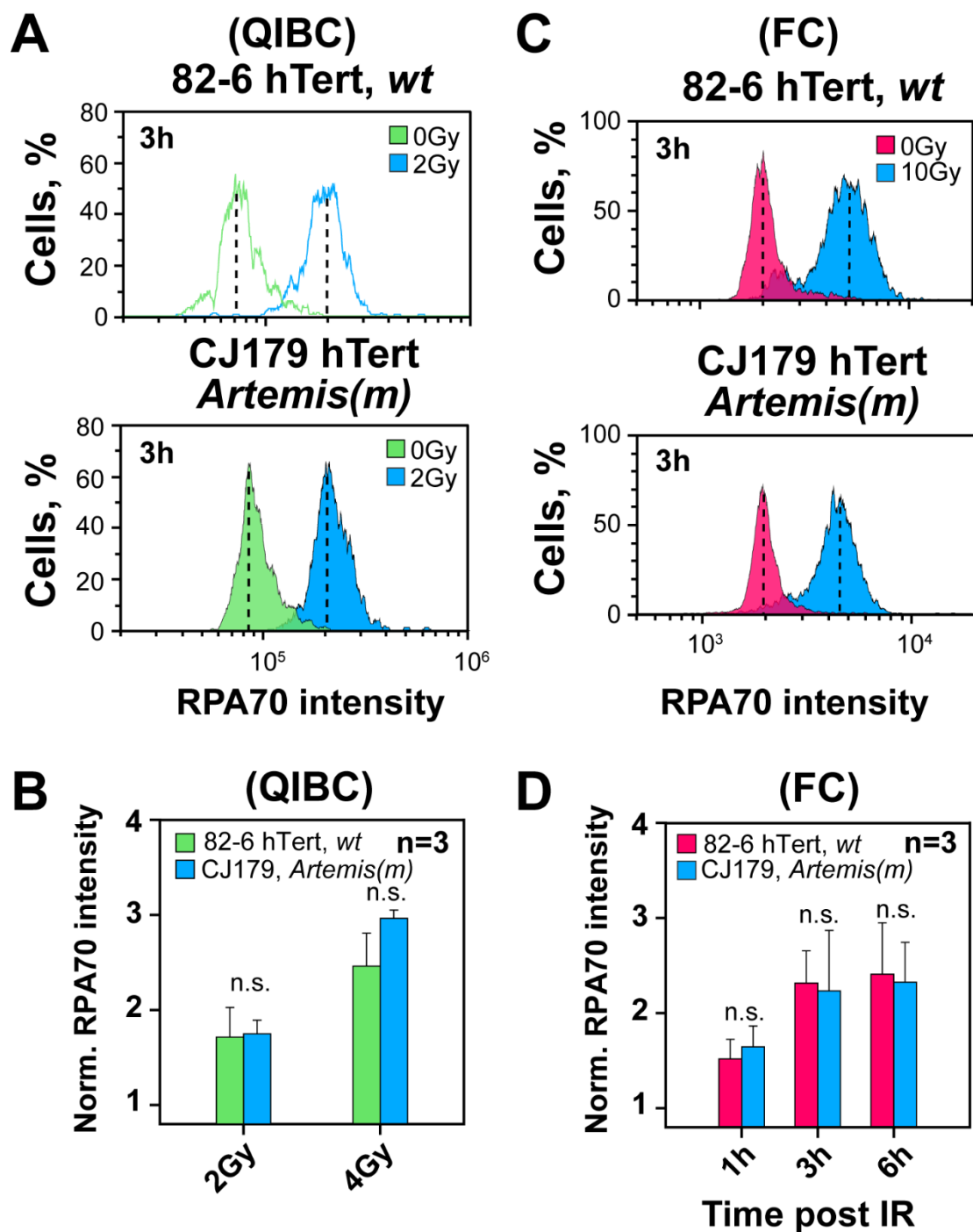


Figure S7. Resection analysis in *Artemis*^{-/-} cells. A) RPA70 signal intensity determined using QIBC 3 h after exposure to 2 Gy or 4 Gy of IR (only data for 2 Gy are shown). B) Quantification of RPA70 signal intensity determined as shown in panel A. C) As in panel A, using FC after exposure to 10 Gy. D) As in panel B, for experiments shown in panel C. Plotted are the mean and SE from three independent experiments.

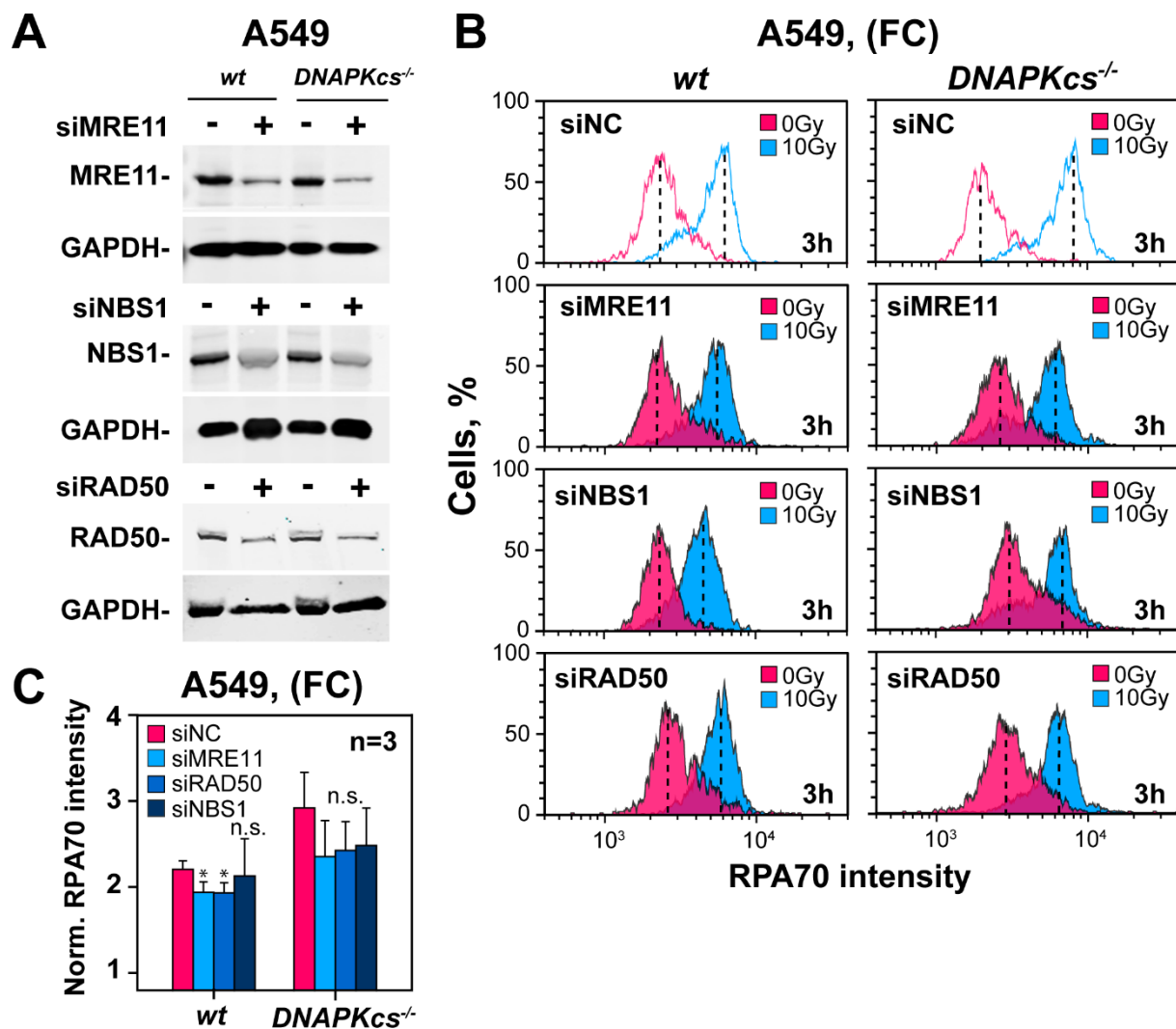


Figure S8. Resection analysis in *wt* and *DNA-PKcs^{-/-}* A549 cells after depletion of components of the MRN complex. A) Western blot analysis showing the levels of MRE11, NBS1 and RAD50 in *wt* and *DNA-PKcs^{-/-}* A549 cells after transfection with the corresponding siRNAs. B) Cell cycle specific FC analysis of RPA70 signal intensity, 3 h after exposure of MRN depleted cells to 10 Gy of IR. C) Quantification of RPA70 signal intensity shown in panel B. The results represent the mean and SE from 3 independent determinations.

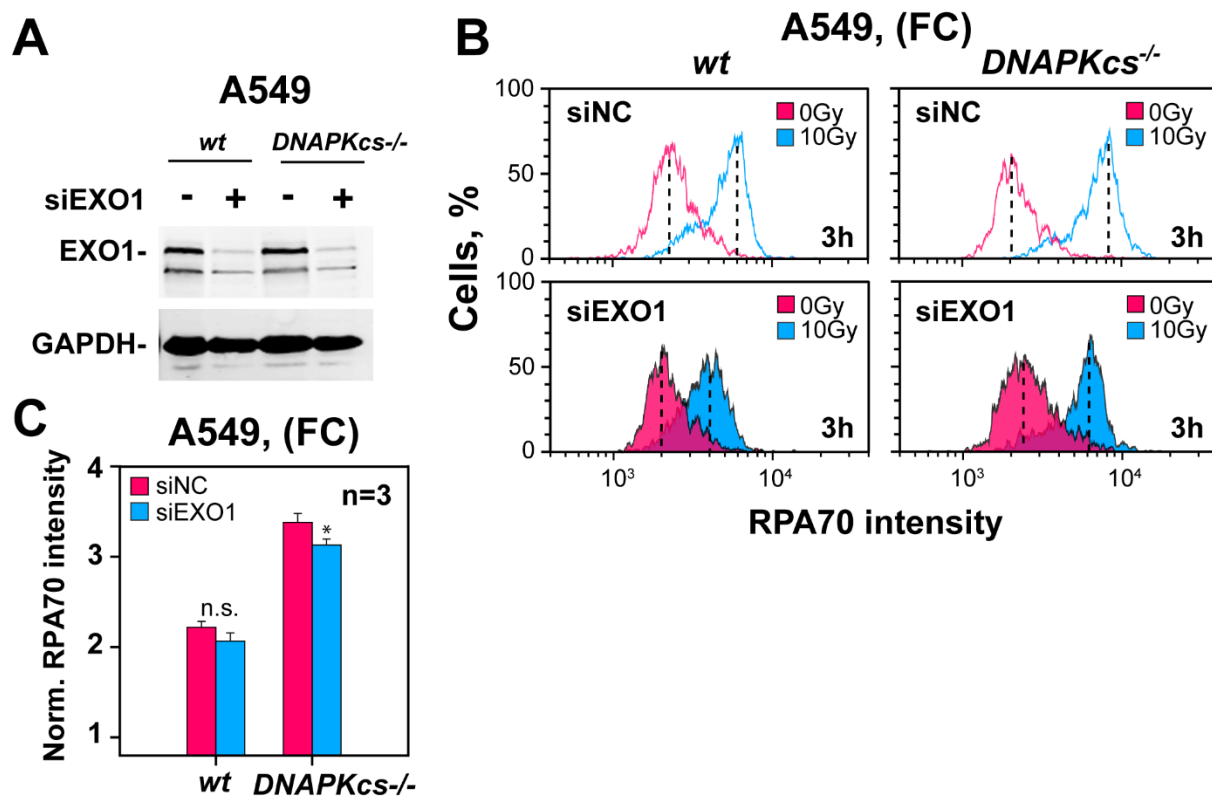


Figure S9. *Effects on resection of EXO1 depletion.* A) Western blot analysis showing the level of EXO1 after transfection with a specific EXO1 siRNAs. B) As in Figure 6B, for cells depleted for EXO1 by transfection with a specific EXO1 siRNA. C) As in Figure 6C, for cells depleted for EXO1.

Table S1. *Sequences of siRNA against human and hamster genes of interests.* For more information see the Materials and Methods.

Target protein	siRNA
Negative control (siNC)	1. UUCUCCGAACGUGUCACGU
EXO1	1. GCCUUUGCUAUCCAAUCCCACG
DNA2	1. AGACAAGGUUCCAGCGCCA
CTIP	1. GCUAAAAC AGGAACGAAUC 2. GAUUCGUUCCUGUUUUAGC
MRE11	1. GAUGCCAUUGAGGAAUAAG
RAD50	1. UAAUGAGACUUGACAAUGA 2. ACACUCUUGGGUACAAUAA 3. ACAGAACUCCUCACUAAGA
NBS1	1. UAACCUUGUUGGCCUGAAGUAGAUG 2. CAGGAUGGCUGUCAGGUUA
BLM	1. GAGCACAUCUGUAAAUUAA 2. CAGGAUGGCUGUCAGGUUA
DNA-PKcs	1. CGGCUAACUCGCCAGUUUA
Human KU80	1. UUCCUUAUUGGCUUGUCGUUU 2. AAACGACAAGCCAUUAAGGAA
Hamster Ku80	1. GAAACUGUCUAUUGCUUAA 2. CCAUAGGGAAGAAGUUUGA 3. GGAUCCUAUGAGUGUUUA

Table S2. Statistical analysis of the results shown in main figures. For more information see the Materials and Methods.

Figure1C 2Gy, wt vs DNAPKcs ^{-/-} P = 0.0013 4Gy, wt vs DNAPKcs ^{-/-} P = 0.0026 Figure1E 10Gy, 1h, wt vs DNAPKcs ^{-/-} P = 0.0494 10Gy, 3h, wt vs DNAPKcs ^{-/-} P = 0.0495 10Gy, 6h, wt vs DNAPKcs ^{-/-} P = 0.0433	Figure3B 2Gy, CHO10B4 vs Xrs6 P = 0.7098 4Gy, CHO10B4 vs Xrs6 P = 0.8009 Figure3D 10Gy, 1h, CHO10B4 vs Xrs6 P = 0.8898 10Gy, 3h, CHO10B4 vs Xrs6 P = 0.8018 10Gy, 6h, CHO10B4 vs Xrs6 P = 0.0717 Figure3G 10Gy, 1h, siNC vs siKU80 P = 0.5974 10Gy, 3h, siNC vs siKU80 P = 0.3473 10Gy, 6h, siNC vs siKU80 P = 0.5025 Figure4A 2Gy, wt vs LIG4 ^{-/-} P = 0.9986 4Gy, wt vs LIG4 ^{-/-} P = 0.1747 Figure4B 2Gy, wt vs XLF(m) P = 0.4116 4Gy, wt vs XLF(m) P = 0.5100 Figure4C 2Gy, wt vs PAXX ^{-/-} P = 0.7155 4Gy, wt vs PAXX ^{-/-} P = 0.6523 Figure4E CHO10B4 vs V3 P = 0.002 CHO10B4 vs XR1 P = 0.196 CHO10B4 vs Xrs6 P < 0.001	Figure5C 2Gy, wt vs LIG4 ^{-/-} P < 0.0001 4Gy, wt vs LIG4 ^{-/-} P = 0.0015 Figure5E 2Gy, wt vs XLF(m) P = 0.0006 4Gy, wt vs XLF(m) P < 0.0001 Figure6C wt, siNC vs siDNA2 P = 0.0002 wt, siNC vs siBLM P = 0.0001 wt, siNC vs siDNA2/siBLM P = 0.0002 DNAPKcs ^{-/-} , siNC vs siDNA2 P < 0.0001 DNAPKcs ^{-/-} , siNC vs siBLM P < 0.0001 DNAPKcs ^{-/-} , siNC vs siDNA2/siBLM P < 0.0001 Figure6E wt, untr vs DNA2i P = 0.0001 DNAPKcs ^{-/-} , untr vs DNA2i P < 0.0001
Figure2C 2Gy, CHOK1 vs XR-C1-3 P = 0.0001 4Gy, CHOK1 vs XR-C1-3 P = 0.0001 2Gy, CHOK1 vs V3 P = 0.0003 4Gy, CHOK1 vs V3 P = 0.0003 2Gy, CHOK1 vs Irs20 P = 0.0063 4Gy, CHOK1 vs Irs20 P = 0.0006 Figure2E 10Gy, 1h, CHOK1 vs XR-C1-3 P < 0.0001 10Gy, 3h, CHOK1 vs XR-C1-3 P = 0.0040 10Gy, 6h, CHOK1 vs XR-C1-3 P = 0.0069 10Gy, 1h, CHOK1 vs Irs20 P = 0.0015 10Gy, 3h, CHOK1 vs Irs20 P = 0.0302 10Gy, 6h, CHOK1 vs Irs20 P = 0.1054		

Table S3. Statistical analysis of the results shown in supplementary figures. For more information see the Materials and Methods.

FigureS2B 1Gy, untr vs DNAPKcsi P = 0.0009 4Gy, untr vs DNAPKcsi P = 0.0266 FigureS2D A549, untr vs DNAPKcsi P = 0.3421 M059K, untr vs DNAPKcsi P = 0.6778	FigureS4D 10Gy, 1h, CHO10B4, siNC vs siKu80 P = 0.8095 10Gy, 3h, CHO10B4, siNC vs siKu80 P = 0.0784 10Gy, 6h, CHO10B4, siNC vs siKu80 P = 0.1835 FigureS4E 10Gy, 1h, CHOK1, siNC vs siKu80 P = 0.8012 10Gy, 3h, CHOK1, siNC vs siKu80 P = 0.9130 10Gy, 6h, CHOK1, siNC vs siKu80 P = 0.2703 FigureS4F 10Gy, 1h, Xrs6, siNC vs siKu80 P = 0.9917 10Gy, 3h, Xrs6, siNC vs siKu80 P = 0.9539 10Gy, 6h, Xrs6, siNC vs siKu80 P = 0.7584	Figure5A 2Gy, wt vs LIG4(m) P = 0.7499 4Gy, wt vs LIG4(m) P = 0.9483 Figure5B 2Gy, wt vs LIG4 ^{-/-} P = 0.9623 4Gy, wt vs LIG4 ^{-/-} P = 0.1187 Figure5SD 10Gy, 1h, wt vs LIG4(m) P = 0.4409 10Gy, 3h, wt vs LIG4(m) P = 0.9884 10Gy, 6h, wt vs LIG4(m) P = 0.0737 Figure5SD, HCT116 10Gy, 1h, wt vs LIG4 ^{-/-} P = 0.4946 10Gy, 3h, wt vs LIG4 ^{-/-} P = 0.4339 10Gy, 6h, wt vs LIG4 ^{-/-} P = 0.8338 Figure5SD, HeLa 10Gy, 1h, wt vs LIG4 ^{-/-} P = 0.1075 10Gy, 3h, wt vs LIG4 ^{-/-} P = 0.0856 10Gy, 6h, wt vs LIG4 ^{-/-} P = 0.1208	FigureS6A 10Gy, 1h, wt vs XLF(m) P = 0.1494 10Gy, 3h, wt vs XLF(m) P = 0.5396 10Gy, 6h, wt vs XLF(m) P = 0.3477 FigureS6B 10Gy, 1h, wt vs PAXX ^{-/-} P = 0.8723 10Gy, 3h, wt vs PAXX ^{-/-} P = 0.6840 10Gy, 6h, wt vs PAXX ^{-/-} P = 0.9533 FigureS6C 10Gy, 1h, wt vs XRCC4(m) P = 0.8898 10Gy, 3h, wt vs XRCC4(m) P = 0.7875 10Gy, 6h, wt vs XRCC4(m) P = 0.0717 FigureS7B 2Gy, wt vs Artemis(m) P = 0.8659 4Gy, wt vs Artemis(m) P = 0.0718 FigureS7D 10Gy, 1h, wt vs Artemis(m) P = 0.4946 10Gy, 3h, wt vs Artemis(m) P = 0.4339 10Gy, 6h, wt vs Artemis(m) P = 0.8338	FigureS8C A549, wt, siNC vs siMRE11 P = 0.0426 A549, wt, siNC vs siNBS1 P = 0.0370 A549, wt, siNC vs siRAD50 P = 0.7767 A549, DNAPKcs ^{-/-} , siNC vs siMRE11 P = 0.1711 A549, DNAPKcs ^{-/-} , siNC vs siNBS1 P = 0.1810 A549, DNAPKcs ^{-/-} , siNC vs siRAD50 P = 0.2751 FigureS9B A549, wt, siNC vs siEXO1 P = 0.0773 A549, DNAPKcs ^{-/-} , siNC vs siEXO1 P = 0.0773
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