Supplemental Data

Homologous Recombination but not Nucleotide Excision Repair Plays a Pivotal Role in Tolerance to DNA-Protein Crosslinks in Mammalian Cells

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Preparation of 150OXA-DPCs—150OXA-DPCs were prepared using two methods (Fig. S1A). In method I for DPCs containing small CLPs (Table S1), proteins were crosslinked to oxanine in 60OXA as described previously (1). The resulting products containing DPCs (60OXA-DPCs) were ligated to 50-mer L1 and 40-mer L2 using 20-mer scaffolds (S1 and S2) and T4 DNA ligase. 150OXA-DPCs were purified by 6% denaturing PAGE. In method II for DPCs containing large CLPs (Table S1), 60OXA was ligated to L1 and L2 to prepare 150OXA. Proteins were crosslinked to oxanine in 150OXA, and the resulting products containing DPCs (150OXA-DPCs) were purified by 10% SDS-PAGE. 150OXA-DPC containing NEIL1 was also digested exhaustively with trypsin or partially with *Acromobacter* protease I to prepare additional DPC substrates. Typical gel separation data of 150OXA-DPCs after crosslinking reactions or protease digestion are shown in Fig. S1B.

Cells—Cells, relevant mutations in repair genes, and sources are summarized in Table S2.

Transfection of GFP Plasmid—pcDNA-EGFP used for transfection assays was a derivative of pcDNA3.1/myc-His A MCS (Invitrogen), and a fragment containing the CMV promoter and the EGFP gene was inserted at the *Nde*I/*Xho*I site (N. Sakamoto unpublished data). MG132 (CALBIOCHEM) was initially dissolved in DMSO at a concentration of 42 mM and added to culture media (DMEM or DMEM + 10% FBS) to final concentrations of 0 (*i.e.*, DMSO alone), 0.5, and 2 μM. The transfection solution containing the Lipofectin Reagent (Invitrogen), pcDNA-EGFP, and DMEM (without or with MG132) was reconstituted as recommended by Invitrogen. WI38VA13 cells (1×10^5 cells) were plated in a 35 mm dish and incubated in DMEM + 10% FBS for 10 h in a 5% CO_2 atmosphere. The medium was changed to DMEM + 10% FBS containing MG132 (0–2 μM), and incubation was continued at 37 °C for 2 h. Cells were washed with DMEM twice and incubated with 1 ml of transfection solution containing pcDNA-EGFP (1 μg) and MG132 (0–2 μM) at 37 °C for 6 h. One ml of DMEM containing 20% FBS and MG132 (final concentration 0–2 μM) was added to the cell culture, and incubation was continued at 37 °C for 16 h. After incubation, cells attached to and detached from the dish were recovered separately, combined, and washed with PBS twice. Cells were

plated onto a 35 mm non-coated glass bottom dish (Matsunami Glass) and observed on an Olympus IX81 microscope. Pictures of cells were taken both in phase contrast and fluorescence modes to count total and GFP positive cells. The fraction (%) of GFP positive cells was calculated as 100 × GFP positive cells/total cells.

TABLE S1
Proteins crosslinked to 150OXA

Abbreviation	Protein (CLP)[a]	Amino acids	MW	Preparation[b]
spermine	spermine[c]	–	202	I
NEIL1-Δ1	hNEIL1 (47–57)	11	1187	II[d]
platelet	platelet factor-4	13	1573	I
dynorphin	dynorphin A	17	2148	I
endorphin	β-endorphin	31	3465	I
midkine	midkine (60–121)	62	6789	II
NEIL1-Δ2	hNEIL1 (30–128)	99	11089	II[d]
histone H2A	histone H2A	130	14091	II
NEIL1-Δ3	hNEIL1 (30–155)	126	14317	II[d]
T4 endo V	T4 endonuclease V	137	16046	II
histone H1	histone H1	215	21841	II
HMG1	HMG1 protein	215	24907	II
NEIL2	hNEIL2 glycosylase	332	36825	II
NEIL1	hNEIL1 glycosylase	390	43710	II

[a] Parenthesized numbers indicate amino acid compositions of truncated proteins.

[b] Methods to prepare 150OXA-DPCs (see supplemental experimental procedures).

[c] Spermine is a polyamine.

[d] Including protease digestion after crosslinking reactions.

TABLE S2

Mammalian cells used in this study

Cell	Mutation	Repair defect	Source and reference
Human cells			
HeLa	WT	none	Lab stock
MRC5SV	WT	none	Lab stock
WI38VA13	WT	none	T. Yagi (2)
XP12ROSV	XPA	NER	K. Tanaka (3)
XP2OSSV	XPA	NER	K. Tanaka (4)
XP4PASV	XPC	NER	T. Yagi (5)
XP6BESV	XPD	NER	K. Tanaka (6)
XP2YOSV	XPF	NER	T. Yagi (2)
FAD423SVT	FANCD1	HR	K. Komatsu (unpublished)
FAD+BRCA2	FANCD1+wtBRCA2	complemented	K. Komatsu (unpublished)
PD20F	FANCD2	HR	T. Taniguchi (7)
PD20F+FAD2	FANCD2+wtFANCD2	complemented	T. Taniguchi (7)
AT5BIVA	ATM	cell cycle check point	Lab stock (8)
AT5+YZ5	ATM+wtATM	complemented	Lab stock (8)
CHO cells			
AA8	WT	none	Lab stock
51D1	RAD51D	HR	L.H. Thompson (9)
51D1.3	RAD51D+wtRAD51D	complemented	L.H. Thompson (9)
irs1SF	XRCC3	HR	J. Thacker (10)
V3	DNA-PKcs	NHEJ	L. Thompson (11)
UV5	ERCC2 (XPD)	NER	A. Yasui (12)
UV41	ERCC4 (XPF)	NER	A. Yasui (13)

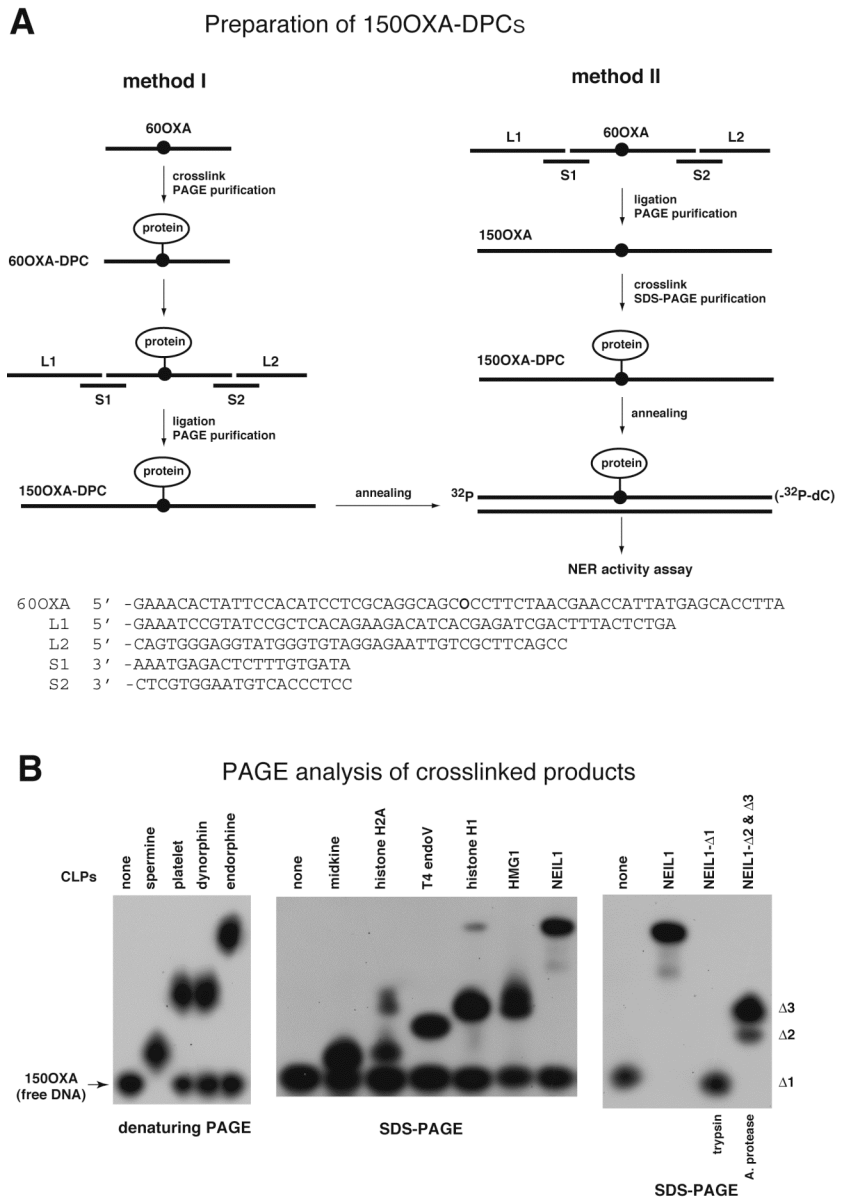

FIGURE S1. **Preparation and PAGE analysis of 150OXA-DPCs.**
A, Preparation of 150OXA-DPCs. 150OXA-DPCs containing small and large CLPs (Table S1) were prepared using methods I and II, respectively, as described in supplemental experimental procedures. Except for the final duplex, the 5'-end ^{32}P (L1) and 3'-end ^{32}P-dC (L2) labels are not shown in the scheme. *B*, PAGE analysis of 150OXA-DPCs. Typical gel separation data of 150OXA-DPCs after crosslinking reactions (left and middle panels) or protease digestion (right panel) are shown. The strand containing DPCs (150OXA) was 5'-end ^{32}P labeled.

SUPPLEMENTAL RESULTS

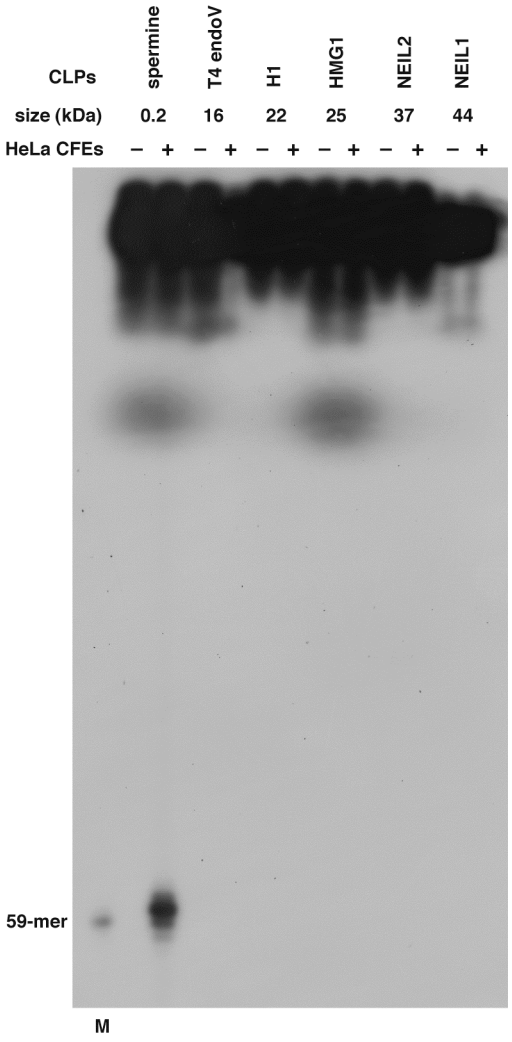

FIGURE S2. **DPC Substrates containing large CLPs are not incised by NER.**
150OXA-DPCs containing the indicated CLPs were 5'-end ^{32}P labeled, and duplex substrates (2 nM) were incubated with HeLa CFEs (100 μg) at 30 °C for 30 min (14). The sample was treated with proteinase K, and products were separated by 10% denaturing PAGE. The leftmost lane indicates a 59-mer marker. No incision occurred for 150OXA-DPCs with large CLPs (T4 endo V, histone H1, HMG1, NEIL2, and NEIL1).

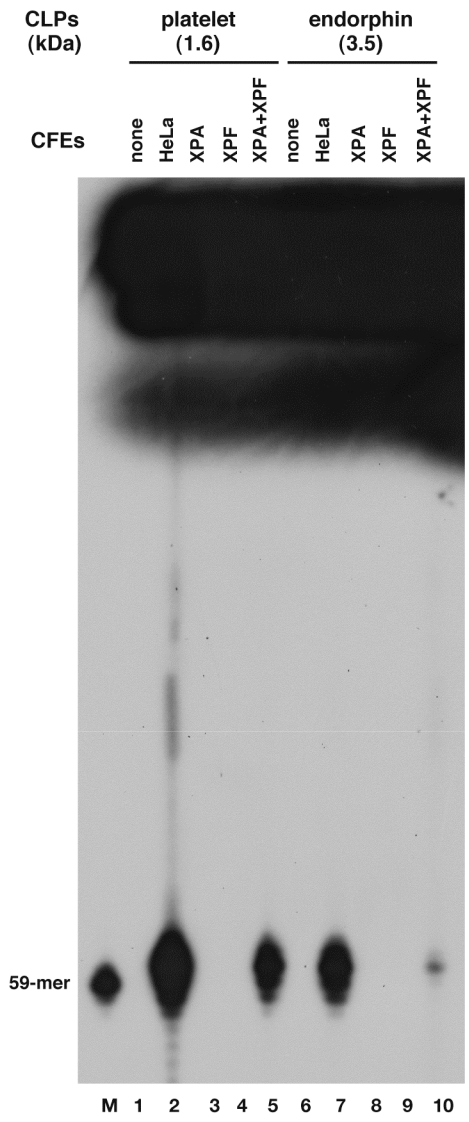

FIGURE S3. **NER incision activity for DPCs is restored when CFEs from XPA and XPF cells are combined.**

150OXA-DPCs containing platelet or endorphin were 5'-^{32}P end labeled, and duplex substrates (2 nM) were incubated with CFEs from HeLa, XPA, and XPF cells (100 μg), or with those from XPA+XPF cells (50 μg each) at 30 °C for 30 min (14). The sample was treated with proteinase K, and products were separated by 10% denaturing PAGE. The leftmost lane indicates a 59-mer marker. No incision occurred for 150OXA-DPCs with CFEs from XPA and XPF cells alone (lanes 3, 4, 8, and 9), but incision occurred with CFEs from XPA + XPF cells (lanes 5 and 10), demonstrating that the incision activity was attributable to NER. The gel was overexposed to show the faint incision band for combined CFEs (lane 10). The yields of incision products for platelet and endorphin with HeLa CFEs (lanes 2 and 7) were comparable to those shown in Fig. 1B.

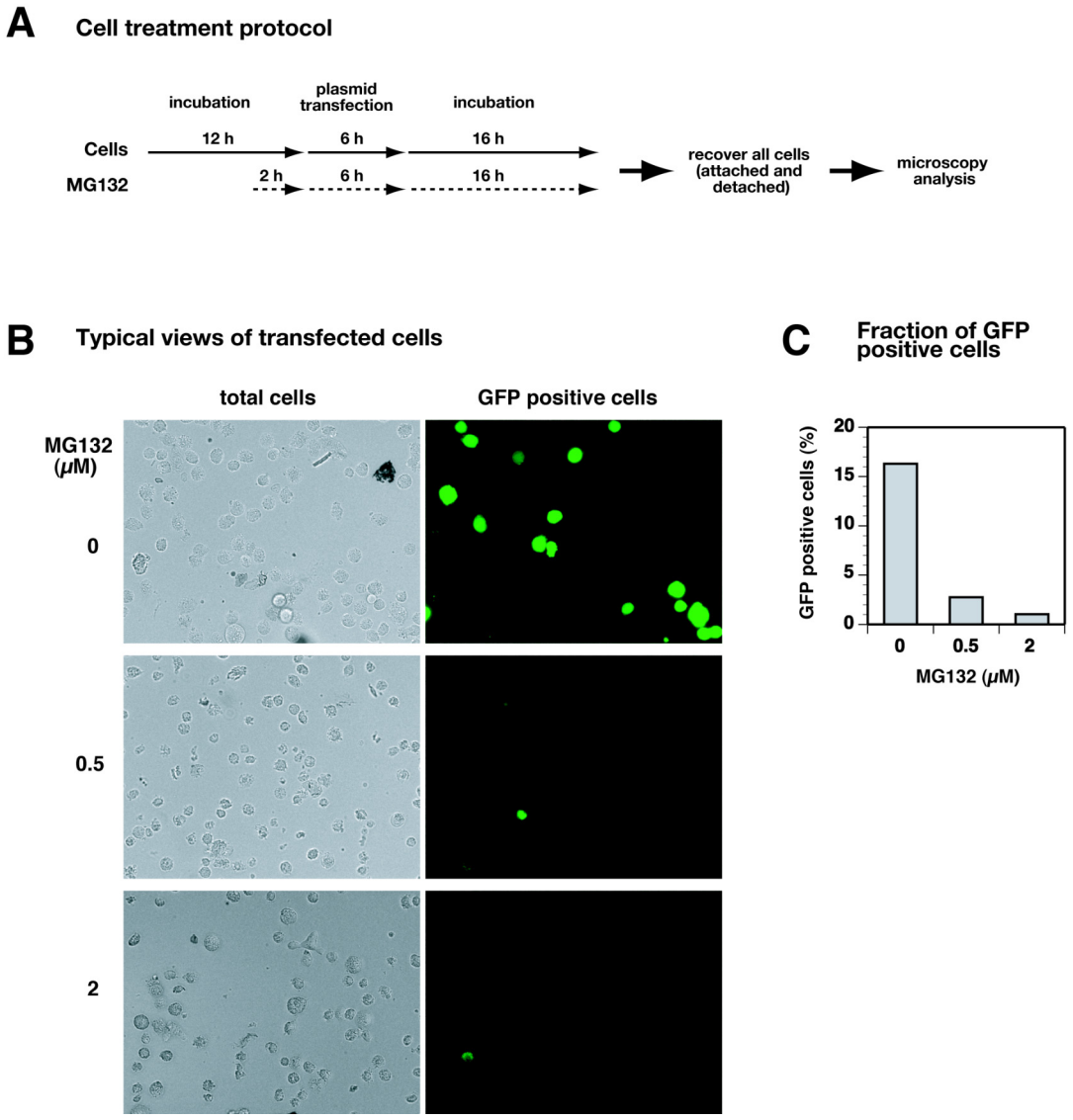

FIGURE S4. **Proteasome inhibitor MG132 impairs the transfection of intact GFP plasmid.** *A*, Protocol of cell treatment. pcDNA-EGFP was transfected into WI38VA13 cells in the presence or absence of the indicated concentrations of MG132 as described in supplemental experimental procedures. After 16 h of transfection, total and GFP positive cells were analyzed. *B*, Typical views of cells observed in phase contrast (total cells) and fluorescence (GFP positive cells) modes. *C*, Fractions (%) of GFP positive cells. The fraction of GFP positive cells decreased markedly in the presence of MG132. Data points are means of two independent experiments.

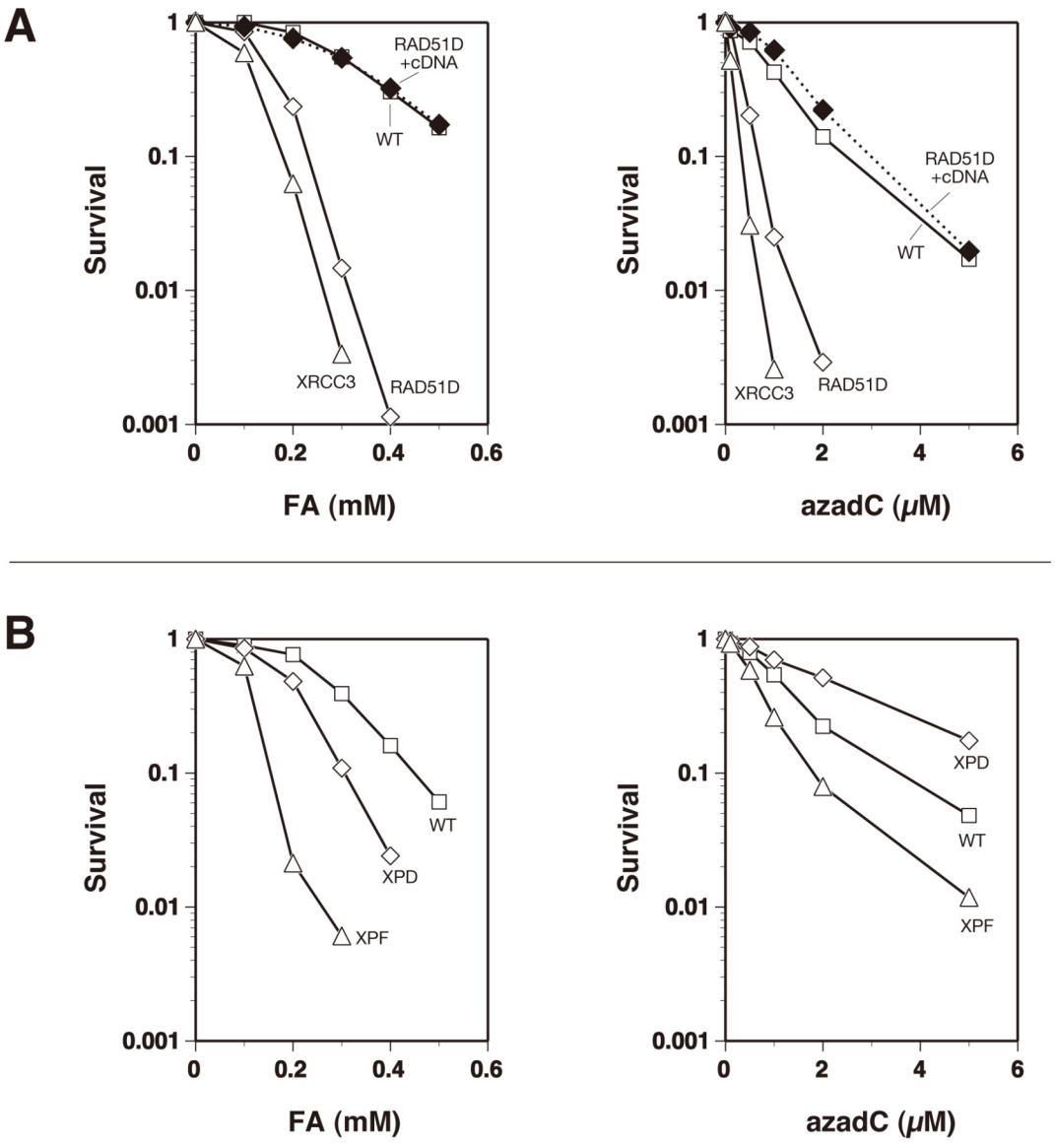

FIGURE S5. **Survival curves of repair-deficient CHO cells treated with FA or azadC.** *A*, Survival curves of HR-deficient cells. AA8 (WT), 51D1 (RAD51D), 51D1.3 (complemented with hamster RAD51D cDNA), and irs1SF (XRCC3). Cells were treated with the indicated concentrations of FA or azadC, and survival was measured by colony formation. Data points are means of three independent experiments. *B*, Survival curves of NER-deficient cells. AA8 (WT), UV5 (ERCC2/XPD), and UV41 (ERCC4/XPF). Cells were treated as in *A*.

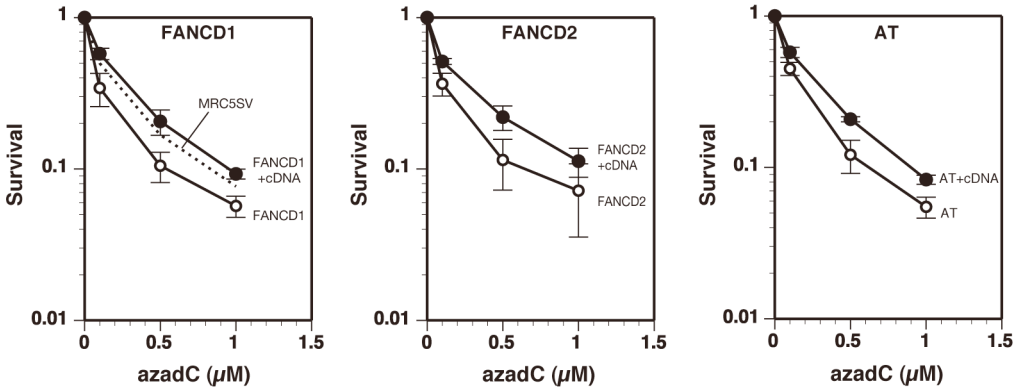

FIGURE S6. **Survival of FANCD1, FANCD2, and AT cells treated with azadC.**
Cells defective in the indicated gene (open symbols), those complemented with respective cDNA (closed symbols), and WT MRC5SV cells (no symbols) were treated with the indicated concentrations of azadC. Cell survival was assayed by colony formation. Data points are means of three or four independent experiments with standard deviation.

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