Supplemental Data

Bcl2 Negatively Regulates DNA Double-Strand Break Repair through a Non-Homologous End Joining Pathway

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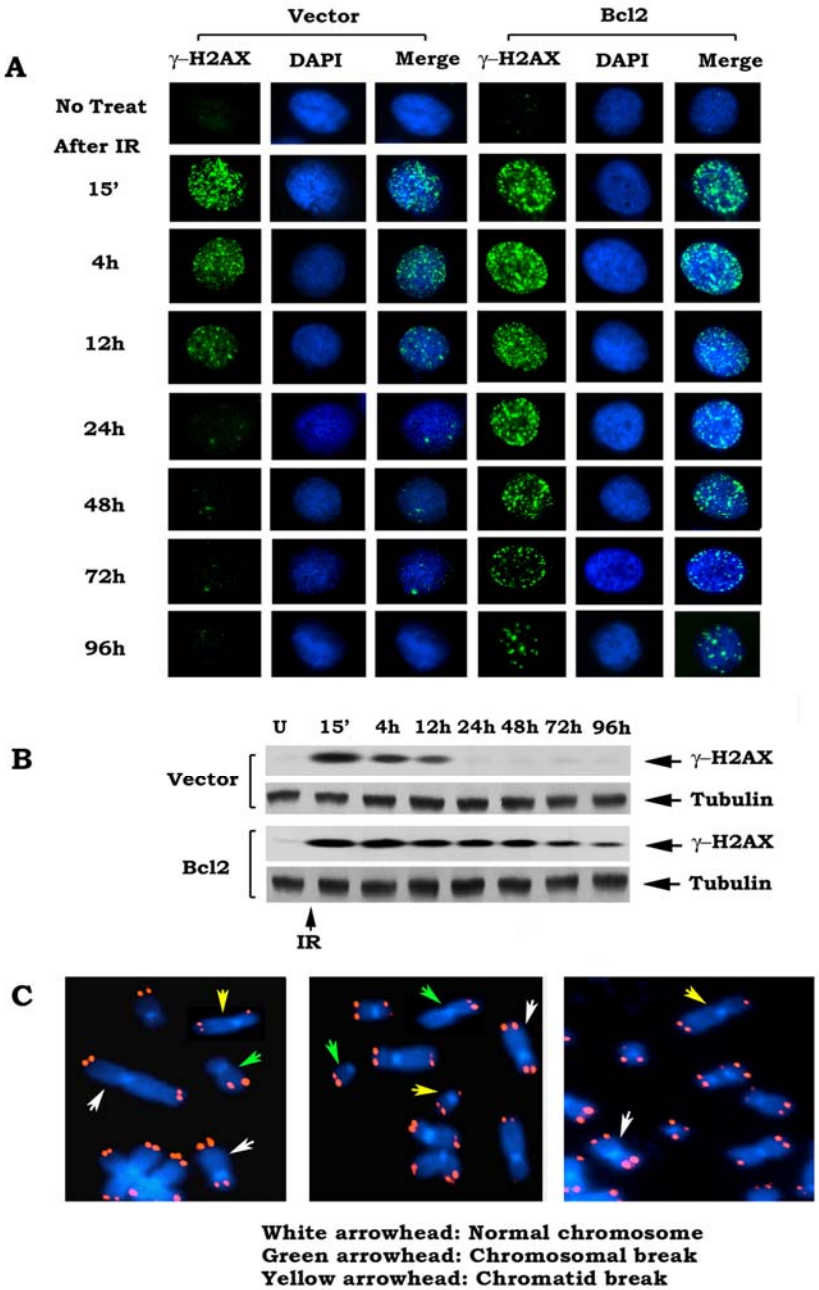

Figure S1. Expression of Bcl2 results in suppression of DSB repair and increased genetic instability. (**A-B**) H1299 cells overexpressing WT Bcl2 or vector control were exposed to 1 Gy of ionizing radiation (IR). Cells were harvested at various time points. DSBs were determined by analysis of γ-H2AX by immunostaining and Western blot. (**C**) Examples of cytogenetic abnormalities analyzed by telomere FISH (T-FISH) in H1299 cells overexpressing Bcl2. DAPI-stained chromosomes are blue. Red dots come from telomere signals. Color-coded arrowheads indicate a normal chromosome and different kinds of cytogenetic abnormalities (white: normal chromosome with four telomere signals; green: chromosomal break; yellow: chromatid break).

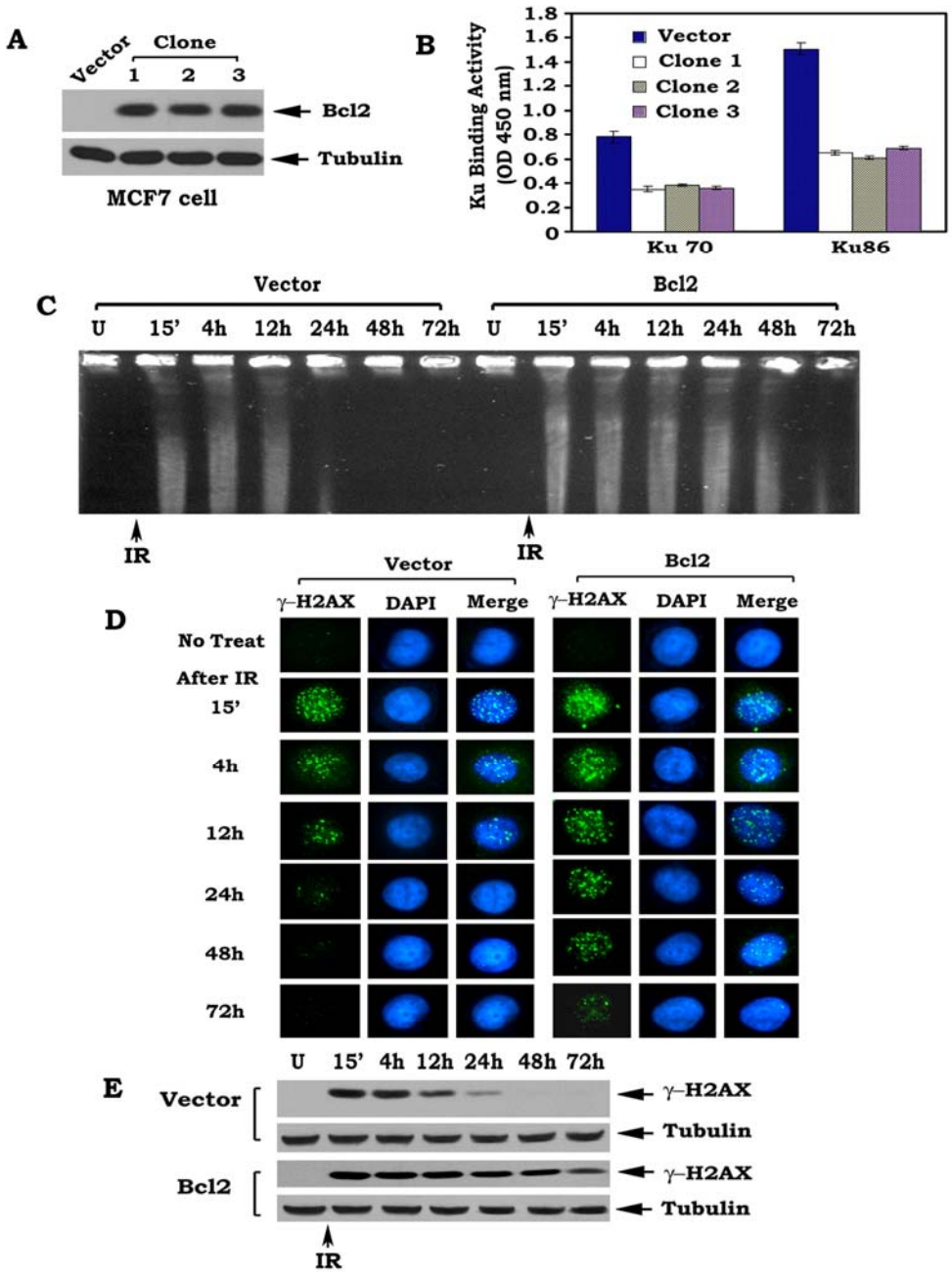

Figure. S2. Overexpression of exogenous Bcl2 down-regulates Ku activity in association with decreased DSB repair and increased genetic instability in MCF7 cells. (**A**) WT Bcl2/pCIneo DNA construct was stably transfected into MCF7 breast cancer cells. Expression levels of Bcl2 were determined by Western blot. (**B**) DNA binding activity of Ku 70 or Ku 86 in MCF7 cells overexpressing Bcl2 or vector control cells was measured using a Ku70/86 DNA Repair Kit. Error bars represent ±S.D. (**C-E**) MCF7 cells overexpressing WT Bcl2 or vector control were exposed to 1 Gy of ionizing radiation (IR). Cells were incubated in normal culture medium for various times. DSBs were determined by PFGE or analysis of γ-H2AX by immunostaining and Western blot.

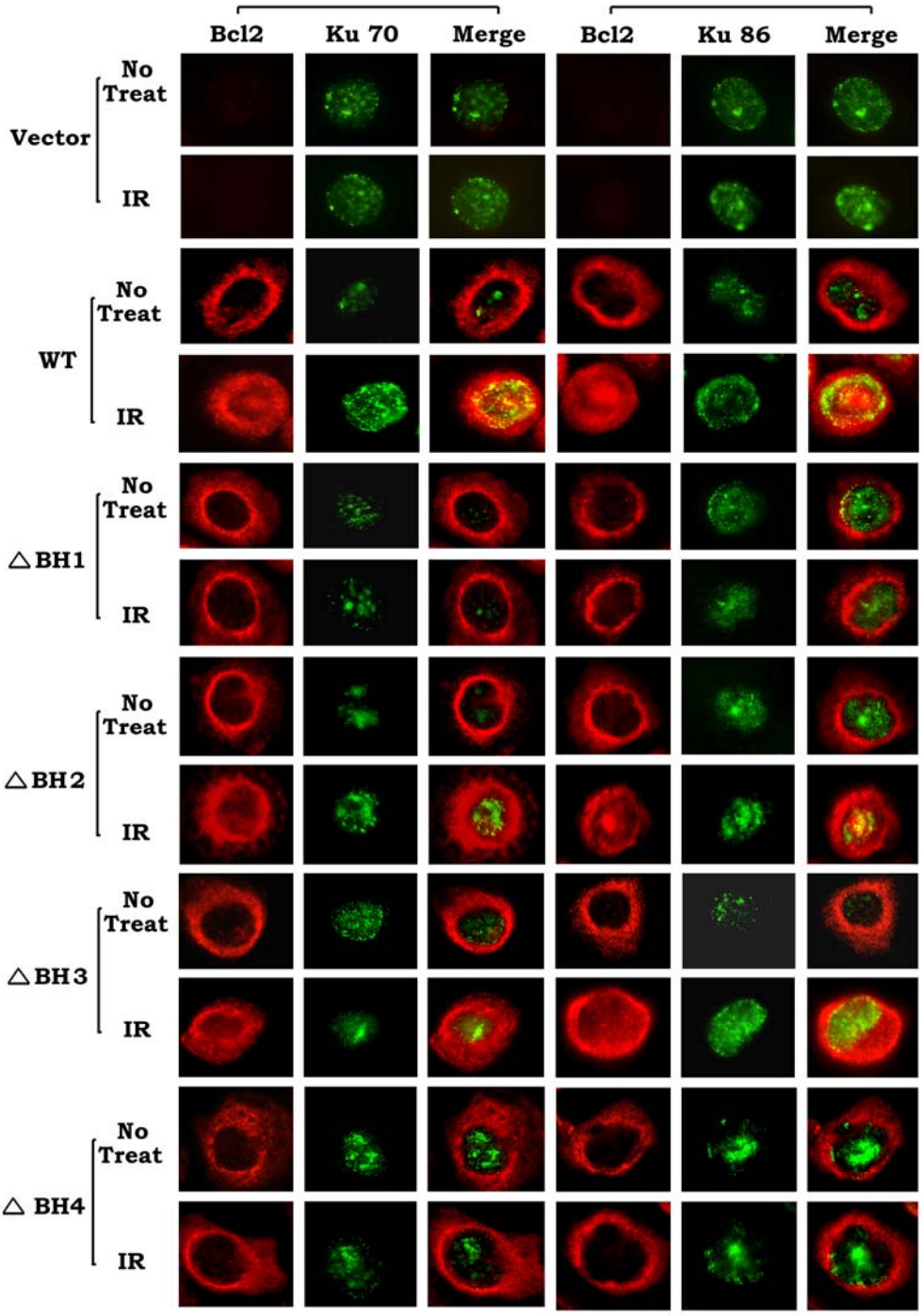

Figure. S3. The BH1 and BH4 domains (*i.e.* Ku binding sites) are required for Ku mediated nuclear sequestration of Bcl2 following IR treatment. H1299 cells expressing WT or each of various Bcl2 BH deletion mutants or vector-only control were exposed to IR (1Gy). Bcl2, Ku70 and Ku 86 were analyzed by immunofluorescent staining.

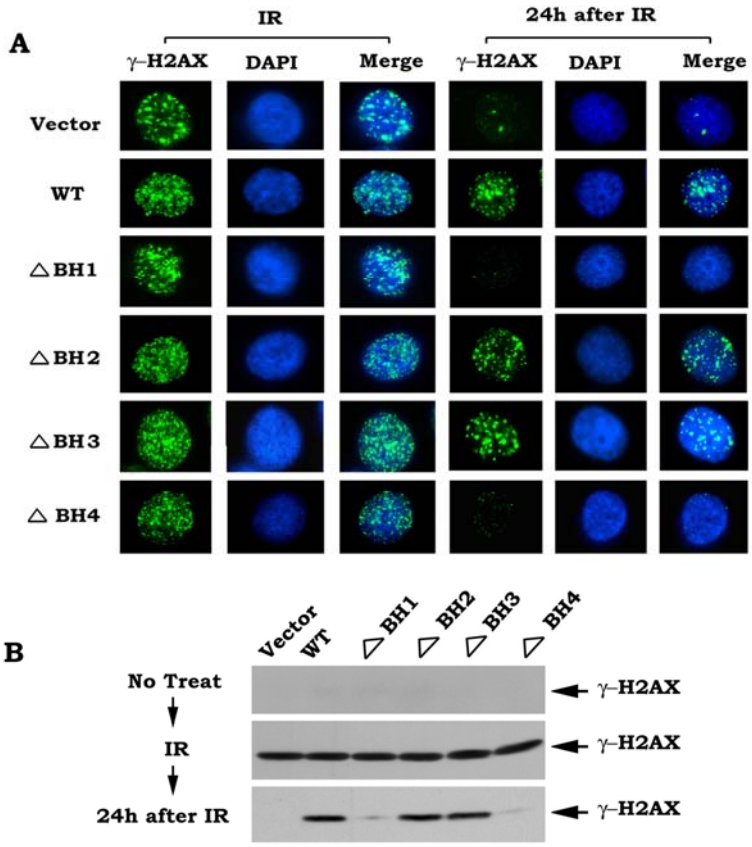

Figure S4. BH1 and BH4 domains are essential for Bcl2 to suppress DSB repair. (**A-B**) H1299 cells expressing WT or each of the BH deletion Bcl2 mutants were exposed to 1 Gy of IR. After 24h, DSBs were measured by analysis of γ-H2AX by immunostaining or Western blot using a γ-H2AX antibody.

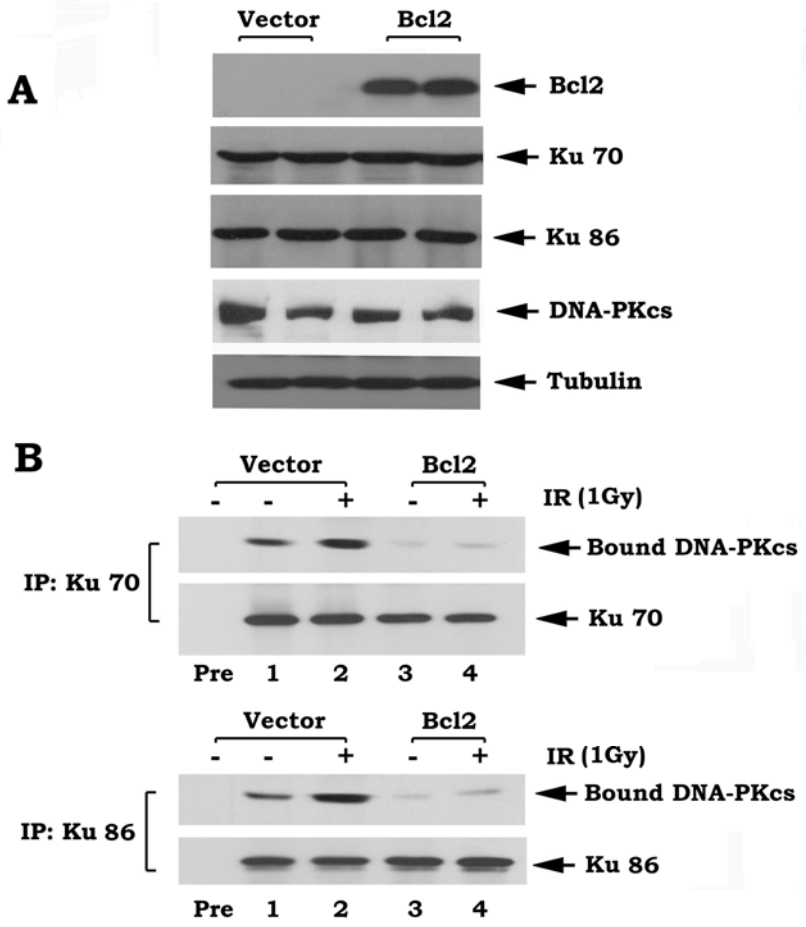

Figure. S5. Overexpresion of Bcl2 suppresses formation of the Ku/ DNA-PKcs complex. (A) Expression levels of Ku 70, Ku 86 and DNA-PKcs in H1299 cell expressing Bcl2 or vector-only control cells were determined by Western blot. (**B**) H1299 cells expressing Bcl2 or vector-only control cells were treated with IR (1 Gy) and disrupted in 1% CHAPS lysis buffer. Co-IP was performed using an agarose-conjugated Ku 70 or Ku 86 antibody, respectively. The Ku-associated DNA-PKcs, Ku 70 and Ku 86 were then analyzed by Western blot. Rabbit preimmune serum (Pre) was used as a control.

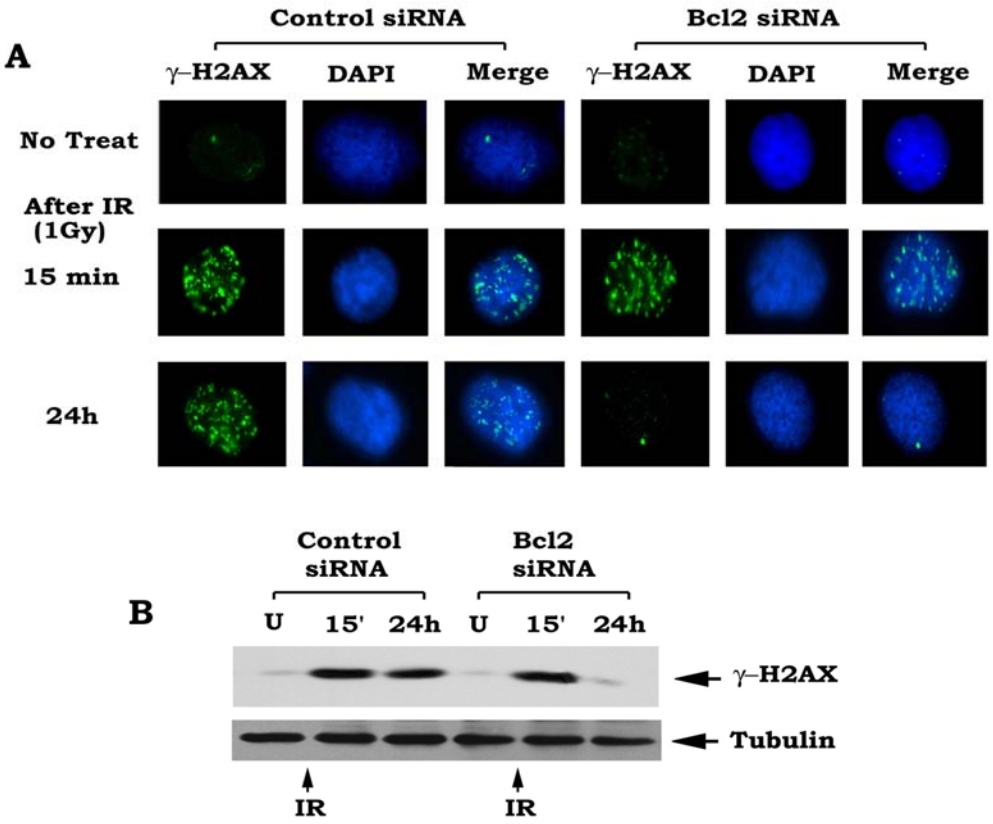

Figure S6. Depletion of endogenous Bcl2 expression by RNAi accelerates DSB repair. (**A-B**) H460 cells expressing Bcl2 siRNA or control siRNA were exposed to 1 Gy of IR. After 24h, DSBs were measured by analysis of γ-H2AX by immunostaining or Western blot using a γ-H2AX antibody.

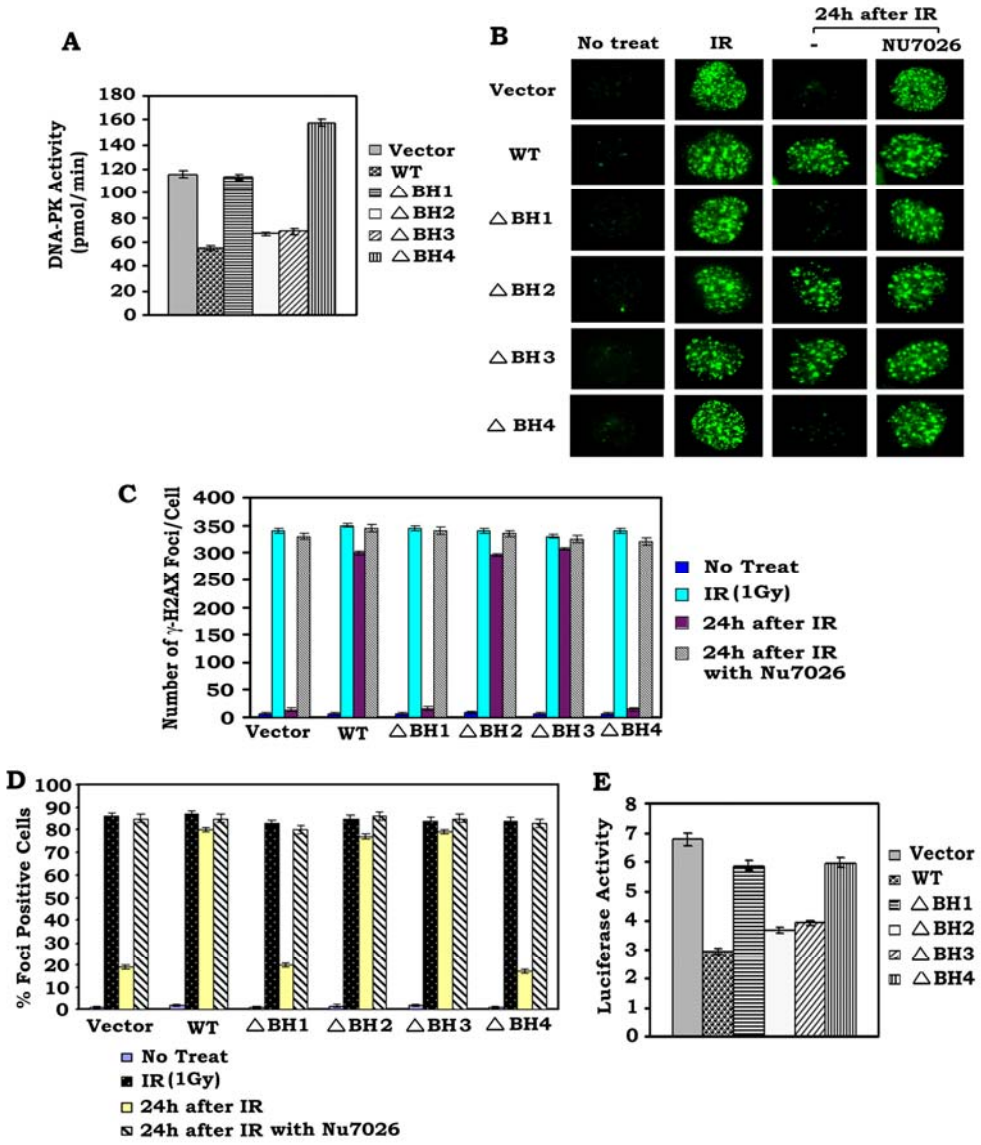

Figure S7. Bcl2 inhibits DNA-PK activity in association with suppression of DNA end-joining and DSB repair, which requires its BH1 and BH4 domains. (**A**) DNA-PK activity was measured using the SignaTECT DNA-PK Assay Kit in H1299 cells expressing similar levels of WT or each of the BH deletion Bcl2 mutants. Error bars represent ±S.D. (**B-D**) H1299 cells expressing WT or each of the BH deletion Bcl2 mutants were exposed to IR (1 Gy) in the absence or presence of NU7026 (10μM). DSBs were measured by immunostaining using a γ-H2AX antibody. The number of γ-H2AX foci per cell was determined on a cell to cell basis by the quantitative analysis of at least 30 randomly chosen cells as described (Balajee et al., 2004). The percentage of γ-H2AX foci positive cells was determined by analyzing 100 randomly chosen cells. Error bars represent ±S.D. (**E**) DNA end-joining activity was measured in H1299 cells expressing WT or each of the BH deletion Bcl2 mutants. Error bars represent ±S.D.

Effect of Bcl2 on RS and Coding Joining

	pJH 200 (RS Joining)				pJH290 (Coding Joining)		
Cell line	(Amp^r + Cam^r)/Amp^r	%	Relative Level	Fidelity	(Amp^r + Cam^r)/Amp^r	%	Relative Level
Expt. I							
Vector	690/28,400	2.43	1.00	11/11 (100%)	354/37,480	0.94	1.00
WT	289/31,250	0.92	0.38	4/10 (40%)	161/39,292	0.41	0.44
△BH1	684/28,700	2.38	0.98	17/17 (100%)	348/39,340	0.88	0.94
△BH2	286/30,800	0.93	0.38	7/15 (47%)	168/39,324	0.43	0.46
△BH3	290/30,160	0.96	0.40	8/16 (50%)	160/37,680	0.42	0.45
△BH4	741/31,500	2.35	0.97	13/13 (100%)	375/40,170	0.93	0.99
Expt. II							
Vector	164/14,480	1.13	1.00	15/15 (100%)	190/70,584	0.27	1.00
WT	70/14,535	0.48	0.42	7/17 (41%)	79/69,854	0.11	0.41
△BH1	156/14,500	1.07	0.95	14/14 (100%)	192/73,185	0.24	0.89
△BH2	72/14,148	0.51	0.45	6/15 (40%)	89/70,909	0.13	0.48
△BH3	68/14,580	0.47	0.42	8/18 (44%)	83/70,148	0.12	0.44
△BH4	169/14,427	1.17	1.03	17/17 (100%)	185/70,975	0.26	0.96
Expt. III							
Vector	139/45,111	0.31	1.00	13/13 (100%)	481/26,082	1.84	1.00
WT	52/45,965	0.11	0.35	8/18 (44%)	208/26,450	0.79	0.43
△BH1	135/47,664	0.28	0.90	16/16 (100%)	482/26,453	1.82	0.99
△BH2	57/45,696	0.13	0.42	9/19 (47%)	216/24,250	0.89	0.54
△BH3	56/45,710	0.12	0.39	7/15 (47%)	194/23,700	0.82	0.45
△BH4	138/45,750	0.30	0.97	10/10 (100%)	496/27,400	1.81	0.98
Expt. IV							
Vector	261/16,340	1.60	1.00	18/18 (100%)	405/34,730	1.17	1.00
WT	124/16,256	0.76	0.48	8/17 (47%)	189/33,925	0.56	0.48
△BH1	233/15,884	1.47	0.92	14/14 (100%)	378/35,280	1.07	0.91
△BH2	129/16,308	0.79	0.49	7/16 (44%)	199/34,260	0.58	0.49
△BH3	136/16,200	0.84	0.53	6/16 (38%)	175/32,750	0.53	0.45
△BH4	268/17,400	1.54	0.96	17/17 (100%)	403/36,190	1.11	0.95

Table S1. RS and coding joining was measured in H1299 cell expressing WT or each of the BH deletion Bcl2 mutants. Four independent transfection experiments were carried out with each cell line.

Supplemental Methods

DNA-PK activity assay. The SignaTECT DNA-PK Assay Kit was used to measure DNA-PK activity (Promega). First, the following reaction mixtures in the presence or absence of activator was prepared (*i.e*. 2.5µl DNA-PK activation buffer or control buffer, 5.0µl DNA-PK in 5 × reaction buffer, 2.5µl DNA-PK biotinylated peptide substrate, 0.2µl BSA(10mg/ml) and 5µl γ^{32}P-ATP). The mixture was preincubated at 30 ^{o}C for 4 min. The whole cell extract sample was diluted in the enzyme buffer and the reaction was initiated by adding the appropriate amount of enzyme sample and incubated at 30 ^{o}C for 5 min. The reaction was stopped by addition of termination buffer. Sample was spotted onto the SAM^{2R} Biotin Capture membrane. The membrane was then washed and dried under a heat lamp for 10 min. DNA-PK enzyme activity was determined by scintillation counting. Each experiment was repeated three times and data represent the mean ± S.D. of three separate determinations.

In vivo DNA end-joining assay. The pGL3 plasmid (Promega), in which expression of the luciferase gene is controlled by the CMV promoter, was used to evaluate correct nonhomologous end-joining activity that precisely rejoins broken DNA ends *in vivo* as described (Shin et al., 2004). The pGL3 plasmid was completely linearized by the restriction endonuclease *Nar*I, which cleaves within the luciferase coding region as confirmed by agarose gel electrophoresis. The linearized DNA was purified and then dissolved in sterilized water. A 20:1 mixture of the linearized pGL3 plasmid and pTK Renilla control luciferase reporter vector (an internal control) were transfected into H1299 cells expressing WT or each of the BH deletion Bcl2 mutants. After 48h, luciferase activity was measured using a dual

luciferase assay system following the manufacture's instruction (Promega). Since the pGL3 reporter plasmid was digested to completion with *Nar*I within the luciferase coding region, only precise DNA end-joining can restore the luciferase activity. Each experiment was repeated three times and data represent the mean ± S.D. of three separate determinations.

Supplemental References

Balajee, A. and Geard, C. (2004).Replication protein A and γ-H2AX foci assembly is triggered by cellular response to DNA double-strand breaks. Experimental Cell Research *300*, 320-334.

Gu, Y., Jin, S., Gao, Y., Weaver, D., and Alt, F.W. (1997).Ku70-deficient embryonic stem cells have increased ionizing radiosensitivity, defective DNA end-binding activity, and inability to support V(D)J recombination. Proc Natl Acad Sci U S A. *94***,** 8076-8081.

Shin, K., Kang, M., Dicterow, E., Kameta, A., Baluda, M., and Park, N. (2004). Introduction of human telomerase reverse transcriptase to normal human fibroblasts enhances DNA repair capacity. Clinical cancer research *10***,** 2551-2560.