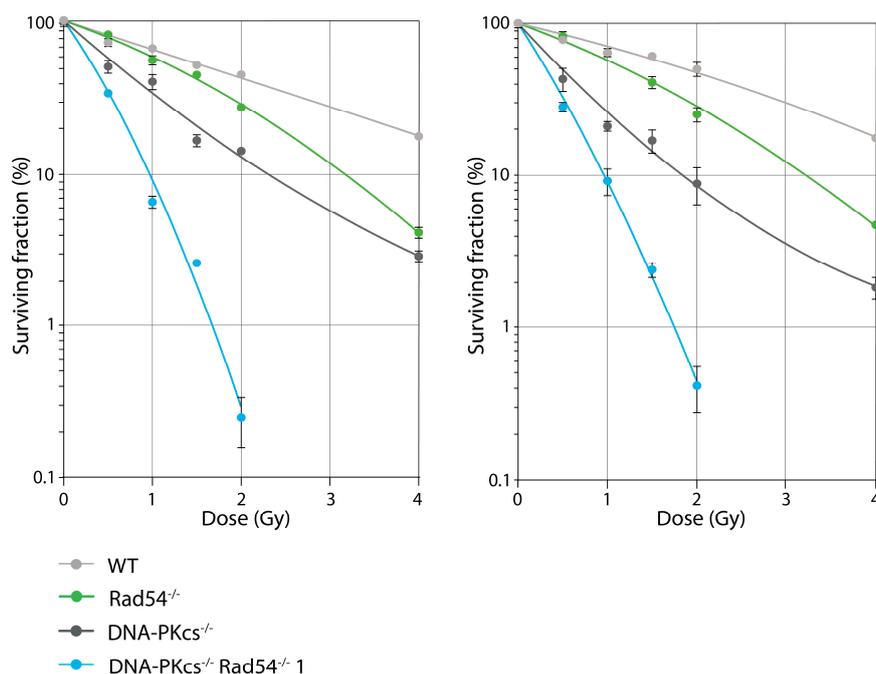


Supplementary Materials

(a)

Cell line	D37	Fold change compared to WT
WT	2.32	
Rad54 ^{-/-}	1.18	1.96
DNA-PKcs ^{-/-}	0.90	2.57
DNA-PKcs ^{-/-} Rad54 ^{-/-} 1	0.45	5.21
DNA-PKcs ^{-/-} Rad54 ^{-/-} 2	0.37	6.28

(b)



Cell line	D37	Fold change compared to WT
WT	2.28	
Rad54 ^{-/-}	1.64	1.39
DNA-PKcs ^{-/-}	0.90	2.54
DNA-PKcs ^{-/-} Rad54 ^{-/-} 1	0.46	4.91

D37	Fold change compared to WT
2.55	
1.64	1.55
0.72	3.54
0.45	5.71

Figure S1. mES DNA-PKcs^{-/-} Rad54^{-/-} cells are hypersensitive to X-ray radiation. (a) Table shows D37 values and fold changes in sensitivity of clonogenic survival shown in Figure 1c. (b) Clonogenic survivals of mES cell lines with indicated genotypes after X-ray irradiation. Error bars represent SEM. The tables display D37 values and sensitivity fold changes of the clonogenic survivals displayed above the tables.

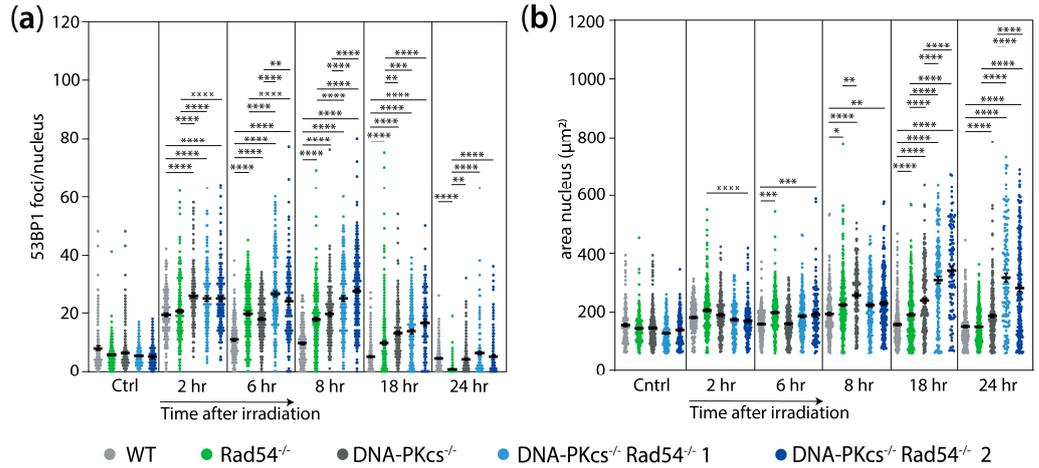


Figure S2. mES DNA-PKcs^{-/-} Rad54^{-/-} cells show impaired 53BP1 foci resolution and increased nuclear size after 2 Gy of X-ray radiation. (a) Quantification of 53BP1 foci per mES nucleus. (b) Quantification of area of mES nuclei. Each dot represents one nucleus. Black bars indicate mean and error bars represent SEM. Asterisks represent following *p*-values: * ≤ 0.05 ; ** ≤ 0.01 ; *** ≤ 0.001 ; **** ≤ 0.0001 .

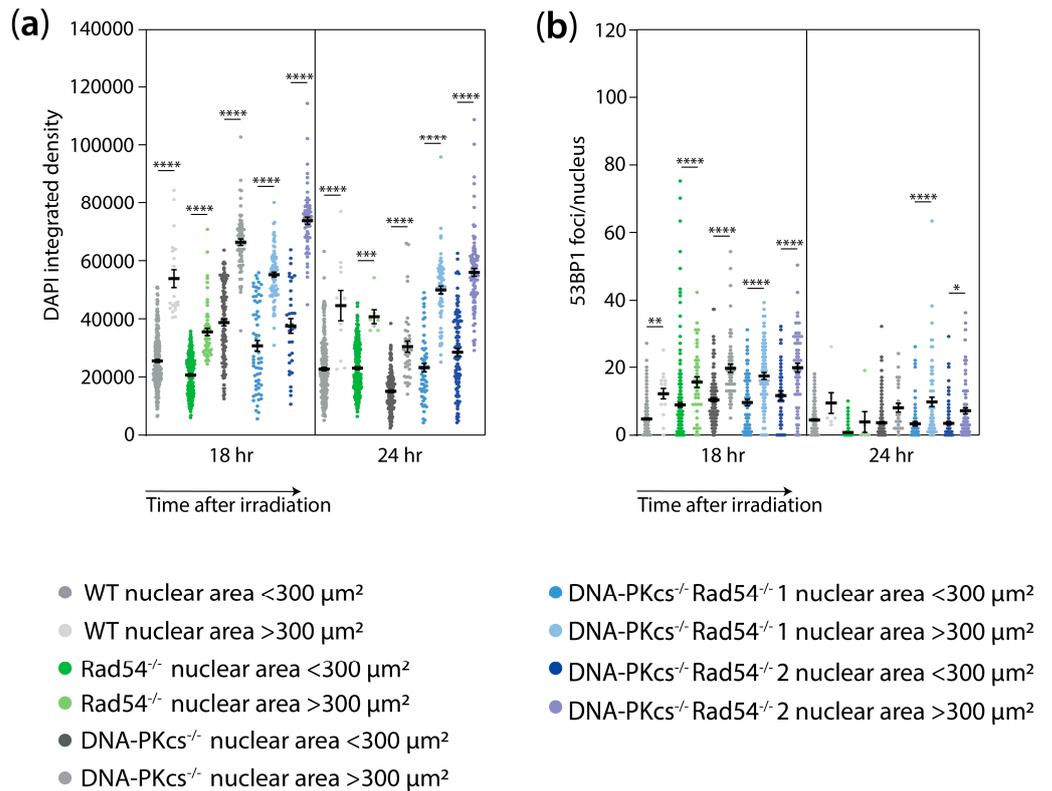


Figure S3. Cells with large nuclei (>300 μm^2) have increased DNA content and more DSBs. (a) Quantification of integrated density of DAPI signal in cells with small (<300 μm^2) and large (>300 μm^2) nuclei at 18 and 24 hours after X-ray irradiation. (b) Quantification of 53BP1 foci per nucleus in cells with small (< 300 μm^2) and large (> 300 μm^2) nuclei at 18 and 24 hours after X-ray irradiation. Each dot represents one nucleus. Black bars indicate mean and error bars represent SEM. Asterisks represent following *p*-values: * ≤ 0.05 ; ** ≤ 0.01 ; *** ≤ 0.001 ; **** ≤ 0.0001 .

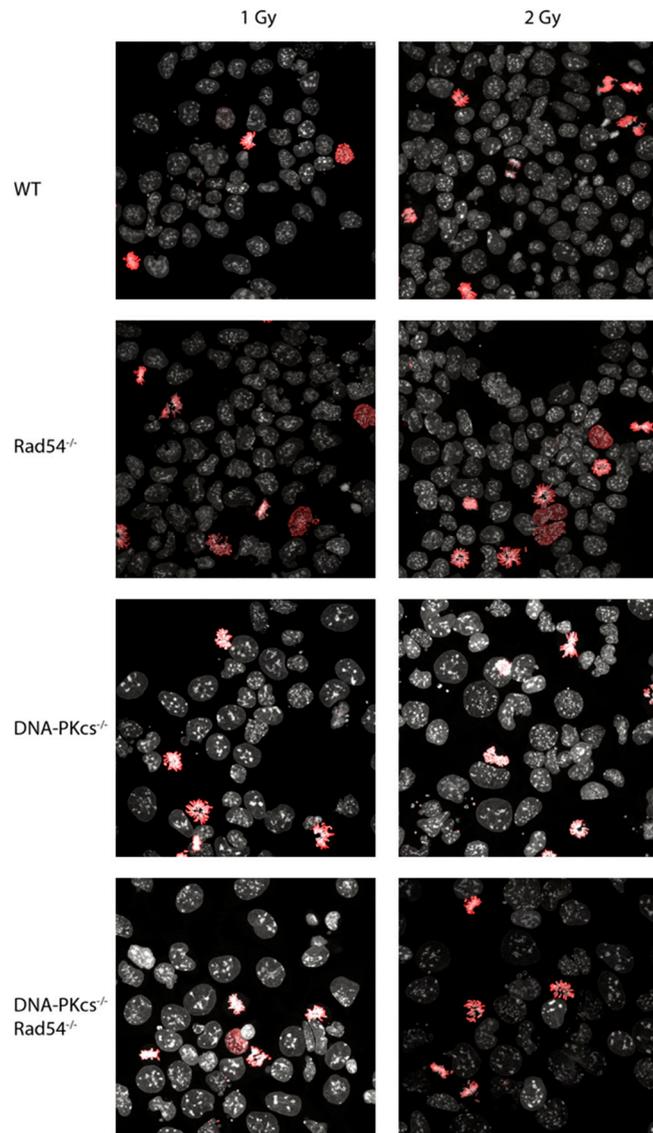
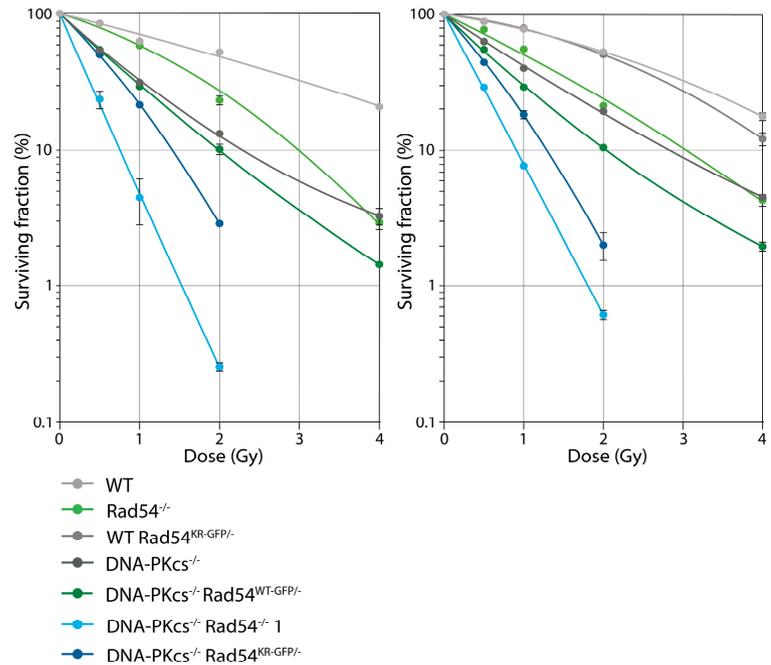


Figure S4. Phospho-H3 staining in mES cells at 24 hours after X-ray irradiation. Representative images of mES cells irradiated with 1 or 2 Gy of X-ray radiation and incubated for 24 hours. After the recovery time, cells were fixed and stained for phospho-H3. DAPI signal is shown in grey and phospho-H3 signal is shown in red.

(a)

Cell line	D37	Fold change compared to WT	Fold change Rad54 KO compared to Rad54 GFP-KI
WT	3.00		
Rad54 ^{-/-}	1.93	1.55	
WT Rad54 ^{KR-GFP/-}	2.49	1.21	1.29
DNA-PKcs ^{-/-}	0.91	3.30	
DNA-PKcs ^{-/-} Rad54 ^{WT-GFP/-}	0.79	3.80	0.87
DNA-PKcs ^{-/-} Rad54 ^{-/-} 1	0.44	6.85	
DNA-PKcs ^{-/-} Rad54 ^{KR-GFP/-}	0.61	4.89	1.40

(b)



Cell line	D37	Fold change compared to WT	Fold change Rad54 KO compared to GFP-KI
WT	2.82		
Rad54 ^{-/-}	1.62	1.74	
WT Rad54 ^{KR-GFP/-}	2.52	1.12	1.56
DNA-PKcs ^{-/-}	0.97	2.89	
DNA-PKcs ^{-/-} Rad54 ^{WT-GFP/-}	0.81	3.49	0.83
DNA-PKcs ^{-/-} Rad54 ^{-/-} 1	0.38	7.32	
	0.64	4.43	1.65

D37	Fold change compared to WT	Fold change Rad54 KO compared to GFP-KI
2.68		
1.49	1.79	
0.88	3.05	
0.82	3.26	0.94
0.32	8.28	
0.68	3.92	2.11

Figure S5. mES DNA-PKcs^{-/-} cells with ATPase defective Rad54 are hypersensitive to X-ray radiation. (a) Table shows D37 values and fold changes in sensitivity of clonogenic survival shown in Figure 4d. (b) Clonogenic survivals of mES cell lines with indicated genotypes after X-ray irradiation. Error bars represent SEM. The tables display D37 values and sensitivity fold changes of the clonogenic survivals displayed above the tables.

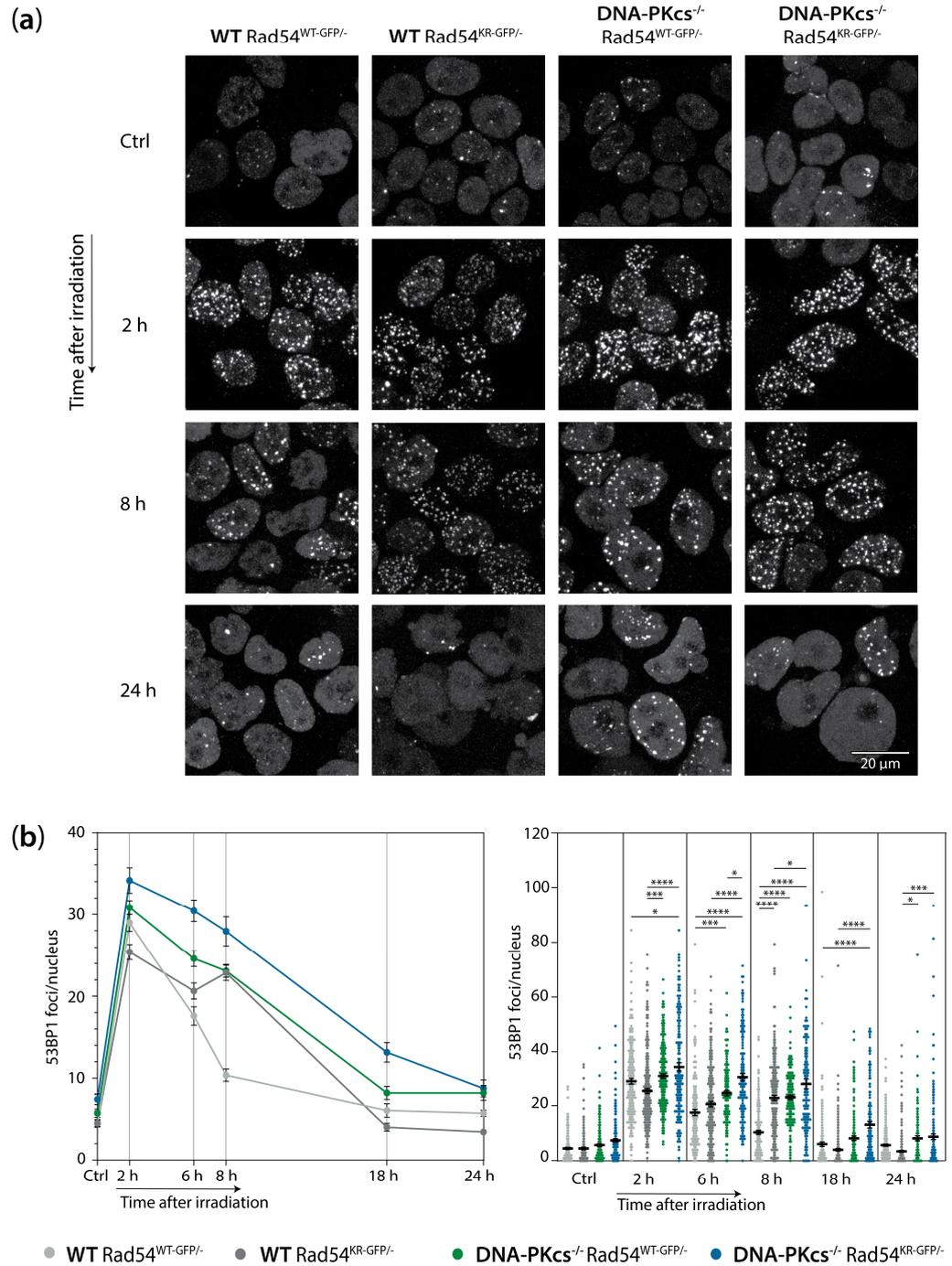


Figure S6. mES cells lacking DNA-PKcs and expressing catalytically dead Rad54 show impaired 53BP1 focus resolution. (a) Representative images of mES cells irradiated with 2 Gy of X-ray radiation and incubated for indicated times. After the recovery time, cells were fixated, stained and imaged for 53BP1. (b) Quantification of 53BP1 foci per mES nucleus. Left graph: dots indicate mean and error bars represent SEM. Right graph: each dot represents one nucleus. Black bars indicate mean and error bars represent SEM. Asterisks represent following *p*-values: * ≤ 0.05 ; *** ≤ 0.001 ; **** ≤ 0.0001 .

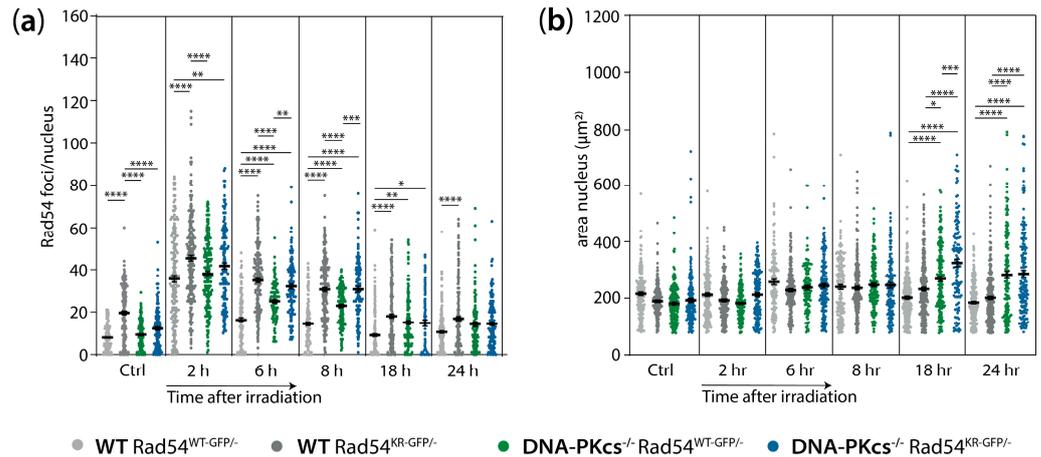


Figure S7. mES cells lacking DNA-PKcs and expressing catalytically dead Rad54 show impaired Rad54 focus resolution and increased nuclear size after 2 Gy of X-ray radiation. (a) Quantification of Rad54 foci per mES nucleus. Rad54 foci disappear in cells with enlarged nuclei (Figure 6), therefore nuclei larger than $400 \mu\text{m}^2$ were excluded from the analysis. (b) Quantification of area of mES nuclei. Each dot represents one nucleus. Black bars indicate mean and error bars represent SEM. Asterisks represent following p -values: * ≤ 0.05 ; ** ≤ 0.01 ; *** ≤ 0.001 ; **** ≤ 0.0001 .

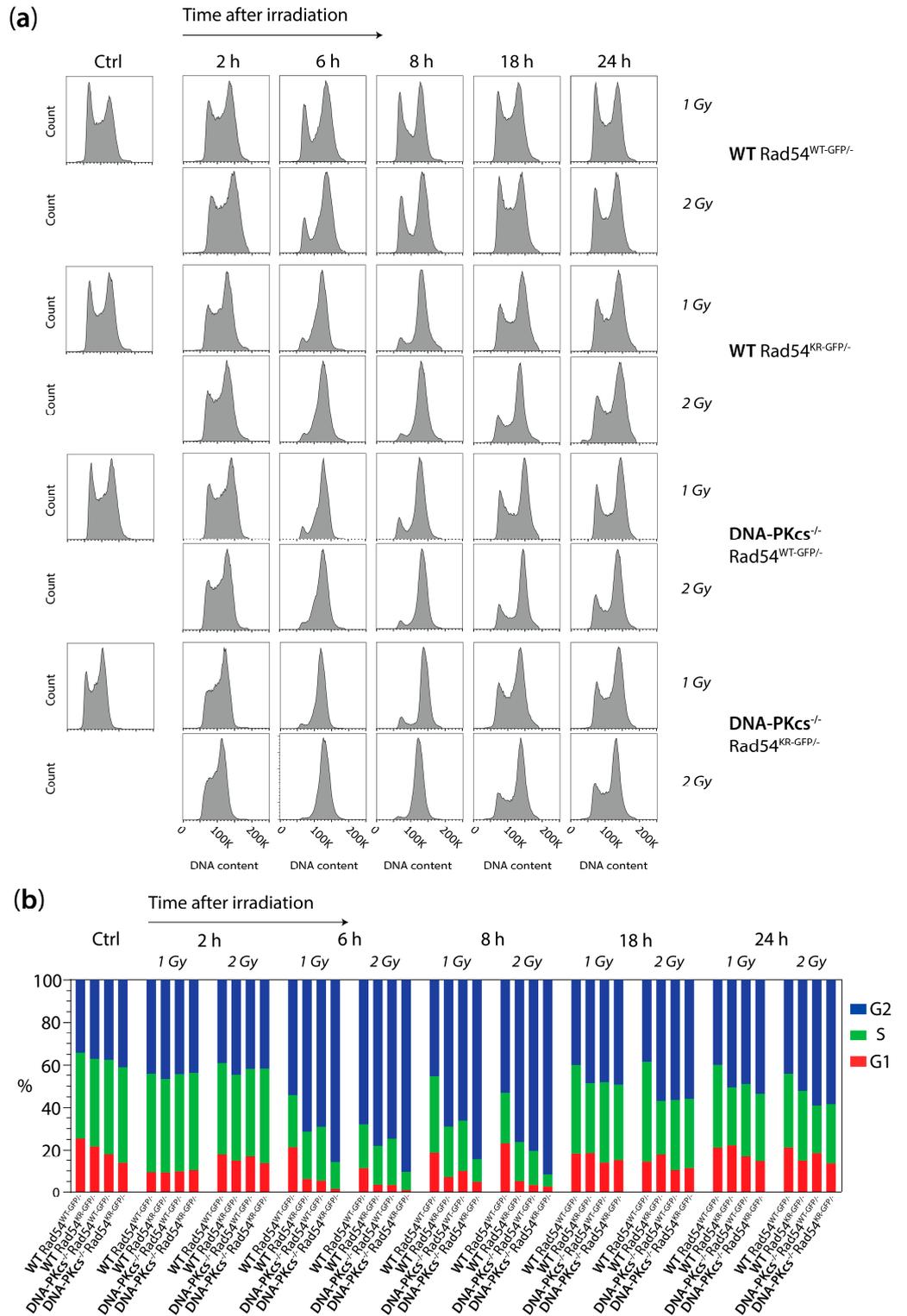


Figure S8. X-ray irradiation results in more persistent G2 phase cell cycle block in mES cells lacking DNA-PKcs and expressing catalytically dead Rad54. (a) mES cells were irradiated with 1 and 2 Gy of X-ray radiation and incubated for indicated times. After recovery time cells were fixed and DNA was stained using Propidium Iodide. Cell cycle distribution was analyzed using flow cytometry. (b) Quantification of percentage of G1, S and G2 phase cells in mES cells irradiated with 1 and 2 Gy of X-ray radiation as shown in Figure 8a.