Supplemental Experimental Procedures

Recombinant Protein Preparations

Wild-type, mutant (S122A, S122E, and C303A), and truncated prodomain of human caspase-2, as well as full-length (910 aa) and truncated death domain of human PIDD, each with a C-terminal FLAG tag, was subcloned into pET28a and expressed in BL21 *E. coli* and purified by His-affinity (Qiagen) and FLAG-affinity (Sigma) columns using standard protocols.

Stable Cell Lines

Human PIDD (910 aa) cloned into pcDNA5/FRT with a C-terminal FLAG tag was transfected into Flp-In^{TM}-293 cells (Invitrogen) or Flp-In-HeLa S3 cells (kindly provided by Drs. Joan and Ron Conaway). Stable HEK293 or HeLa cell lines expressing FLAG-PIDD were selected with 300 μg/ml of hygromycin and used for immunoprecipition and mass spectrometry analysis.

Human catalytic site mutant (C303A) caspase-2 cloned into pcDNA5/FRT with an N-terminal FLAG tag was expressed into Flp-In^{TM}-293 cells. Stable cell lines were selected and used for mapping phosphorylation sites by mass spectrometry.

Mutations of S122A and C303A were each introduced into pcDNA3.1-caspase-2-WT (435 aa, GenBank: AAP35904) by QuickChange Site-Directed Mutagenesis Kit (Stratagene). The resultant caspase-2 variants along with the caspase-2-WT constructs were each cloned into pRevTRE to generate pRevTRE-Caspase-2-WT, pRevTRE-Caspase-2-S122A, and pRevTRE-Caspase-2-C303A. These constructs were transfected into the retrovirus packaging Plat E cells to produce retroviruses expressing pRevTRE-vector, pRevTRE-Caspase-2-WT, pRevTRE-

Caspase-2-S122A, or pRevTRE-Caspase-2-C303A. Each of the resultant retroviruses was infected into caspase-2 (-/-) MEFs immortalized by SV40 large T antigen. The resultant isogenic stable MEF clones were designated as caspase-2 (-/-) + Vector, caspase-2 (-/-) + WT, caspase-2 (-/-) + S122A, and caspase-2 (-/-) + C303A. pRevTRE-vector and pRevTRE-PIDD were also infected into the SV40-immortalized caspase-2 (-/-) MEFs to generate stable cell lines of caspase-2 (-/-) + Vector and caspase-2 (-/-) + PIDD.

A pair of RAIDD wild-type and knockout primary MEF cells (kindly provided by Dr. Tak M. Mak), PIDD wild-type and knockout primary MEF cells, DNA-PKcs wild-type and knockout primary MEF cells, Ku80 wild-type and knockout primary MEF cells were immortalized by SV40 large T antigen.

Chemicals

Biotinylated-VAD-fmk (b-VAD-fmk) was purchase from Sigma; Caspase-3 inhibitor II (Z-D(OMe)E(Ome)VD(OMe)-FMK), DNA-dependent protein kinase inhibitor II (NU7026) and ATM kinase inhibitor from CalBiochem.

Antibodies and Immunoblotting

A polyclonal antibody was generated (Rockland, Inc) by immunizing rabbits with purified recombinant truncated PIDD (residues 755-895,140 aa) and purified on protein G columns (Millipore). The specificity of this antibody was verified by siRNA-mediated knockdown of PIDD expression as well as by ectopic expression of PIDD. A phospho-S122-specific polyclonal antibody was generated (Pacific Immunology Inc.) with phosphorylated synthetic peptide

DYDLpSLPFPVC representing the sequences at and surrounding serine 122 and purified by peptide-affinity and protein A agarose column (Millipore). Antibodies against DNA-PKcs (H-163), p53 (DO-1), and p21 (H-164) were purchased from Santa Cruz; anti-phospho-histone H3 (ser10, 3H10)-FITC conjugate from Upstate; caspase-2 monoclonal antibody (11B4) from Chemicon; caspase-3, active caspase-3, BIP and mouse RAIDD antibodies from Cell Signaling; RIP1 monoclonal (ab56815) antibody from Abcam; RAIDD polyclonal antibody from MBL International Corporation; Ku80 monoclonal (S10B1) and Ku70 monoclonal (N3H10) antibodies from Lab Vision; mouse Ku80 antibody from Novus Biologies; ATM monoclonal (MAT3-4G10/8) antibody from Sigma; PIDD monoclonal (anto-1) antibody from Alexis Biochemicals; FLAG M2 monoclonal antibody from Sigma; PARP-1 monoclonal (C2-10) antibody from BD Pharmingen. Immunoblotting was performed in 50 μg nuclear or cytosolic extracts.

Immunoprecipitation

Two mg of nuclear extract was pre-cleared with protein G agarose and incubated with antibodies plus protein G agarose beads (Roche) at 4 ^{o}C overnight. The immunoprecipitates were washed four times and subjected to SDS-PAGE and immunoblotted with specific antibodies.

For ethidium bromide (EB) treatment, EB was added into nuclear lysates at final concentration of 10 or 50 μg/ml and the nuclear lysates were incubated on ice for 30 min. The mixture was utilized for co-immunoprecipitation with Flag M2 monoclonal antibody conjugated agarose beads. The original concentration of EB was maintained during the followed washing steps. For micrococcal nuclease treatment, the immunoprecipitates bound to the beads after four washes were suspended in 50 μ1 of digestion buffer (50 mM NaCl/10 mM Tris, pH 7.0/4 mM CaCl2) and incubated with micrococcal nuclease (10 or 50 units, NEB) at 37 °C for 1 hr. The co-

immunoprecipitation samples were washed twice with 1 ml of digestion buffer prior to SDS/PAGE.

Identification of PIDD-Interacting Proteins and Mapping of Caspase-2 Phosphorylation Sites by Multidimensional Protein Identification Technology (MudPIT)

FLAG tagged-PIDD-stable HEK293 cells were left untreated or γ-radiated with 80-Gy IR. Two hrs after IR the cells were harvested for nuclear extract preparation, from which PIDD was immunoprecipitated with anti-FLAG M2 antibody. PIDD-interacting proteins were identified by MudPIT (See supplemental materials for details). To map the phosphorylation sites on caspase-2, HEK293 cells stably expressing a FLAG tagged, catalytic site mutant caspase-2 were either left untreated, irradiated with 80 Gy IR, or etoposide-treated (100 μM). Two hrs following treatment the cells were harvested for nuclear extract preparation. FLAG-caspase-2 (C303A) protein was purified by anti-FLAG-M2-immunoprecipitation and subjected to MudPIT identification for phosphorylated peptides (See supplemental materials for details).

Labeling Caspases in Live Cells with b-VAD-fmk

To label active caspases in viable cells, $2X10^7$ HeLa, HEK293, or caspase-2 wild-type and deficient MEF cells grown in culture dishes were fed with different amounts of biotinylated-VAD-fmk (b-VAD-fmk, Sigma) and incubated for 1 hr at 37°C. Cells were washed three times before harvest. Cell pellets were lysed at 4 °C in 1ml of low salt buffer [10 mM Hepes pH7.9, 300 mM sucrose, 1.5 mM $MgCl_2$, 10 mM KCl supplemented with 1 mM dithiothreitol, protease inhibitor (Sigma), and phosphatase inhibitor (set I and II) cocktails (Sigma)]. Plasma membranes were broken by passing through a 25-gauge syringe 30 times and checked with 0.2% trypan blue

staining under a light microscope. The cell lysate was centrifuged at 500 g for 5 minutes at 4 °C and the upper layer of the supernatant was collected as the cytosolic fraction. The nuclear pellet was washed twice with the low salt buffer, followed by resuspension in high salt buffer [20 mM HEPES pH7.9, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 100 mM KCl, 25% glycerol, 1% Triton X-100 plus the protease and phosphatase inhibitors, and disrupted by four cycles of freeze-thaw and a brief sonication. The nuclear fraction was obtained following centrifugation at 12,080 g for 30 minutes at 4 °C. Nuclear extracts from equal cell numbers were incubated with 30 µl of streptavidin conjugated agarose beads (Sigma) overnight with rotation at 4 °C. The agarose beads were washed four times with washing buffer (20 mM HEPES pH7.9, 300 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol, and 1% Triton X-100). The b-VAD-binding proteins were eluted from the beads by the addition of 40 µl of 2X SDS sample loading buffer and heated at 95 °C for 5 minutes.

GST Pulldown

cDNA encoding different regions of DNA-PKcs (amino acid residues 1-403, 500-1025, 1000-1025, 1500-2000, 1878–2182, 2261-2700, and the 3747-4128 PI3K domain) were PCR-amplified and cloned into pGEX vector. Bacterial cell lysates expressing GST or GST-DNA-PKcs fusion proteins were incubated with Glutathione Sepharose 4B beads (Amersham Biosciences) at 4 °C for 1 hour and washed three times with GST binding buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Nonidet P-40). Recombinant proteins of the caspase-2 prodomain or the DD of PIDD were added to the glutathione beads and incubated at 4 °C for 2 hrs, followed by three washes with GST binding buffer. The bound proteins were resolved on SDS-PAGE and analyzed by immunoblotting.

In Vitro DNA-PK Kinase Assay

The hot kinase assay was carried out at 30°C for 30 minutes in 10 μl reaction volume containing 10 nM DNA-PKcs, 10 nM Ku (both purified from HeLa cells), 20 or 40 nM caspase-2, 1 μg sheared salmon sperm DNA, and 0.16 μM [γ-^{32}P]ATP (6000 Ci/mmol) in the reaction buffer (25 mM Tris-HCl pH 7.9, 25 mM $MgCl_2$, 1.5 mM DTT, 50 mM KCl, and 10% glycerol) (Lee et al., 2002). The cold kinase reactions were carried out under the same conditions but with 10 mM to replace [γ-^{32}P]ATP. PIDD at 10 nM or 20 nM was added in some reactions. Reactions were stopped and samples were resolved by 12% SDS-PAGE. Protein phosphorylation was detected by PhosphorImager for the hot or immunoblotting for the cold reaction products.

Gel Filtration Chromatography

HeLa nuclear extracts were fractionated using FPLC protein purification system (Pharmacia) on a Superose 6 column (Amersham Biosciences) at 4°C equilibrated with buffer A (20 mM Hepes-KOH, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, pH 7.9) plus 150 mM NaCl. HeLa nuclear extracts (2 mg) were loaded and eluted from the column with the same buffer at a flow rate of 0.4 ml/min and 400μl fractions were collected. The column was calibrated with gel filtration standards (Bio-Rad Laboratories).

Annexin V-FITC/Propidium Iodide (PI) Apoptosis Assay

Cells unirradiated or irradiated with 4 Gy of IR were washed with PBS, resuspended in annexin V binding buffer containing PI and annexin V (Annexin V kit, BD Pharmingen™), and

incubated for 15 min at room temperature in dark. Cellular fluorescence was monitored with a CyAn flow cytometer (Dako). A total of 30,000 cells were analyzed for each sample.

Silencing of PIDD or Caspase-2 Expression by Small Interfering RNA (siRNA)

siRNA duplexes with 3'dTdT overhangs corresponding to PIDD mRNA [CAGACTGTTCCTGACCTCAGA, named siPIDD(Tinel and Tschopp, 2004) or caspase-2 mRNA (GUUGUUGAGCGAAUUGUUA, named siC2) were designed by Dharmacon siDESIGN®Center (without sequence match to other genes in the database). Both siC2 and the control siRNA siControl (UAAGGCUAUGAAGAGAUAC) were synthesized by Dharmacon. U2OS or HEK293 cells were transfected with these siRNA duplexes by Gene Silencer siRNA transfection reagent (Gene Therapy Systems) for two successive rounds at 48-hour intervals, and knockdown efficiency was examined in nuclear extracts by immunoblotting.

***In Vivo* DNA End-joining Assay**

The plasmid-based DNA end-joining *in vivo* assay was performed as described previously (Wang et al., 2006), in which the green florescent protein (GFP) vector (pEGFP-N1, Clontech, Palo Alto, CA) was utilized as a reporter. The pEGFP-N1 vector was linearized using *Bsr*GI, to cut within the *GFP* coding region. The linearized plasmid DNA was separated by agarose gel electrophoresis and purified on Qiagen spin columns (Qiagen). The completeness of restriction digestion was verified by negative colony growth on LB agar plates after being electro-transformed into *Escherichia coli* (*E. coli*). Immediate and parallel transfection into mammalian cells either wild type or deficient of genes of interest with the linearized or uncut (positive control) plasmids (2 μg) was done in 6-well plates using LipofectAMINE 2000 (Invitrogen)

following manufacturer's instruction and the transfectants were harvested 48 hours later and GFP intensity in the live cells was detected using a flow cytometer.

Forty-eight hours after transfection the mammalian cells were harvested after three-time-washes with phosphate buffer saline (PBS, pH7.4). Plasmid DNA was extracted from those cells using SV Minipreps DNA Purification System (Promega). Then the bacterially methylated DNA, which has not replicated in mammalian cells, was digested with *Dpn*I (total 23 sites in pEGFP-N1 vector), followed by electro-transformation into competent *E. coli* ElectroMAX DH10B (Invitrogen). Transformants from religated circular plasmids were selected on LB agar plates containing kanamycin (25 μg/ml). Kanamycin-resistant colonies were picked for plasmid DNA preparations and the religated junctions were sequenced with the primers which are situated upstream of the restriction site.

In Vivo Precise NHEJ Assay Using _LacZ_ Reporter

We adopted a plasmid-based assay that provides readout of NHEJ. Plasmid DNA cut and linearized within the *LacZ* gene by restriction digestion can be repaired by NHEJ after transfected into mammalian cells. Once these plasmids are retrieved from those transfected cells and subsequently transformed in DH10B cells, faithful ligation of the original plasmid ends by mammalian NHEJ machinery will restore the *lacZ* reading frame and result in blue colonies on X-gal agar medium. However, unfaithful ligation after insertion or deletion of nucleotides will usually result in a frame-shift mutation that inactivates the *lacZ* gene and results in white colonies.

The pBluescript II KS (+) plasmid was modified using the strategy similar to the previously described (Aniukwu et al., 2008) with the insertion of a 1800 bp-fragment from another plasmid into pBluescript II KS (+) plasmid at the *Eco*RV site. This modified plasmid (pBluescript II KS (+)-1800- *Eco*RV) was linearized with *Eco*RV digestion and the linearized plasmid was gel-purified and used for the *in vivo* NHEJ assay as described previously (Buck et al., 2006; Gong et al., 2005) with some modifications. Briefly, MEF cells were transfected with the linearized plasmid using lipofectamine following manufacturer's instructions (Invitrogen, Inc.). After cultured for additional 24 hours, the recircularized / repaired plasmids were retrieved from those MEF cells using plasmid extraction kit (Promega, Inc.) and transformed to *Escherichia coli* DH10B cells (Invitrogen, Inc.) by electroporation. Bacterial cells were plated and incubated at 37°C for 16 hours. Blue and white colonies were counted and the percentage of blue over the total colonies, which represents the precise DNA end-joining, was calculated. Four independent experiments were performed for each pair of MEF cell lines.

Statistical Analysis

Data were expressed as means ±S.D.. Differences were analyzed by two-tailed Student's t test and p values < 0.05 were considered significant.

MudPIT Analysis of Protein Complexes

TCA-precipitated proteins purified from PIDD FLAG immunoprecipitations were resuspended in 30 µl of 100 mM Tris-HCl, pH 8.5, 8 M urea, reduced with 5 mM TCEP [Tris(2-Carboxylethyl)-Phosphine Hydrochloride, (Pierce)], and alkylated with 10 mM Iodoacetamide (Sigma). As described in (Washburn et al., 2001), a two-step digestion procedure was used.

Endoproteinase Lys-C (Roche) was added to 0.5 µg for at least 6 hours at 37°C, and the sample was diluted to 2 M urea with 100 mM Tris-HCl, pH 8.5. Calcium chloride was added to 2 mM and the digestion with trypsin (0.5 µg) was left to proceed overnight at 37°C while shaking. The reaction was quenched by adding formic acid to 5% and the peptide mixture was loaded onto a 100 µm fused-silica microcapillary column packed with 8 cm of reverse phase material (Aqua, Phenomenex), followed with 3 cm of 5-µm Strong Cation Exchanger (Partisphere SCX, Whatman), followed by 2 cm of 5-µm C_{18} reverse phase (McDonald et al., 2002). The loaded microcapillary column was placed in-line with a Quaternary Agilent 1100 series HPLC pump. Overflow tubing was used to decrease the flow rate from 0.1 ml/min to about 200–300 nl/min. Fully automated 7-step chromatography runs were carried out (Florens and Washburn, 2006). Three different elution buffers were used: 5% acetonitrile, 0.1% formic acid (Buffer A); 80% acetonitrile, 0.1% formic acid (Buffer B); and 0.5 M ammonium acetate, 5% acetonitrile, 0.1% formic acid (Buffer C). Peptides were sequentially eluted from the SCX resin to the reverse phase resin by increasing salt steps, followed by an organic gradient. The last two chromatography steps consisted of a high salt wash with 100% Buffer C followed by the acetonitrile gradient. The application of a 2.5 kV distal voltage electrosprayed the eluting peptides directly into a Deca-XP ion trap mass spectrometer equipped with a nano-LC electrospray ionization source (ThermoFinnigan). Full MS spectra were recorded on the peptides over a 400 to 1,600 *m*/*z* range, followed by three tandem mass (MS/MS) events sequentially generated in a data-dependent manner on the first, second, and third most intense ions selected from the full MS spectrum (at 35% collision energy). Mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (ThermoFinnigan). SEQUEST (Eng et al., 1994) was used to match MS/MS spectra to peptides in a database of

82242 sequences, consisting of 40873 human proteins (downloaded from NCBI on 2006-03-03), 177 usual contaminants such as human keratins, IgGs, and proteolytic enzymes, and 105 epitope-tagged proteins. To estimate false discovery rates (FDR), each non-redundant protein entry was randomized (keeping the same amino acid composition and length) and the resulting 41121 "shuffled" sequences were added to the database used for the SEQUEST searches. The validity of peptide/spectrum matches was assessed using the SEQUEST-defined parameters, cross-correlation score (XCorr) and normalized difference in cross-correlation scores (DeltCn). Spectra/peptide matches were only retained if they had a DeltCn of at least 0.08 and, minimum XCorr of 1.8 for singly-, 2.5 for doubly-, and 3.5 for triply-charged spectra. In addition, the peptides had to be fully tryptic and at least 7 amino acids long. Combining all runs, proteins had to be detected by at least 2 such peptides, or 1 peptide with 2 independent spectra, leading to spectral FDRs below 1%. DTASelect (Tabb et al., 2002) was used to select and sort peptide/spectrum matches passing this criteria set. Peptide hits from multiple runs were compared using CONTRAST (Tabb et al., 2002) and an in-house developed script (*contrast-report*). To estimate relative protein levels, spectral counts of non-redundant proteins were normalized (Zybailov et al., 2006) using an in-house developed script (*contrast-report-add-nsaf*): for each protein *k* detected in a particular MudPIT analysis, Normalized Spectral Abundance Factors (NSAFs) were calculated as follow :

$$(NSAF)_k = \frac{(SpC / Length)_k}{\sum_{i=1}^{N} (SpC / Length)_i}$$

Defining Post-Translational Modifications (PTMs) Sites on Caspase 2

Preparation of peptide mixtures by multiple digestions — Proteins were purified by nuclear

extraction and anti-FLAG affinity purification from cells expressing FLAG-caspase 2 C303A. In all, three different biological conditions were analyzed: i) cells irradiated with 80 gamma radiation, ii) cells treated with 100mM Etoposide for 2 hours, and iii) untreated cells. Negative controls from cells expressing an empty FLAG vector were analyzed in parallel.

The method loosely follows that of (MacCoss et al., 2002) with several new implementations (Florens and Washburn, 2006). TCA-precipitated protein samples were solubilized in 30µl of freshly made 0.1 M Tris-HCl (pH 8.5), 8M urea, and 5mM TCEP. After 30 minutes at room temperature, freshly made 0.5M IAM was added to a final concentration of 10mM, and the samples were left in the dark at room temperature for another 30 minutes. For the subtilisin A (Calbiochem) digestion, urea was diluted to 4.8 M by adding 20µl 0.1M Tris-HCl (pH 8.5). Subtilisin A (0.1 µg) was added to an approximate enzyme-to-protein ratio of 1:50 (wt/wt) and left to incubate for 2 hours at 37°C. For the elastase (Calbiochem) digestion, urea was diluted to 2M with 0.1M Tris-HCl (pH 8.5) and elastase 1:50 (wt/wt) was added for 6 hours at 37°C. For the trypsin digestion, endoproteinase Lys-C (Roche) was first added at a 1:100 enzyme-to-protein ratio, for at least 6 hours at 37°C. Urea was then diluted to 2M with 0.1M Tris-HCl (pH 8.5), $CaCl_2$ was added to 0.5mM, and trypsin 1:100 (wt/wt) was added for over 12 hours at 37°C. For the proteinase K digestion, the pH was raised to 11.5 by adding 1M sodium carbonate to 100mM. Proteinase K was then added to an enzyme-to-substrate ratio of 1:100 (wt/wt) at 37°C for 4 hrs (Wu and Irizarry, 2004). For the Endoproteinase Glu-C digestion, urea was diluted to 1M with 100mM Tris-HCl (pH 7.8) and Endoproteinase Glu-C (Roche) was added to 1:100 (wt/wt) at 25°C for 6 hours. All enzymatic digestions were quenched by adding formic acid to 5%.

Data Acquisition — Each differently-digested sample was analyzed multiple independent times by Multidimensional Protein Identification Technology (MudPIT) using a Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA) interfaced with a quaternary Agilent 1100 quaternary pump (Agilent Technologies, Palo Alto, CA). Peptide mixtures were pressure-loaded onto triphasic 100μm fused-silica columns as described above. During the course of a fully automated chromatography, seven to ten 120-minute cycles of increasing salt concentrations followed by organic gradients slowly released peptides directly into the mass spectrometer. Each full MS scan (from 400 to 1600m/z) was followed by five MS/MS events using data-dependent acquisition where the 1st most intense ion was isolated and fragmented by collision-induced dissociation (CID), followed by the 2nd to 5th most intense ions.

Data Analysis — RAW files were extracted into ms2 file format (McDonald et al., 2004) using RAW_Xtract (obtained from J. Venable, Yates' lab, Scripps Research Institute). MS/MS spectra were queried for peptide sequence information on a 112-node dual processor Beowulf Linux cluster with two master nodes dedicated to SEQUEST analyses (Eng et al., 1994). To avoid the computational bottleneck associated with both differential modification searches and datasets acquired on linear ion traps, MS/MS spectra were analyzed in a recursive manner as follows.

To account for carboxamidomethylation by IAM, +57 Da were added statically to cysteine residues for all the searches. First, ms2 files were searched without specifying differential modifications against the database of 82242 amino acid sequences described above. This SEQUEST "ASAP" (All Spectra against All Proteins) step allowed establishment of a list of proteins detected in the samples. The same ms2 files were also searched for post-translational modifications (PTMs) against a database containing only the sequences for the proteins of interest, i.e. Capsase 2 C303A and WT caspase 2 isoform 1 preproprotein

(gi|39995059|ref|NP_116764.2|). In SEQUEST "ASFP" (All Spectra against Few Proteins), six differential modification searches were set up to query the small protein database for peptides containing: 1) methylated lysines, serines, and threonines (+14) AND oxidized methionines (+16); 2) oxidized methionines, and hydroxylated lysines and aspartates (+16); 3) dimethylated lysines and arginines (+28), AND oxidized methionines; 4) trimethylated/acetylated lysines, serines and threonines (+42), AND oxidized methionines; 5) phosphorylated lysines, serines,and tyrosines (+80) AND oxidized methionines; 6) ubiquitinated lysines (+ 114.1), AND oxidized methionines. The maximum number of modified amino acids per differential modification in a peptide was limited to 4.

After this first round of searches, an in-house developed script, *sqt-merge* (Zybailov et al., 2005), was used to combine the 7 sets of SEQUEST output files (sqt files) generated from the normal search (i.e. without modifications) and PTMs searches described above into one set. The *sqt-merge* script read in sqt files from normal and differentially modified searches generated from the same ms2 files, then merged and ranked the spectrum/peptide matches based on cross-correlation scores (XCorrs). Normalized differences in XCorrs (DeltCn) values were re-calculated. This merging step allowed only the best matches out of *n* differential SEQUEST queries to be ranked first. The peptide matches contained in the merged sqt files were compiled and sorted into protein-level information using DTASelect (Tabb et al, 2002). The protein lists were established based on conservative filtering criteria as previously reported (DeltCn of at least 0.08, minimum XCorr of 1.8 for +1, 2.5 for +2, and 3.5 for +3 spectra and minimum peptide length of 7 amino acids).

For the second round of searches, only spectra passing these criteria and matching modified peptides were selected (-m 0 –t 0 DTASelect parameters), and their coordinates written out into

smaller ms2 files using the "-- copy" utility of DTASelect. Because these subset ms2 files contained at best a few hundred MS/MS spectra, they could be subjected to the same six differential searches against the complete human database, without having to pay too high of a computer-hour price (SEQUEST "MSAP": Modified Spectra against All Proteins). Again *sqt-merge* was used to bring together the results generated by these different searches. This step allowed us to check that spectra matching modified peptides from Caspase 2 sequences did not find a better match against the larger protein database. DTASelect was used to create reports listing all detected proteins and the modified residues on Caspase 2. All spectra matching modified peptides were visually assessed. For example, peptides containing phosphoSerine or phosphoThreonine were expected to display a large neutral loss peak -98m/z off the precursor ion mass.

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Supplemental Figure Legends

Figure S1. The Association of PIDD with DNA-PKcs

(A) PIDD is associated with DNA-PKcs in the cell nucleus and with RAIDD in the cytoplasm. PIDD was immunoprecipitated by anti-FLAG-M2 antibody from cytoplasmic and nuclear extracts prepared from HEK293 cells stably expressing FLAG-PIDD. The immunoprecipitated samples were separated by SDS-PAGE for immunoblotting (IB) of PARP1 (nuclear marker), caspase-3 (cytoplasmic marker), FLAG (PIDD), DNA-PKcs and RAIDD. PIDD was shown in its full-length, PIDD-C, and PIDD-CC forms.

(B) The association of PIDD with DNA-PKcs is insensitive to ethidium bromide or micrococcal nuclease. **Same** nuclear extracts as in (A) were immunoprecipitated (IP) by anti-FLAG-M2 antibody in the absence or presence of ethidium bromide (EB) or micrococcal nuclease (MN). The immunoprecipitated samples were analyzed by IB of PIDD and DNA-PKcs. PIDD was shown in its autoclaved forms of PIDD-C (51 kD) and PIDD-CC (40 kD).

Figure S2. *In Vivo* Association of Endogenous Caspase-2, DNA-PKcs, and PIDD in Wild-type but Not Caspase-2-deficient Cell Nucleus

The isogenic wild-type [C2 (-/-) + WT] and caspase-2-deficient [C2 (-/-) + Vector] MEF lines ($2X10^7$) were left untreated or exposed to 80 Gy of IR. Eight hrs after IR, cells ($2X10^7$) were labeled with b-VAD-fmk (4 μM) for 1 hr. Nuclear extracts were prepared from these cells and subjected to streptavidin bead-pulldown. Supernatant and b-VAD-

fmk-bound complex were analyzed by IB with antibodies against caspase-2, PIDD, DNA-PKcs, and RAIDD. The caspase-2-dependency for the three proteins (endogenous caspase-2, DNA-PKcs, and PIDD) to be associated in live cell nuclei in the z-VAD-fmk pulldown was shown by the absence of recruitment of DNA-PKcs and PIDD in caspase-2-deficient cells whereas the association of the three was readily detected in wild-type cells.

Figure S3. Association of Caspase-2, DNA-PKcs, and PIDD in Live PIDD-stable HEK293 Cell Nucleus

PIDD-stable HEK293 cells were incubated with increasing amounts of cell-permeable b-VAD-fmk (1-5 μM) or DMSO (0) for 1 hour and nuclear extracts were subjected to streptavidin bead-pulldown. Proteins bound to the beads were eluted and immunoblotted using antibodies against the indicated proteins.

Figure S4. Nulcear DNA-PKcs, PIDD, and Caspase-2 Were Co-eluted in High Molecular Weight Fractions

(A) DNA-PKcs, PIDD, and caspase-2 in untreated HeLa nuclear extracts were co-eluted in high molecular weight fractions. HeLa nuclear extracts (2 mg) from control cells (-IR) were loaded on a Superose 6 high-resolution analytical size-exclusion column. An aliquot (30 μl) of each eluted fraction was subjected to immunoblotting. The column was calibrated with Amersham gel filtration standards containing blue dextran (2000 kD), bovine thyroglobulin (670 kD), ferritin (440 kD), aldolase (158 kD), bovine serum albumin (67 kD), and chymotrypsinogen A (25 kD).

(B) The same experiments described in (A) were done with nuclear extracts from irradiated HeLa cells (+IR, 80 Gy)**.**

Figure S5. Specificity of Polyclonal Antibody against Caspase-2 Phospho-S122 Site

(A) Phosphorylated synthetic peptide used for phospho-caspase-2 antibody production.

(B) Anti-pS122 immunoblotting of an aliquot of nuclear caspase-2 used for MudPIT PTM analysis. HEK293 cells stably expressing a catalytic mutant caspase-2 (C303A) were left untreated or exposed to 80 Gy IR and cultured for an additional 2 hours. Nuclear extracts were immunoprecipitated with FLAG-M2-agarose, and the immuno-complexes were immunoblotted with the caspase-2 polyclonal anti-phospho-S122 antibody alone at a 1:500 dilution (lanes 1and 2), with the antibody pre-mixed with the phospho-peptides at a 1:200 molar ratio (lanes 3 and 4), or with the antibody pre-mixed with the same amount of non-phospho-peptide (lanes 5 and 6). Only the phospho-peptides could block the immuno-signals in irradiated samples (upper). The same membrane was stripped and reblotted with anti-caspase-2 antibody (clone 11B4) (lower) in the absence of peptide addition.

(C) The same experiments described in (B) were repeated in samples treated with etoposide (ETOP) at 100 μM for 2 hours.

Figure S6. Caspase-2 Phosphorylation at S122 Site Is Abolished in DNA-PKcs or Ku80 Knockout Cells

(A) Caspase-2 phosphorylation at S122 Site is abolished in DNA-PKcs knockout cells. The immortalized DNA-PKcs wild-type (WT) or knockout (KO) MEF cells were left untreated or exposed to 4 Gy IR and cultured for additional 2 or 6 hrs. Nuclear extracts were immunoblotted with the indicated antibodies

(B) Caspase-2 phosphorylation at S122 site is abolished in Ku80 knockout cells. The immortalized Ku80 WT or KO MEF cells were treated the same as in (A). Nuclear extracts were immunoblotted with the indicated antibodies.

Figure S7. Detection of Apoptosis

(A) Detection of apoptosis in wild-type and S122A mutant caspase-2 cells. Isogenic caspase-2-deficient MEFs stably expressing wild-type (C2 (-/-) + WT) or S122A mutant (C2 (-/-) + S122A) caspase-2 were left untreated or irradiated with 10 Gy IR for 2 hrs. Apoptotic cells were scored as Annexin-V-positive and PI (Propidium Iodide)-negative. Each bar represents the mean ±S.D. of three independent experiments (n=3).

(B) Apoptotic measurement in DNA-PKcs deficient or proficient cells. DNA-PKcs-deficient human glioma M059J and isogenenic DNA-PKcs-proficient ML-L24 [with exogenous (exo) wild-type (WT) DNA-PKcs expressed back] cells were left untreated or exposed to 80 Gy IR and cultured for additional 24 hrs. Apoptotic cells were scored as Annexin-V-positive and PI-negative. Each bar represents the mean ±S.D. of three independent experiments (n=3).

Figure S8. IR Induces PIDD Accumulation in Cell Nucleus

(A) IR induces PIDD accumulation in HEK293T cell nucleus. HEK293T cells were left untreated or irradiated with 80 Gy. Cells were harvested at the indicated times post IR. Cytoplasmic and nuclear extracts were immunoblotted for the indicated proteins. PIDD was predominantly present in cytoplasm in HEK293T cells. No cleavage of PARP-1 and caspase-3 (C3) were observed in cells within 36 hrs of IR treatment. Both proteins also served as loading controls.

(B) IR induces PIDD accumulation in U2OS cell nucleus. The same experiments described in (A) were repeated in U2OS cells. PIDD was predominantly present in the nuclear faction of U2OS cells.

Figure S9. PIDD Requires Ku80 to Promote S122 Phosphorylation and Nuclear Caspase-2 Cleavage

Increasing amounts of pcDNA-FLAG-PIDD (0 – 0.4 μg) were transfected into immortalized Ku80 WT and KO MEF cells for 24 hrs. Nuclear extracts were immunoblotted with the indicated antibodies.

Figure S10. Apoptotic Measurements

(A) Measurement of apoptosis after PIDD overexpression in HEK293T cells. HEK293T cells were transfected with increasing amounts of pcDNA-FLAG-PIDD (0 - 0.50 μg) for 24 hrs and scored for apoptosis. Each bar represents the mean ±S.D. of three independent experiments (n=3).

(B) Detection of apoptosis after PIDD overexpression in DNA-PKcs proficient and deficient cells. DNA-PKcs-deficient M059J and isogenenic DNA-PKcs-proficient ML-L24 were transfected with increasing amounts of pcDNA-FLAG-PIDD (0 - 1.6 μg) for 24 hrs and scored for apoptosis. Each bar represents the mean ±S.D. of three independent experiments (n=3).

Figure S11. IR-induced Nuclear Caspase-2 Cleavage Occurs Prior to Cell Death in HEK293T Cells

(A) Time course of nuclear caspase-2 cleavage induced by IR in HEK293T cells. The cells were left untreated or irradiated with 80 Gy. Cells were harvested at the indicated times post IR and nuclear extracts were immunoblotted with the indicated antibodies.

(B) Analysis of cell death by trypan blue staining in HEK293T cells treated as in (A). Each bar represents the mean ±S.D. of three independent experiments (n=3).

Figure S12. IR-induced Nuclear Caspase-2 Cleavage Occurs Prior to Cell Death in MJ-L24 Cells

(A) Time course of nuclear caspase-2 cleavage induced by IR in MJ-L24 cells. The cells were left untreated or irradiated with 80 Gy. Cells were harvested at the indicated times post IR and nuclear extracts were immunoblotted with the indicated antibodies.

(B) Analysis of cell death by trypan blue staining in MJ-L2 treated as in (A). Each bar represents the mean ±S.D. of three independent experiments (n=3).

Figure S13. The Three Components of DNA-PKcs-PIDDosome Are Not Required for HeLa Cell Death Induced by IR

(A) DNA-PKcs is not required for HeLa cell death induced by IR. HeLa cells were pre-incubated with 10 μM DNA-PKcs kinase inhibitor NU7026 or DMSO for 1 hr, and left untreated or followed by irradiation with 80 Gy. Cells were harvested at the indicated times post IR and cell death were counted by trypan blue staining. Each bar represents the mean ±S.D. of three independent experiments (n=3).

(B) DNA-PKcs is not required for MEF cell death induced by IR. The immortalized DNA-PKcs WT and KO MEF lines were treated with 80 Gy of irradiation. Cells were harvested at the indicated times post IR and cell death were counted by trypan blue staining. Each bar represents the mean ±S.D. of three independent experiments (n=3).

(C) Caspase-2 is not required for MEF cell death induced by IR. Isogenic wild-type [C2 (-/-) + WT] and caspase-2-deficient [C2 (-/-) + Vector] MEF lines were treated and analyzed the same as in (B). Each bar represents the mean ±S.D. of three independent experiments (n=3).

(D) PIDD is not required for MEF cell death induced by IR. Immortalized wild-type and PIDD KO MEF cells were treated and analyzed the same as in (B). Each bar represents the mean ±S.D. of three independent experiments (n=3).

Figure S14. Caspase-2 Contributes to a G2/M DNA Damage Checkpoint in U2OS Cells

(A) Silencing caspase-2 expression in human U2OS cells. U2OS cells were transfected with control siRNA (siControl) or siRNA targeting caspase-2 (siC2) for two rounds at

48 hr intervals. These transfected cells were left untreated or exposed to 4 Gy IR followed by additional culture for 1 and 2 hrs. Silencing of caspase-2 expression was analyzed in nuclear extracts by immunoblotting with an anti-caspase-2 antibody (11B4, upper).

(B) Analysis of caspase-2 G2/M checkpoint function. U2OS cells treated identically as in (A) were fixed and double-stained with PI and FITC-conjugated anti-p-H3 antibody. The percentage of mitotic cells was analyzed by flow cytometry

(C) Time course of apoptosis in U2OS cells. U2OS cells were harvested by trypsinization at the indicated time post 4 Gy IR. Apoptotic cells were scored as Annexin-V positive and PI negative populations. Each bar represents the mean ±S.D. of three independent experiments (n=3).

Figure S15. Effect of PIDD Overexpression on Caspase-2 Activation

(A) HeLa cells stably expressing an empty vector (Vector-stable) or FLAG-tagged PIDD (PIDD-stable) were left untreated or exposed to 4 Gy IR and cultured for 1 and 2 hrs. Nuclear extracts were immunoblotted with the indicated antibodies.

(B) Caspase-2-deficient MEFs [C2 (-/-)] stably expressing an empty vector (Vector-stable) or FLAG-tagged PIDD (PIDD-stable) were left untreated or exposed to 4 Gy IR and cultured for additional 2 or 6 hrs. Nuclear extracts were immunoblotted with antibodies against PIDD and DNA-PKcs.

Figure S16. The Three Components of DNA-PKcs-PIDDosome Play Roles in NHEJ *In Vivo*

(A) DNA-PKcs functions *in vivo* in DNA end-joining. *In vivo* plasmid-based end-joining assay using the pEGFP-N1 vector linearized by *Bsr*GI as substrate was performed in immortalized DNA-PKcs wild-type and knockout MEF cells. Cells transfected with unlinearized vector served as positive controls whereas mock-transfected cells (no pEGFP) as negative controls. The percentage of GFP-postive cells was analyzed by flow cytometry.

(B) PIDD affects DNA end-joining *in vivo* assayed the same as in (A).

(C) Caspase-2 affects DNA end-joining *in vivo* assayed as in (A).

(D) Both S122 and C303 sites are involved in caspase-2 DNA end-joining activity *in vivo* assayed as in (A).

Figure S17. *In Vivo* Precise NHEJ Assay in WT and KO MEF Cell Lines

(A) Map of reporter plasmid pBluescript II KS (+)-1800-*Eco*RV, in which the introduced 1800-nt-fragment contains an *Eco*RV restriction site at each end and this fragment interrupts the *lacZ* coding region.

(B) Blunt end (*Eco*RV) precise end-joining assay. The plasmid in (A) was transfected into various MEF cell lines as indicated. Twenty four hours after transfection, the plasmids were retrieved from these MEFs and transformed to bacteria DH110B for blue/white colony selection. The y-axis indicates percentage of blue clones which represent precise end-joining, and the x-axis indicates cell lines used in

these assays. Each bar represents the mean ±S.D. of three independent experiments (n=3). *, $p < 0.05$ (two-tailed Student's t test).

Figure S1

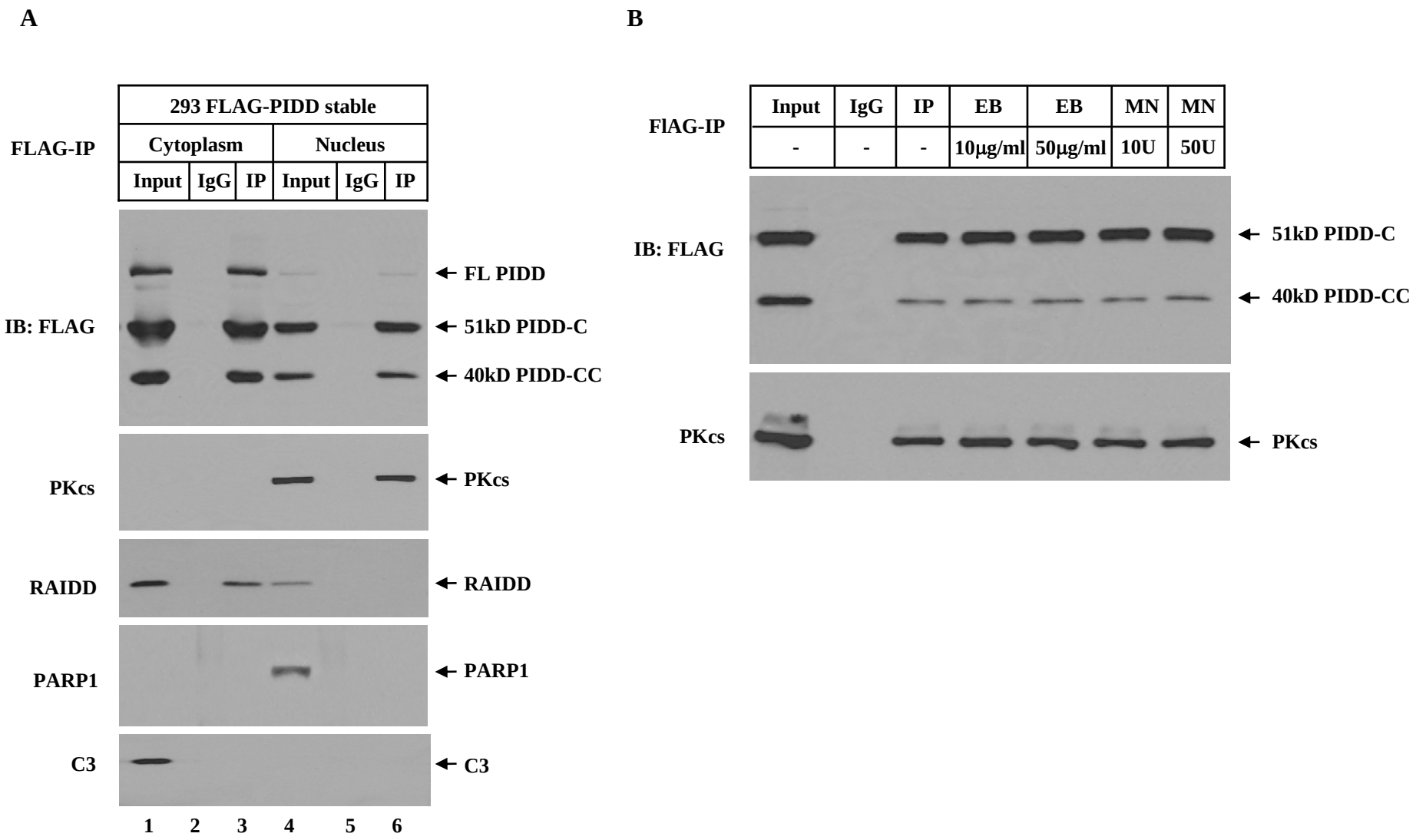

A
293 FLAG-PIDD stable
FLAG-IP
Cytoplasm
Nucleus
Input
IgG
IP
Input
IgG
IP
IB: FLAG
FL PIDD
51kD PIDD-C
40kD PIDD-CC
PKcs
PKcs
RAIDD
RAIDD
PARP1
PARP1
C3
C3
1 2 3 4 5 6
B
FlAG-IP
Input
IgG
IP
EB
EB
MN
MN
-
-
-
10μg/ml
50μg/ml
10U
50U
IB: FLAG
51kD PIDD-C
40kD PIDD-CC
PKcs
PKcs

Figure S2

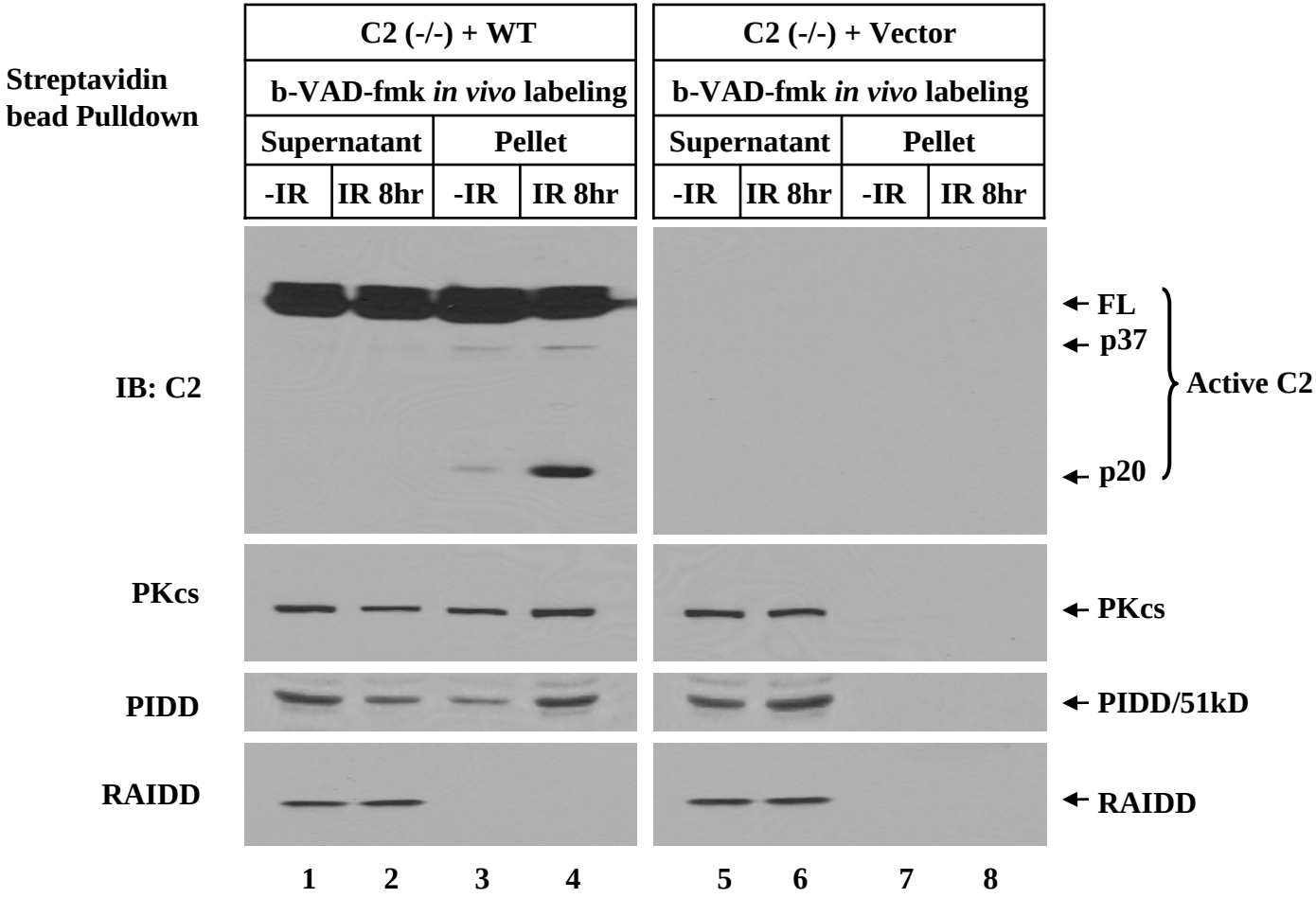

Streptavidin bead Pulldown
C2 (-/-) + WT
C2 (-/-) + Vector
b-VAD-fmk *in vivo* labeling
b-VAD-fmk *in vivo* labeling
Supernatant
Pellet
Supernatant
Pellet
-IR
IR 8hr
-IR
IR 8hr
-IR
IR 8hr
-IR
IR 8hr
IB: C2
FL
p37
p20
Active C2
PKcs
PKcs
PIDD
PIDD/51kD
RAIDD
RAIDD
1
2
3
4
5
6
7
8

Figure S3

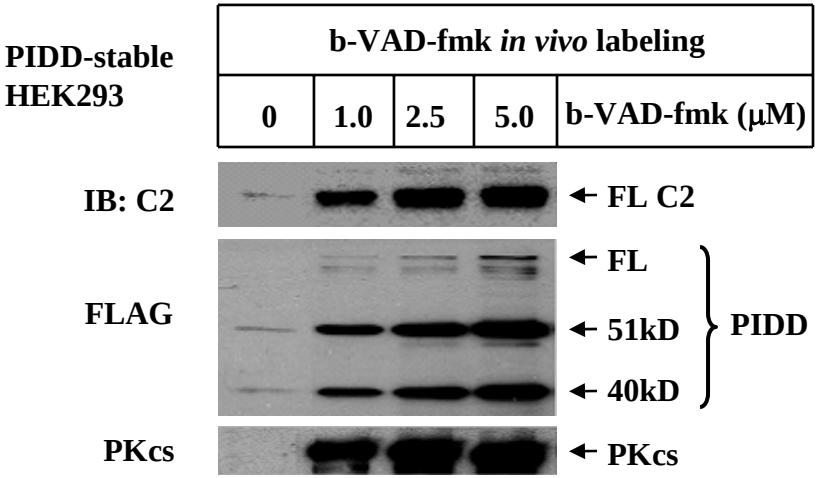

PIDD-stable HEK293
b-VAD-fmk *in vivo* labeling
0
1.0
2.5
5.0
b-VAD-fmk (μM)
IB: C2
FL C2
FLAG
FL
51kD
40kD
PIDD
PKcs
PKcs

Figure 4

A

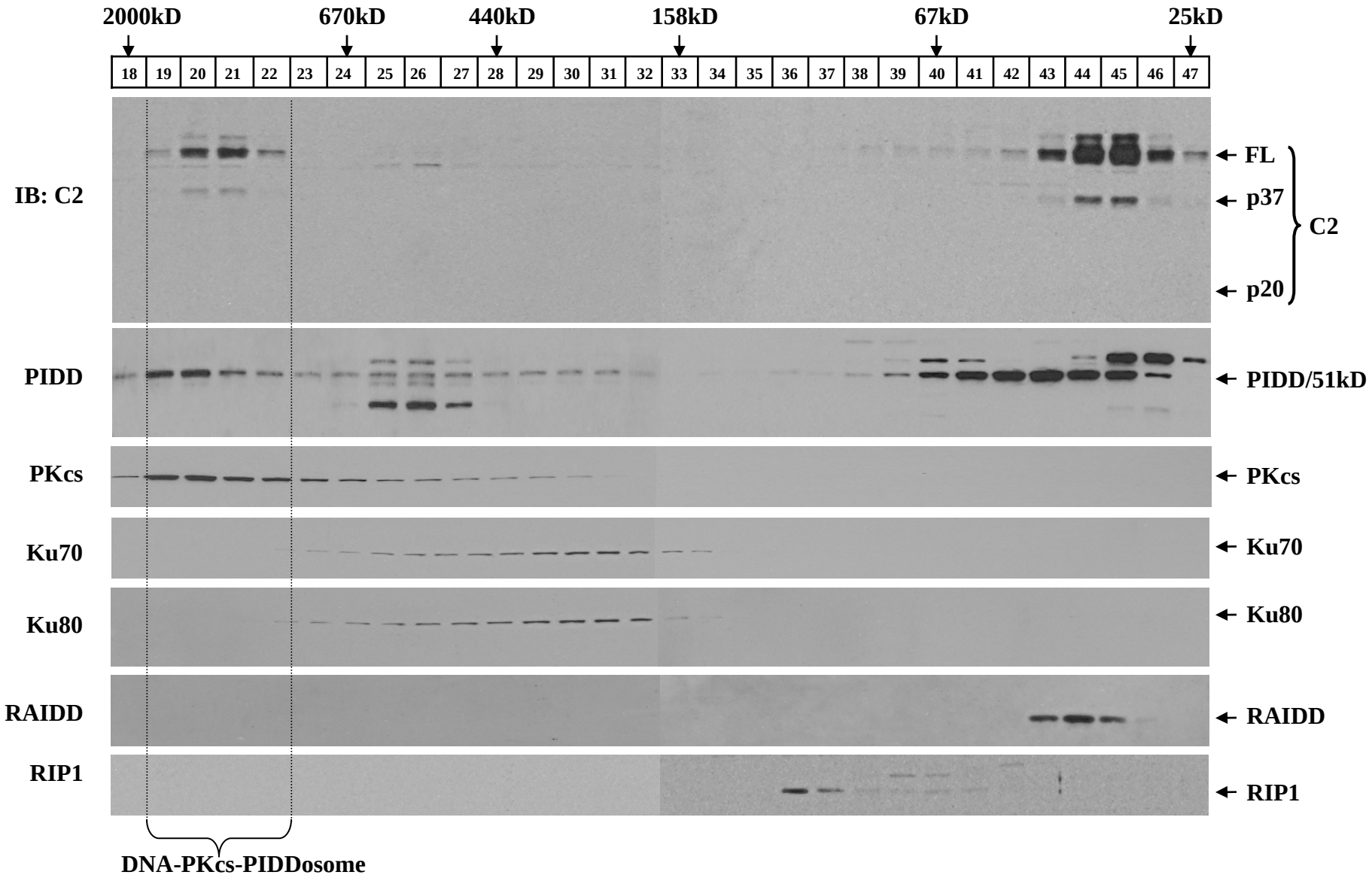

2000kD
670kD
440kD
158kD
67kD
25kD
18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47
IB: C2
FL
p37
p20
C2
PIDD
PIDD/51kD
PKcs
PKcs
Ku70
Ku70
Ku80
Ku80
RAIDD
RAIDD
RIP1
RIP1
DNA-PKcs-PIDDosome

Figure 4

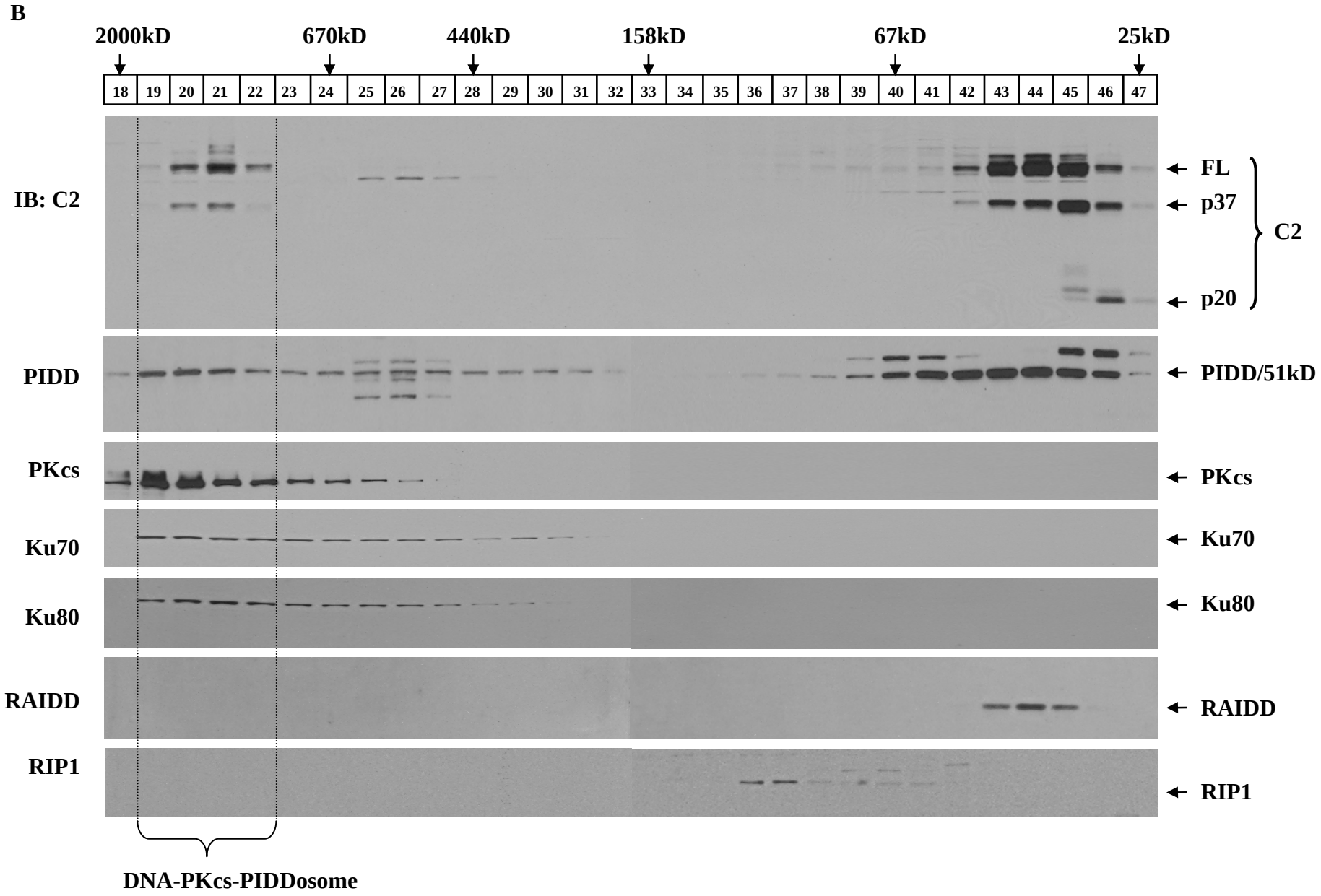

B
2000kD
670kD
440kD
158kD
67kD
25kD
18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47
IB: C2
FL
p37
p20
C2
PIDD
PIDD/51kD
PKcs
PKcs
Ku70
Ku70
Ku80
Ku80
RAIDD
RAIDD
RIP1
RIP1
DNA-PKcs-PIDDosome

Figure S5

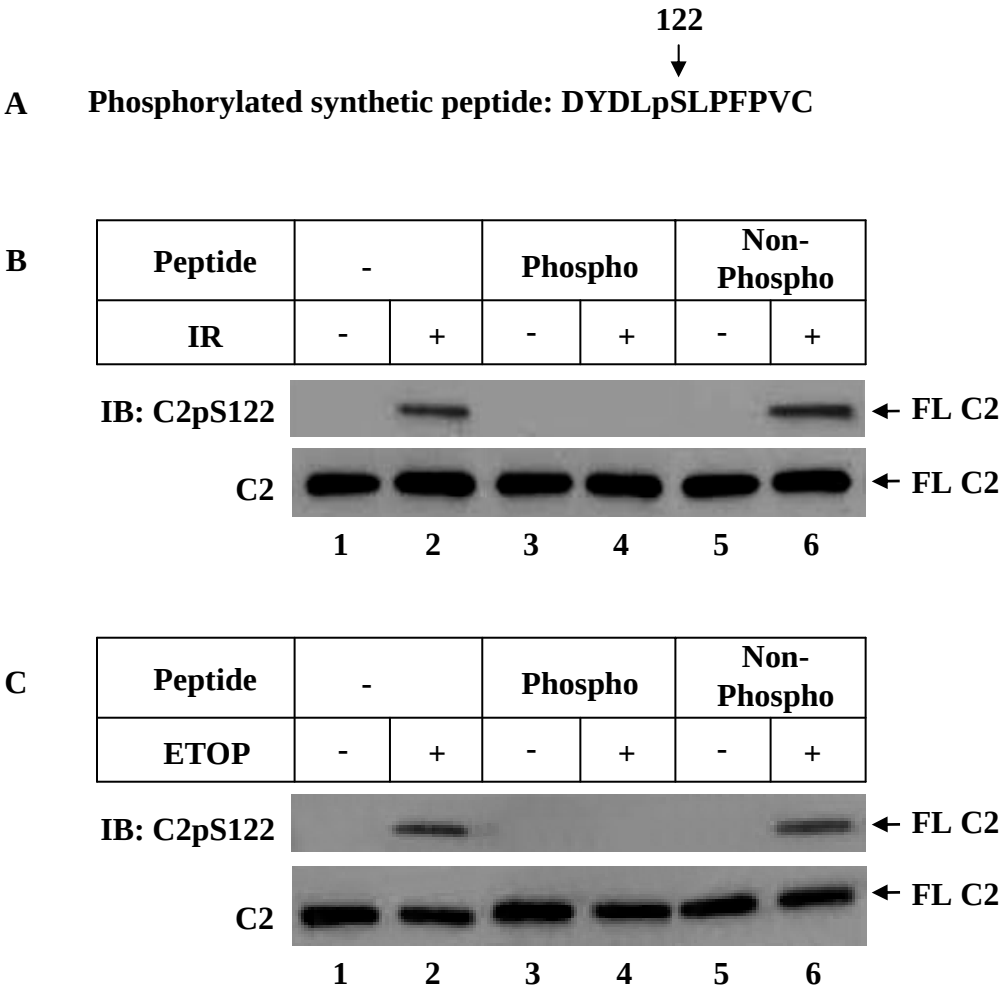
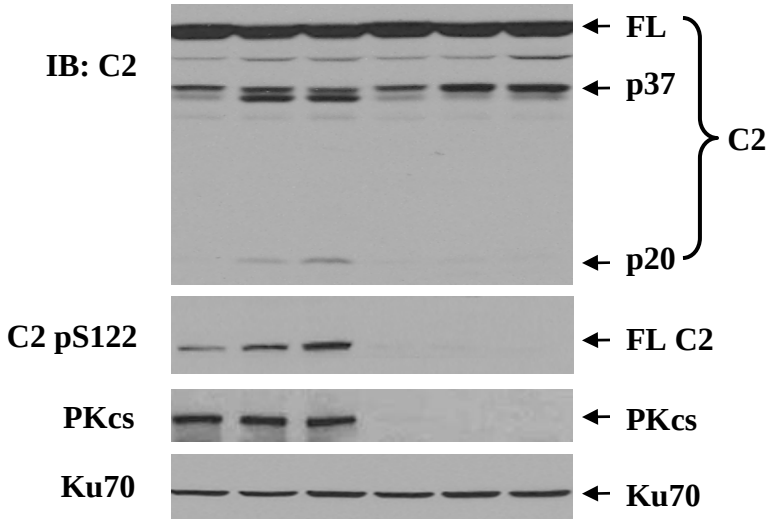

122
A
Phosphorylated synthetic peptide: DYDLpSLPFPVC
B
Peptide
-
Phospho
Non-Phospho
IR
- + - + - +
IB: C2pS122
FL C2
C2
FL C2
1 2 3 4 5 6
C
Peptide
-
Phospho
Non-Phospho
ETOP
- + - + - +
IB: C2pS122
FL C2
C2
FL C2
1 2 3 4 5 6

Figure S6

A

Cell lines	PKcs WT			PKcs KO		
hr Post IR (4Gy)	-IR	2	6	-IR	2	6



B

Cell lines	Ku80 WT MEF			Ku80 KO MEF		
hr Post IR (4Gy)	-	2	6	-	2	6

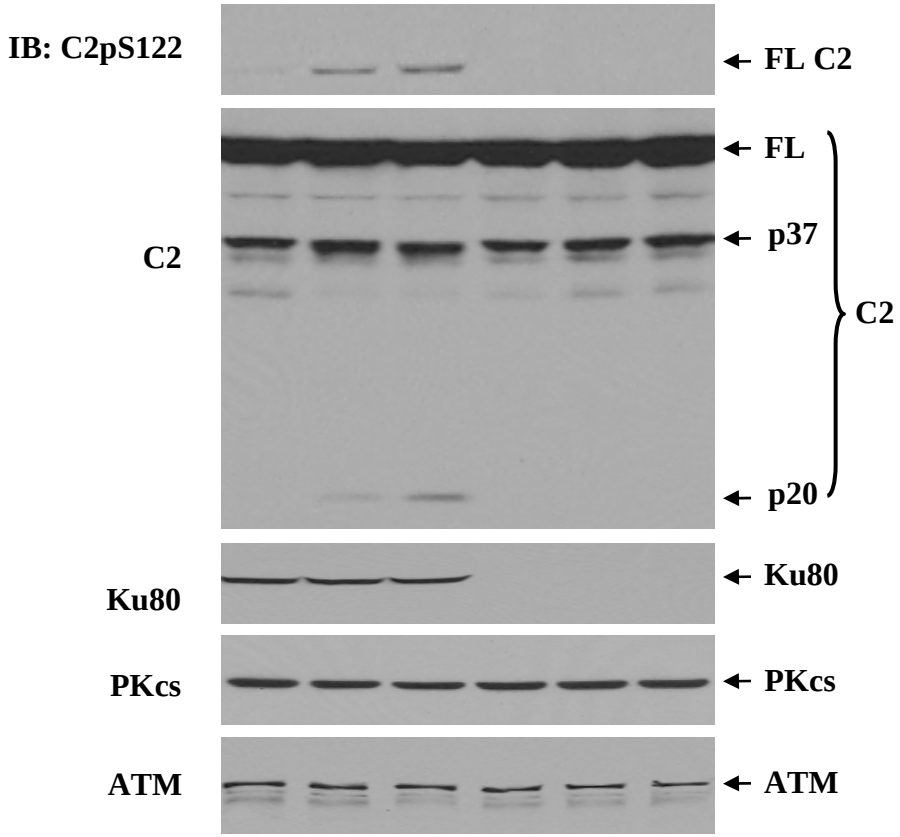
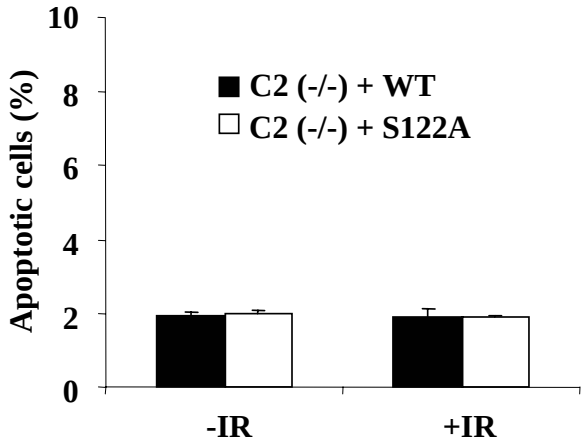


Figure S7

A


Apoptotic cells (%)
10
8
6
4
2
0
C2 (-/-) + WT
C2 (-/-) + S122A
-IR
+IR

B

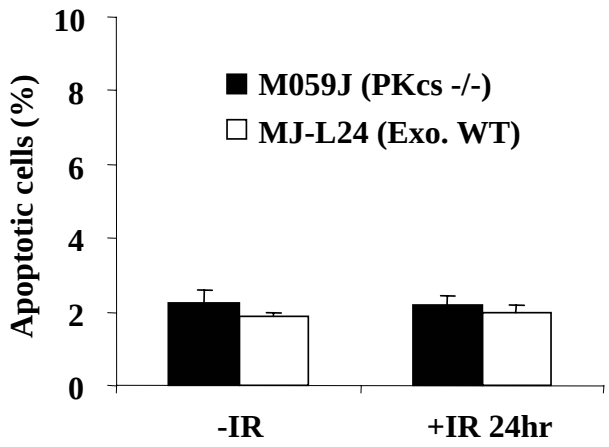

Apoptotic cells (%)
10
8
6
4
2
0
M059J (PKcs -/-)
MJ-L24 (Exo. WT)
-IR
+IR 24hr

Figure S8

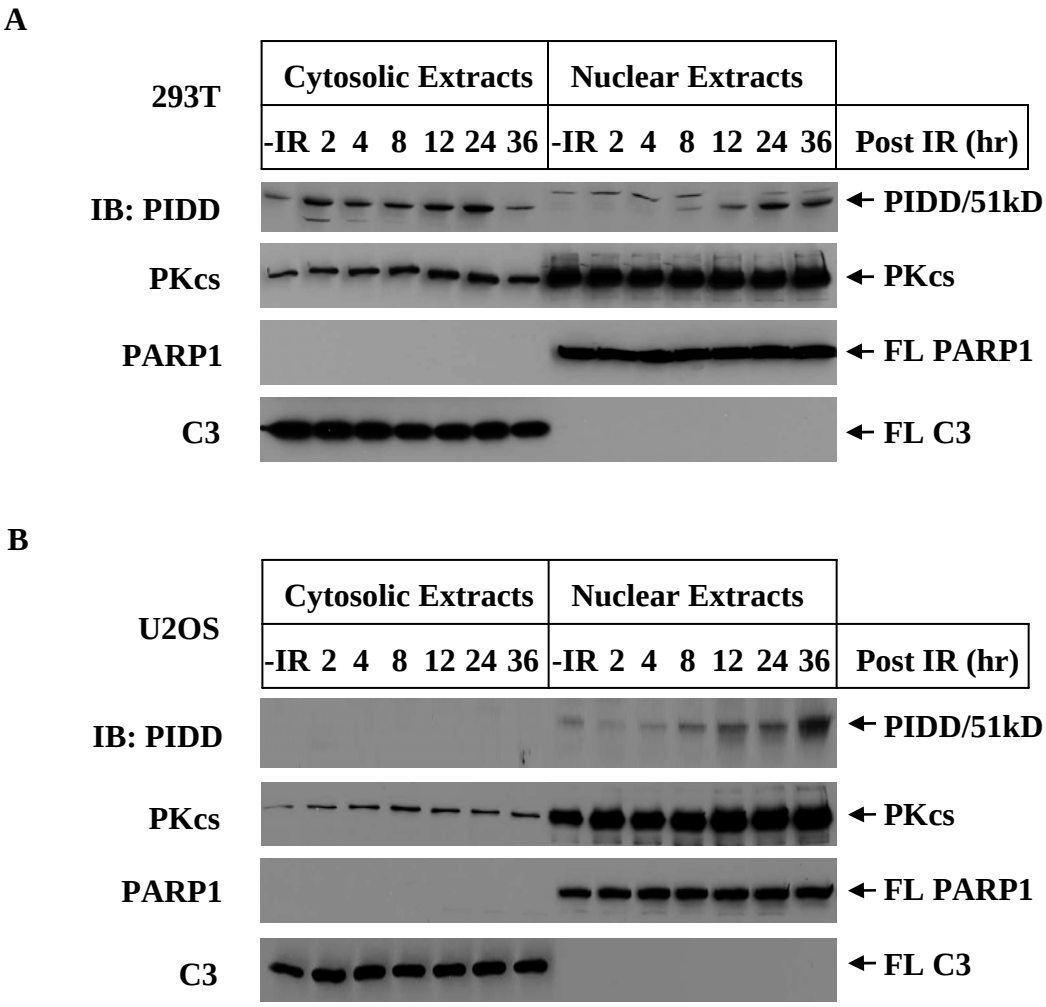

A
293T
Cytosolic Extracts
Nuclear Extracts
-IR 2 4 8 12 24 36
-IR 2 4 8 12 24 36
Post IR (hr)
IB: PIDD
PIDD/51kD
PKcs
PKcs
PARP1
FL PARP1
C3
FL C3
B
U2OS
Cytosolic Extracts
Nuclear Extracts
-IR 2 4 8 12 24 36
-IR 2 4 8 12 24 36
Post IR (hr)
IB: PIDD
PIDD/51kD
PKcs
PKcs
PARP1
FL PARP1
C3
FL C3

Figure S9

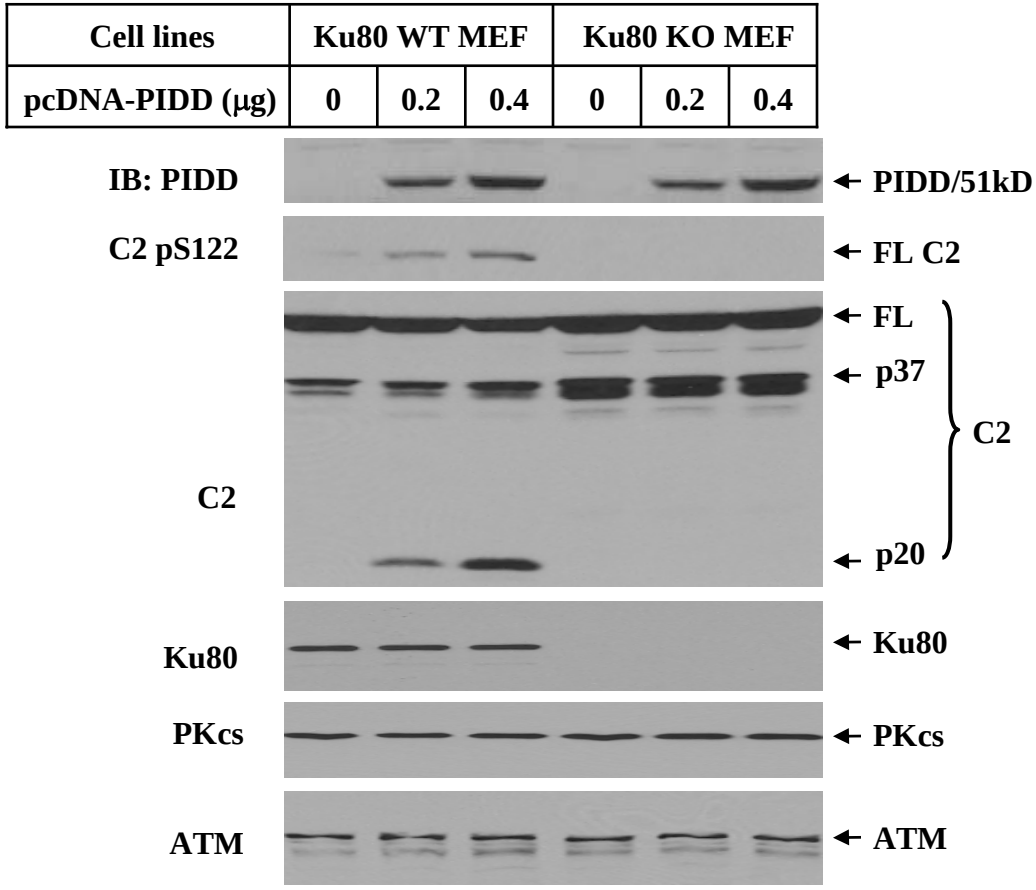

Cell lines
Ku80 WT MEF
Ku80 KO MEF
pcDNA-PIDD (μg)
0
0.2
0.4
0
0.2
0.4
IB: PIDD
PIDD/51kD
C2 pS122
FL C2
C2
FL
p37
p20
C2
Ku80
Ku80
PKcs
PKcs
ATM
ATM

Figure S10

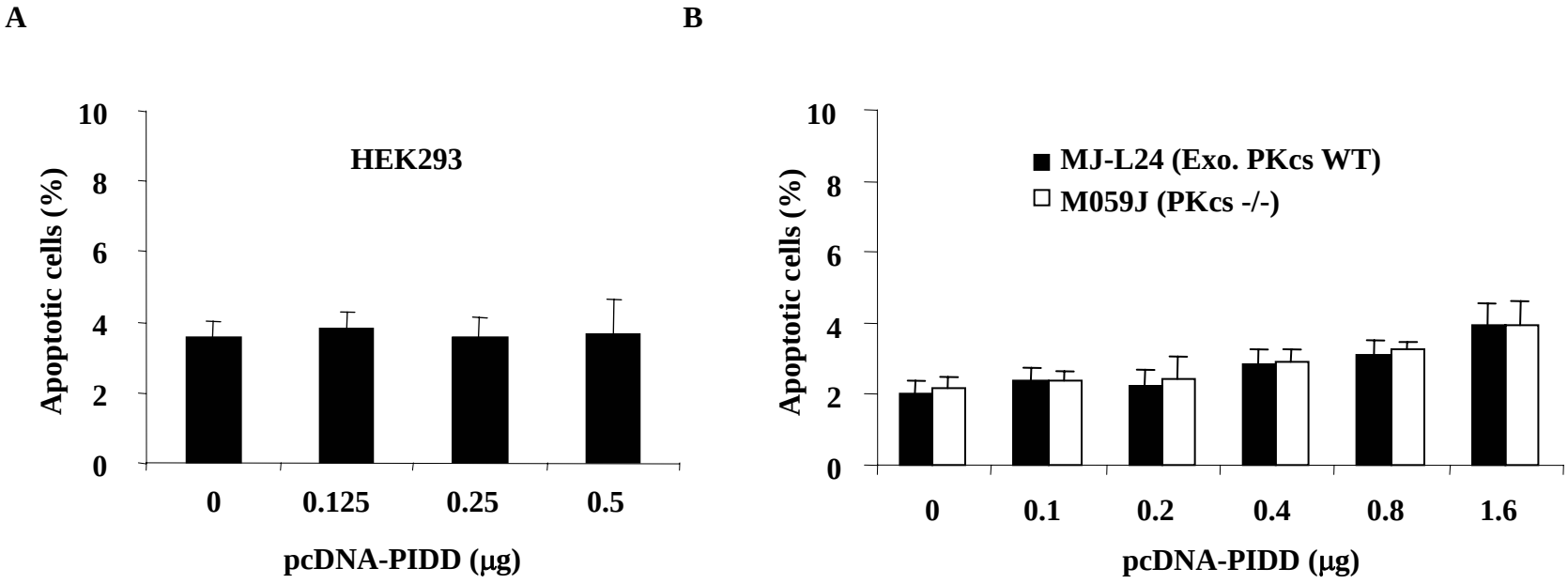
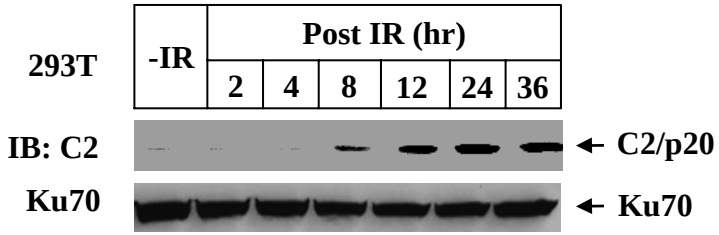

A
B
HEK293
Apoptotic cells (%)
10
8
6
4
2
0
0
0.125
0.25
0.5
pcDNA-PIDD (μg)
MJ-L24 (Exo. PKcs WT)
M059J (PKcs -/-)
0
0.1
0.2
0.4
0.8
1.6

Figure S11

A



B

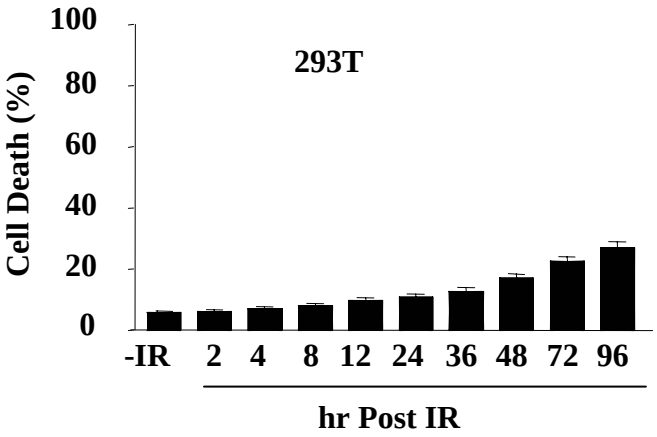


Figure S12

A

B

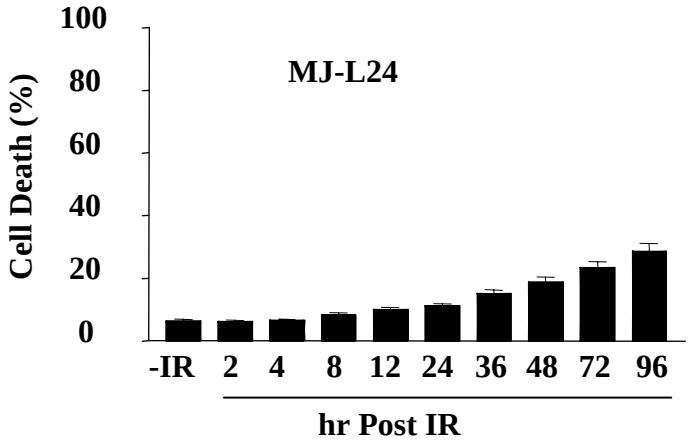


Figure S13

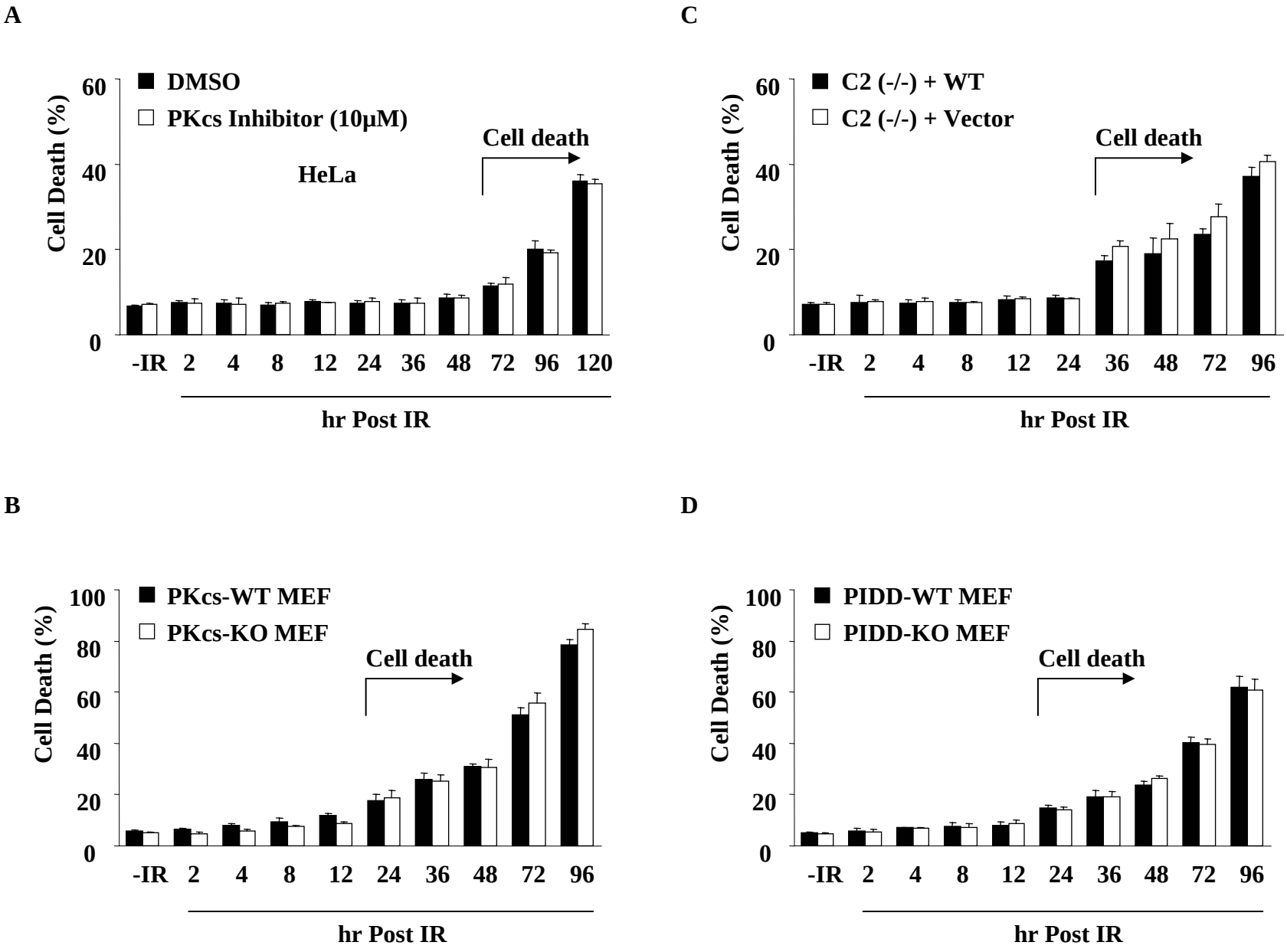

A
DMSO
PKcs Inhibitor (10μM)
HeLa
Cell death
Cell Death (%)
60
40
20
0
-IR 2 4 8 12 24 36 48 72 96 120
hr Post IR
C
C2 (-/-) + WT
C2 (-/-) + Vector
Cell death
Cell Death (%)
60
40
20
0
-IR 2 4 8 12 24 36 48 72 96
hr Post IR
B
PKcs-WT MEF
PKcs-KO MEF
Cell death
Cell Death (%)
100
80
60
40
20
0
-IR 2 4 8 12 24 36 48 72 96
hr Post IR
D
PIDD-WT MEF
PIDD-KO MEF
Cell death
Cell Death (%)
100
80
60
40
20
0
-IR 2 4 8 12 24 36 48 72 96
hr Post IR

Figure S14

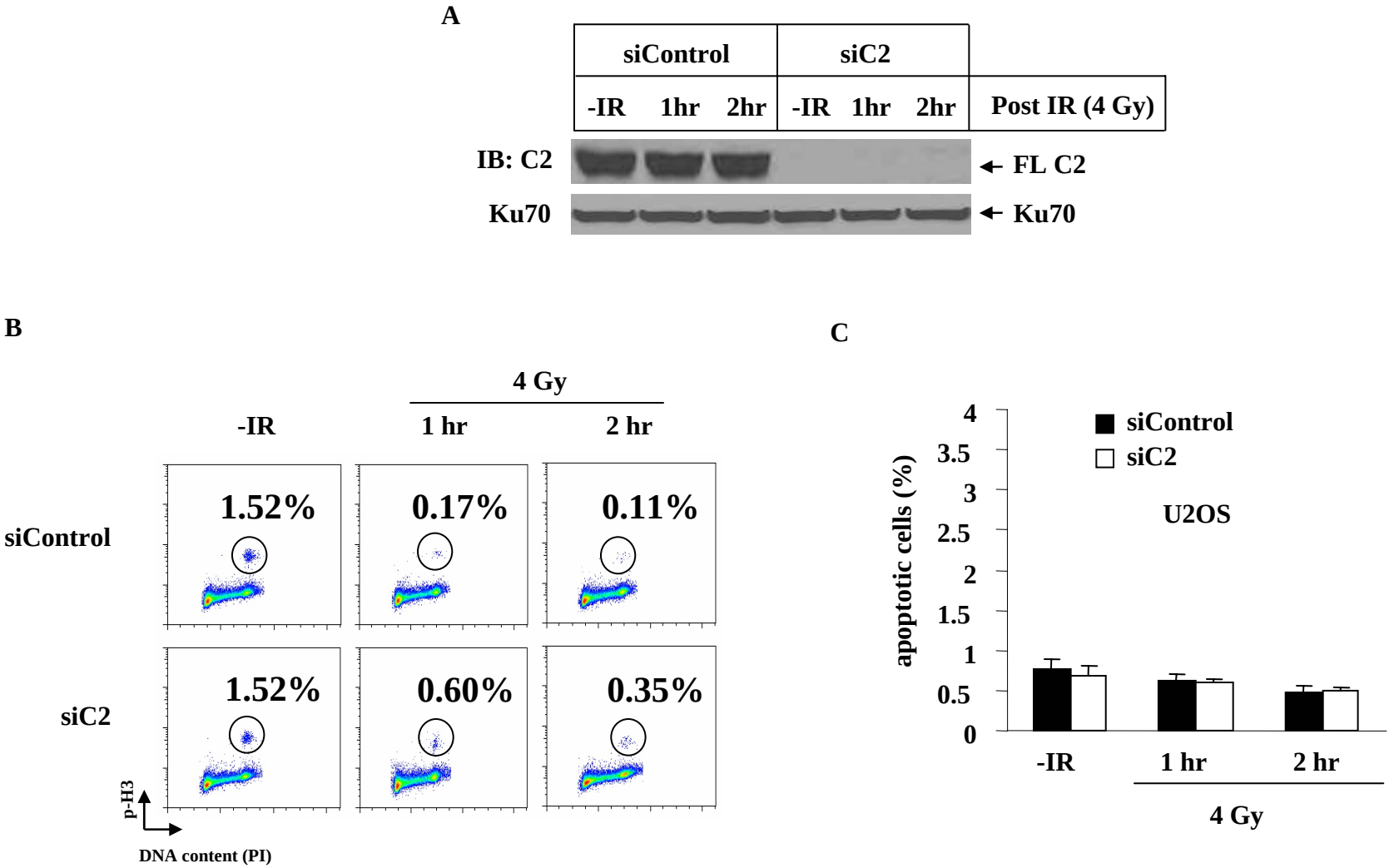
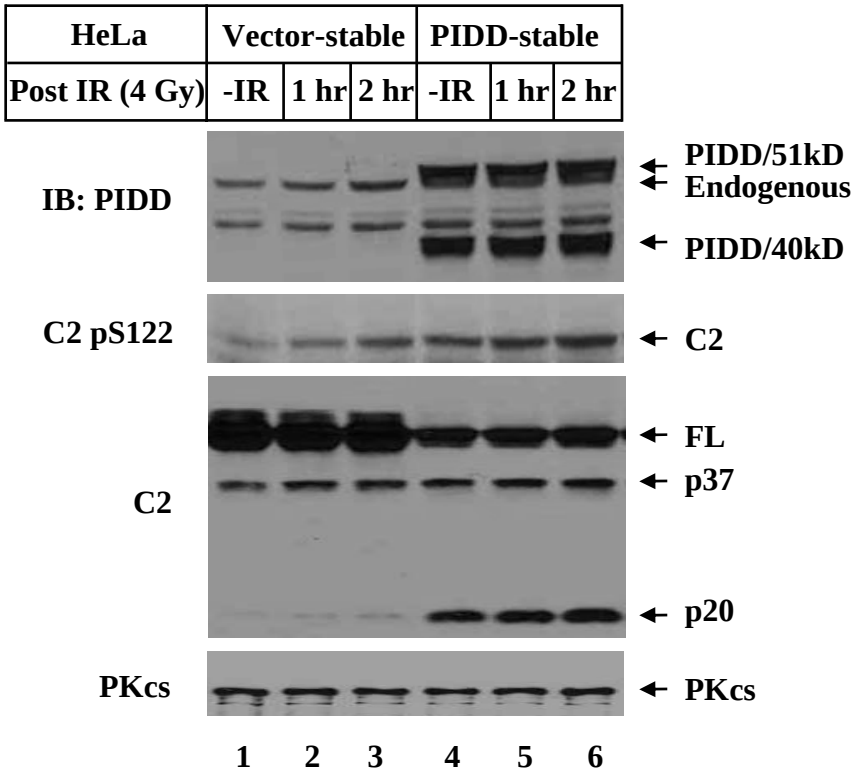

A
siControl
siC2
-IR
1hr
2hr
-IR
1hr
2hr
Post IR (4 Gy)
IB: C2
FL C2
Ku70
Ku70
B
4 Gy
-IR
1 hr
2 hr
siControl
siC2
1.52%
0.17%
0.11%
1.52%
0.60%
0.35%
p-H3
DNA content (PI)
C
siControl
siC2
U2OS
apoptotic cells (%)
4
3.5
3
2.5
2
1.5
1
0.5
0
-IR
1 hr
2 hr
4 Gy

Figure S15

A



B

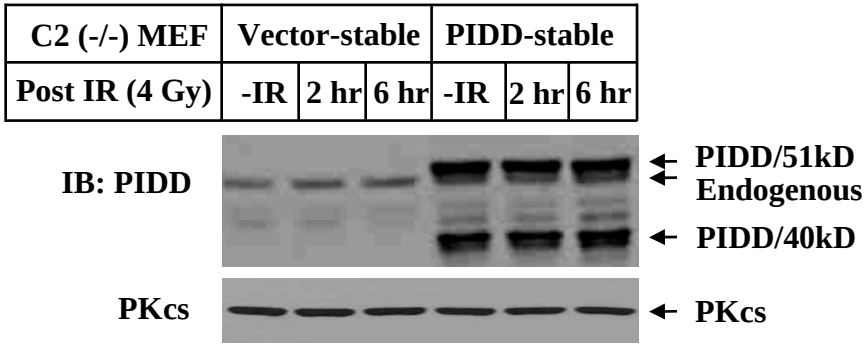


Figure S16

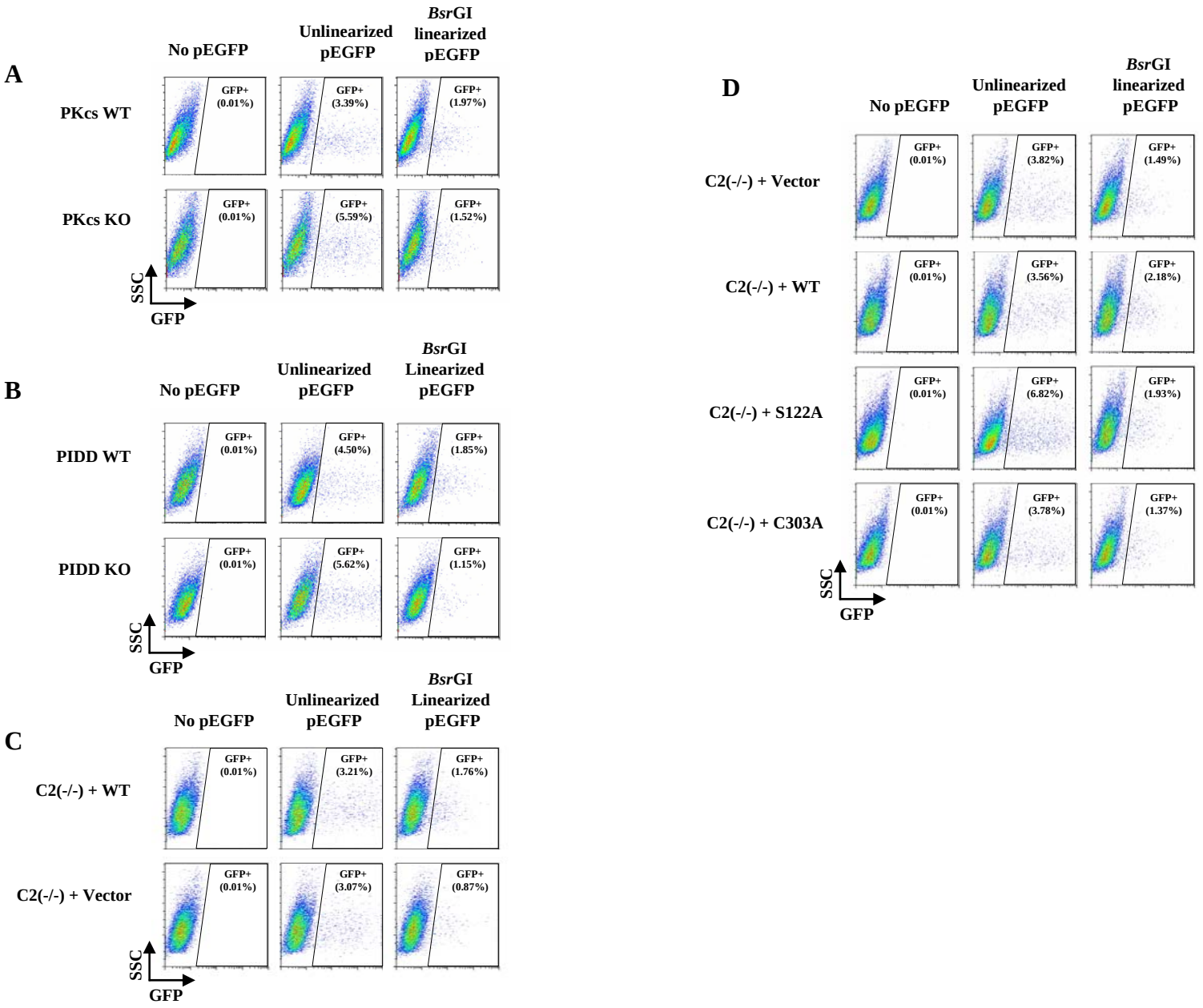
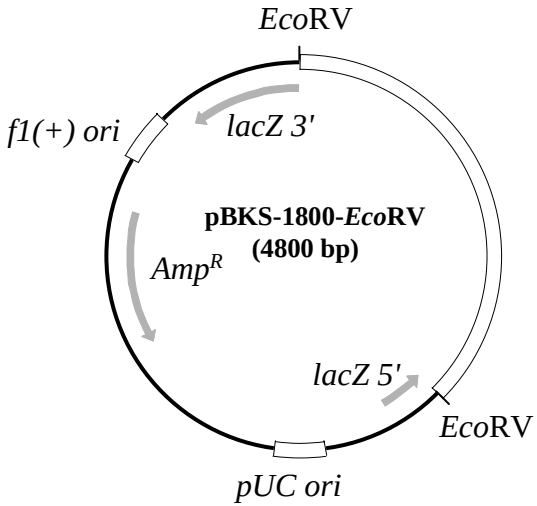

A
No pEGFP
Unlinearized pEGFP
BsrGI linearized pEGFP
PKcs WT
PKcs KO
GFP+ (0.01%)
GFP+ (3.39%)
GFP+ (1.97%)
GFP+ (0.01%)
GFP+ (5.59%)
GFP+ (1.52%)
SSC
GFP
B
No pEGFP
Unlinearized pEGFP
BsrGI Linearized pEGFP
PIDD WT
PIDD KO
GFP+ (0.01%)
GFP+ (4.50%)
GFP+ (1.85%)
GFP+ (0.01%)
GFP+ (5.62%)
GFP+ (1.15%)
SSC
GFP
C
No pEGFP
Unlinearized pEGFP
BsrGI Linearized pEGFP
C2(-/-) + WT
C2(-/-) + Vector
GFP+ (0.01%)
GFP+ (3.21%)
GFP+ (1.76%)
GFP+ (0.01%)
GFP+ (3.07%)
GFP+ (0.87%)
SSC
GFP
D
No pEGFP
Unlinearized pEGFP
BsrGI linearized pEGFP
C2(-/-) + Vector
C2(-/-) + WT
C2(-/-) + S122A
C2(-/-) + C303A
GFP+ (0.01%)
GFP+ (3.82%)
GFP+ (1.49%)
GFP+ (0.01%)
GFP+ (3.56%)
GFP+ (2.18%)
GFP+ (0.01%)
GFP+ (6.82%)
GFP+ (1.93%)
GFP+ (0.01%)
GFP+ (3.78%)
GFP+ (1.37%)
SSC
GFP

Figure S17

A



B