

Supplementary Information

Materials and Methods

Cells and Reagents

HEK293 cells were obtained from Korean Type Culture Collection (KTCC). Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS, Invitrogen). Doxorubicin (Dox), hydroxyurea (HU), etoposide (Ets), cycloheximide (CHX), interferon α (IFN- α), and 2-aminopurin (2-AP) purchased from Sigma Co. MG132 (A.G.Scientific, Inc.) was used for the experiments.

Plasmid Constructions

Wild type and mutant human Cdc2 cDNAs were prepared by RT-PCR using the primer pairs whose sequences are shown in Table S1. The cDNAs were cloned into pcDNA3-Flag and pcDNA3 plasmid at *EcoRI/XhoI* and *BamHI/XhoI* sites. Human PKR cDNA was cloned into both pcDNA3-Flag and pcDNA3-Myc plasmids at *NotI/XbaI*. Flag- or Myc-tagged PKR kinase dead forms (PKR/K296R) were generated by PCR-based mutagenesis with the mutagenic primer pairs shown in Table S1. The ubiquitin-expressing plasmid (pcDNA3-HA-Ub) used in a previous paper (Lee et al, 2009) was kindly provided by Dr. J. H. Song (Sungkyunkwan University). Quantitative RT-PCR was performed using the Hot-start PCR kit (Intron Biotech, Korea) with the following primer pairs: *cdc2*; forward, 5'-CTGGGGTTCAGCTCGTTACTC-3', reverse: 5'-AAATTCGTTTGGCTGGATCA-3', *gapdh*; forward, 5'-GACATCAAGAAGGTGGTGAA-3', reverse, 5'-TGTCATACCAGGAAATGAGC - 3'.

Immunoprecipitation and Western Blot Analysis

Immunoprecipitation was performed as described previously (Wu et al, 2007) with minor modifications. Cells were lysed by cell lysis buffer [50 mM Tris-HCl pH7.5, 150 mM NaCl, 0.5% Triton X-100, 1mM DTT and protease inhibitor cocktail (PIERCE. Co), 1mM Na₃VO₄ and 10mM NaF]. When necessary, cell lysates were incubated with 500 units of lambda-phosphatase (New England Biolabs) at 30 °C for 1 h. Cell extracts were immunoprecipitated with the appropriate antibodies at 4 °C for 4 h. Cell extracts or immunoprecipitants were subjected to SDS-PAGE, and protein bands were transferred onto PVDF membrane (Millipore Corp.). Western blot analyses were performed as described previously (Lee et al, 2007; Yoon et al, 2009) with appropriate antibodies: anti-human PKR (Cell Signaling), anti-Cdc2 (Cell Signaling or Santacruz), anti-Cyclin B1(Santacruz), anti-Cdk2 (Santacruz), anti-Cdk4 (Santacruz), anti-HA (Santacruz), anti-Myc (Cell Signaling), anti-FLAG (Sigma, M2), HRP-conjugated anti-ubiquitin (Santacruz, P4D1) and/or anti-phosphotyrosine

(anti-Y; p-Tyr-100 pan phosphotyrosine antibody, Cell Signaling), anti-pT14-Cdc2 and anti-pY15-Cdc2 (Cell Signaling) antibodies. Proteins were visualized using the enhanced chemiluminescence (ECL) detection system according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Pulse Chase Assay

Pulse chase (metabolic labeling) assay was performed as described previously (Tworkowski et al, 2002) with minor modification. In brief, cells were starved for 2 h in methionine/cysteine-free medium supplemented with 5% dialyzed FBS (Gibco BRL), metabolically labeled for 1h with 100 μ Ci/ml 35 S-protein labeling mix (PerkinElmer Life Sciences) in methionine/cysteine-free medium, washed twice, and were then incubated in pre-warmed complete medium. At the indicated time points, the cells were harvested and immunoprecipitated with anti-Cdc2 antibody. Proteins were subjected to SDS-PAGE, and then visualized by autoradiogram.

Recombinant Proteins

cDNAs of PKR, Cdc2 (wild-type and mutants) and eIF2 α (wild type and S51A mutant) were amplified by PCR, and cloned into the GEX-5X-1 plasmid (Amersham Pharmacia Biotech) and pRSETc (Invitrogen). *Escherichia coli* BL21-Codon Plus (DE3)-RIL (Stratagene) was transformed with recombinant plasmids (pGEX5X-1-PKR and/or pGEX5X-1-Cdc2 and pRSETc-eIF2 α). Luria Bertani (LB) broth cultures (50 ml) supplemented with 50 mg/ml ampicillin (Sigma) were shaking incubated overnight at 37 °C. Recombinant proteins were induced by treating the culture with isopropyl- β -D-1-thiogalactopyranoside (Sigma) for 5 h to a final concentration of 1 mM. Bacteria were pelleted by centrifugation at 5,000 rpm at 4 °C for 10 min and resuspended in 4 ml of EBC buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40) supplemented with 5 mM DTT (Sigma), 2 mg/ml lysozyme (Sigma), protease inhibitor cocktail (Roche Applied Sci. Inc., 1 mg/ml leupeptin, 2 mg/ml aprotinin), and 50 mg/ml phenylmethylsulfonyl fluoride (Sigma). After incubation for 15 min, cells were sonicated three times on ice, and the lysates were centrifuged at 12,000 rpm at 4 °C for 15 min. GST-fusion proteins were purified as described previously (Huang & Paudel, 2000) with glutathione sepharose 4BTM (Amersham Pharmacia Biotech) from the soluble fraction of bacteria. 6XHis-attached *wt* and *mt* eIF2 α expressed in *E. coli* were purified by using Ni-NTA agarose bead (QIAGEN). In case of *in vitro* kinase assays with GST-free Cdc2, GST-tags were removed by treating recombinant GST-Cdc2 proteins with Factor Xa as reported previously (Nagai & Thogersen, 1984).

In vitro Kinase assay

In vitro kinase assay was performed as described previously (Jammi & Beal, 2001) with minor modifications. In brief, 1 μ g of purified recombinant GST-PKR was reconstituted and pre-

activated in 10 μ l of kinase reaction buffer (50 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 50 mM KCl, 1 μ g/ml poly(I):(C) and 20 mM ATP) at 30 °C for 1 h in the presence or absence of 10 μ Ci of [γ -³²P]ATP. After incubation, 10 μ l of GST-Cdc2 solution (50 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 50 mM KCl, 20 mM ATP, 10 μ Ci [γ -³²P]ATP, 1 μ g/ml of poly(I):(C) and 1 μ g of GST-Cdc2) was added to the PKR mixture, and then incubated at 30 °C for additional 1h. Each reaction was terminated and separated on a 10% SDS-PAGE. Cdc2 phosphorylation was visualized by autoradiogram or Western blot analysis using anti-pY antibody. Cdc2 immune complex kinase assay was performed as described previously (Park et al, 2005; Poon et al, 1996) with minor modification. In brief, cells were lysed in lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl (pH8.0), 150 mM NaCl, 50mM KCl and protease inhibitor cocktail). A total of 1mg of protein was used for each immunoprecipitation assay. Cdc2 was co-immunoprecipitated with 1 μ g of an anti-cyclin B1 antibody (Cell Signaling). Cdc2 immune complex kinase assay was performed with 5 μ g of histone H1 and 5 μ Ci of [γ -³²P]ATP in 20 μ l of kinase buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 1 mM DTT) at 30 °C for 30 min. The kinase reactions were subjected to 10% SDS-PAGE, and then Cdc2 kinase activities were visualized by autoradiogram.

Preparation of anti-pY4-Cdc2 peptide-derived antiserum

The synthetic human Cdc2 phosphopeptide MEDpYTKIE (pY represents phosphotyrosine) was provided by Peptron Inc. Antiserum against phospho-Tyr-4 Cdc2 peptide was prepared by immunizing rabbits three times with KLH (keyhole limpet hemocyanin)-conjugated Cdc2 phosphopeptide. The serum obtained from the immunized rabbits was purified by using the SulfoLink^R Immobilization Kit for Peptides (Thermo scientific) according to the manufacturer's protocol. In brief, flow through antiserum was pooled after adsorption to the unphosphorylated form of the Cdc2 control peptide conjugated to KLH (KLH-MEDYTKIE). The antiserum was further purified using affinity chromatography charged with the phosphopeptide, MEDpYTKIE. The specificity of purified antiserum was examined by Western blot analysis with ovalbumine (OVA)-conjugated unphosphorylated Cdc2 peptide and OVA-conjugated pY4-Cdc2 peptide as shown in **supplementary Fig S2**.

Sample Preparation for Tandem Mass Spectrometry Analysis (MS/MS)

After in vitro kinase assay, The Cdc2 fraction extracted from the gel was digested by using sequencing-grade modified trypsin (Promega, Madison, WI) at a ratio of 1:20 (enzyme: protein). The tryptic peptides were extracted with 50% Acetonitrile (ACN) in 5% formic acid (FA), and were then dried using a SpeedVac. Dried peptides were reconstituted with 1% FA in H₂O.

Tandem Mass Spectrometry and Data Analysis

This study was performed as described previously (Hur et al, 2008), with minor modifications. The tryptic peptides were applied to a 12 cm x 75 μ m C₁₈ reverse-phase column packed with 5 μ m C₁₈ beads. The column was in line with an LTQ ion-trap mass spectrometer (Thermo Fisher Scientific Inc, San Jose, CA). Liquid chromatography was carried out at a flow rate of 0.25 μ l/min. The peptides were eluted in buffer A with a gradient of 3–40% buffer B (0.1% formic acid in 90% acetonitrile) over 80 min using an Ultimate 3000 nano-HPLC system (Dionex LC Packings, Idstein, Germany). The electrospray voltage was set at 2.1kV, and the threshold for triggering MS/MS was 250. The normalized collision energy for MS/MS was 35% of the main RF amplitude, and the duration of activation was 30 msec. Tandem mass spectrometry (MS/MS) was performed in a data-dependent manner in which each full MS scan was followed by consecutive MS/MS scans on the TOP five most intense ions from the full MS scan. The repeat count of peak for dynamic exclusion was 1, and its repeat duration was 30 s. The dynamic exclusion duration was set for 180 sec, and exclusion mass width was 1.5 Da. The list size of dynamic exclusion was 50. The resulting MS/MS data were searched by TurboSEQUENT 3.3 algorithm against the targeted human Cdc2 protein database (GI:30582846). Modifications were set as a fixed modification of 57 Da on Cysteine, 16 Da on Methionine and a dynamic modification of 80 Da on Serine, Threonine and Tyrosine.

Cell Cycle Analysis

Cell cycle analysis was carried out as described previously (Yoon et al, 2009). In brief, cells were pre-treated with genotoxic agents, harvested, washed in cold phosphate-buffered saline (PBS), fixed in 70% ethanol for 1 h, and then stained with a propidium iodide (PI) solution containing RNaseA (Sigma) for 30 min at room temperature in the dark. Samples were then analyzed by FACS Calibur (BD Bioscience) with CellQuest software.

References

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Table S1. Sequences of each primer set used for cloning

Primer name	forward	reverse
hCDC2 wild type	5'-ACTAGAATTCATGGAAGATTATACC AAAATAGAG-3'	5'-ACTACTCGAG TACATCTTCTT AATCTGATTGTC-3'
hCDC2 Δ 1	5'-ACTAGAATTCGAAGATTATACCAA AATA-3'	Same as above
hCDC2 Δ 1-2	5'-ACTAGAATTC GATTATACCAAAAT AGAG -3'	Same as above
hCDC2 Δ 1-3	5'-ACTAGAATTCTATACCAAAATAGAG AAA-3'	Same as above
hCDC2 Δ 1-4	5'-ACTAGAATTCACCAAAATAGAGAA AATT-3'	Same as above
hCDC2 Δ 1-5	5'-ACTAGAATTCAAAATAGAGAAAA TTGGA -3'	Same as above
hCDC2 Y4A	5'-ACTAGAATTCATGGAAGATGCAACC AAAATAGAGAAAATTGGA-3'	Same as above
hCdc2Y4D	5'-ACTAGAATTCATGGAAGATGATCCA AAATAGAGAAAATTGGA-3'	Same as above
hCdc2Y4E	5'-ACTAGAATTCATGGAAGATGAAACC AAAATAGAGAAAATTGGA-3'	Same as above
hCdc2Y4F	5'-ACTAGAATTCATGGAAGATTTTACC AAAATAGAGAAAATTGGA-3'	Same as above
hCdc2 K6R	5'-ACTAGAATTCATGGAAGATTATACC CGTATAGAGAAAATTGGA-3'	Same as above
hCdc2del3	5'-ACTAGAATTCATGGAATATACCAAAA TAGAG-3'	Same as above
hCdc2del4	5'-ACTAGAATTCATGGAAGATACCAAAA TAGAG-3'	Same as above
hCdc2del5	5'-ACTAGAATTCATGGAAGATTATAAAA TAGAG-3'	Same as above
hPKRK296R	5'-AGACGTGTTAAATATAATAACGAGA A-3'	5'-AATAACGTAAGTCTTTCCGTC A-3'
hPKR	5'-ACTAGCGGCCGCAAATGGCTGGTGA T CTTTCAGCA-3'	5'-ACTATCTAGACTAACATGTGT GTCGTTCAAT-3'
hCdc2wt-Flag	5'-AATCGGATCCACCATGGATGGAA GATTATACCAAAATAGAGTTT-3'	5'-ATGCCTCGAGTCAGATCTT ATCGTCGTCATCCTTGTAATCCAT CTTCTTAATCTGATTGTCCAAATC -3'
hCdc2Y4F-Flag	5'-AATCGGATCCACCATGGATGGAA GATTTTACCAAAATAGAGTTT-3'	Same as above
hCdc2Y4E-Flag	5'-AATCGGATCCACCATGGATGGAA GATGAAACCAAAATAGAGTTT-3'	Same as above

Supplementary Figures

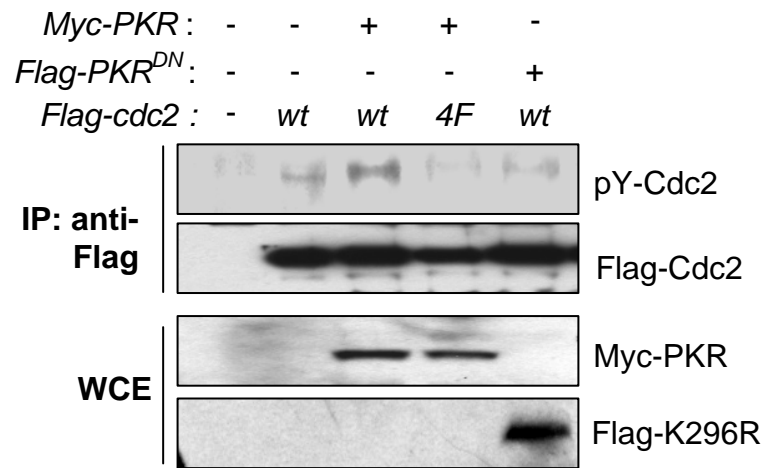


Figure S1: Ex vivo Cdc2 phosphorylation by PKR. HEK293 cells were co-transfected with wild type (wt) or mutant (Y4F) *Flag-cdc2* plasmid together with Flag-tagged wild type or kinase dead form (K296R) of PKR. After 48 h, cell lysates were immunoprecipitated with anti-Flag mAb, and then Cdc2 phosphorylation were visualized by Western blot analysis with mAb (Cell Signaling). WCE were analyzed with anti-Myc, anti-Flag mAbs.

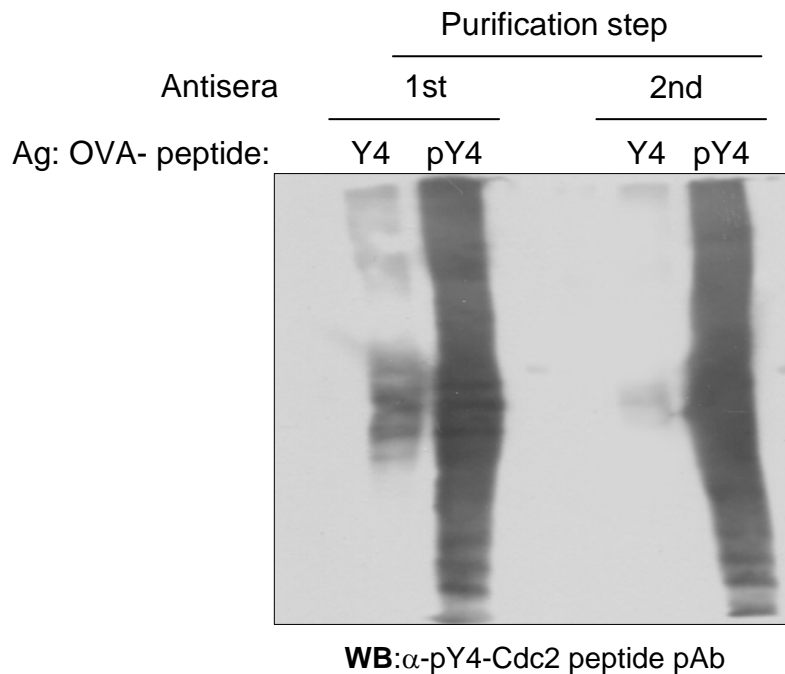


Figure S2. OVA-conjugated control Cdc2 peptide (Y4) and OVA-conjugated pY4-Cdc2 peptide (pY4) were separated on SDS-PAGE, and then examined by Western blot analysis with rabbit anti-pY4-Cdc2 peptide antiserum prepared and purified as described in Supplementary *Methods*.

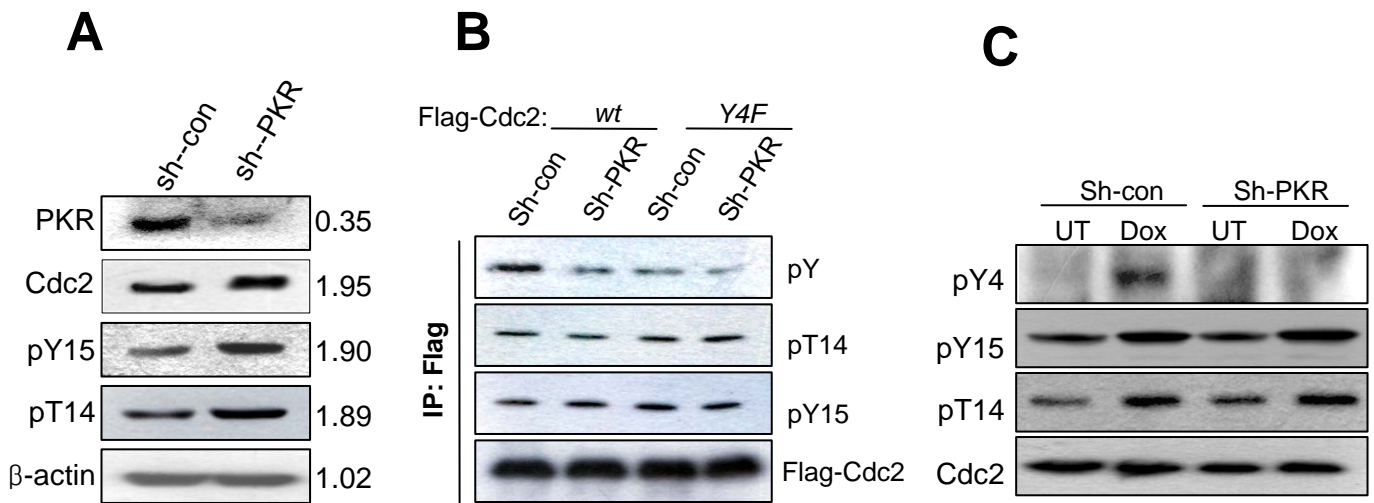


Figure S3. (A) Cell extracts from PKR-normal (*sh-con*) or PKR^{KD} (*sh-PKR*) HEK293 cells were subjected to Western blot analysis with appropriate antibodies. Relative band intensities indicated were assessed by MCID analysis program (www.micd.co.uk). (B) *sh-con* and *sh-PKR* HEK293 cells were transfected with wild type (*wt*) or mutant (*Y4F*) *Flag-cdc2* plasmid. After 48 h, cell lysates were immunoprecipitated with anti-Flag mAb. Following *Flag-Cdc2* normalization, Cdc2 phosphorylations were assessed by Western blot analysis with appropriate phospho-specific mAbs. (C) *sh-con* and *sh-PKR* HEK293 cells were treated with 1 μ M doxorubicin (Dox) for 12 h. After normalization with total Cdc2, Cdc2 phosphorylations were assessed by Western blot with appropriate phospho-specific mAbs or pY4-Cdc2-peptide-specific serum generated in our Lab.

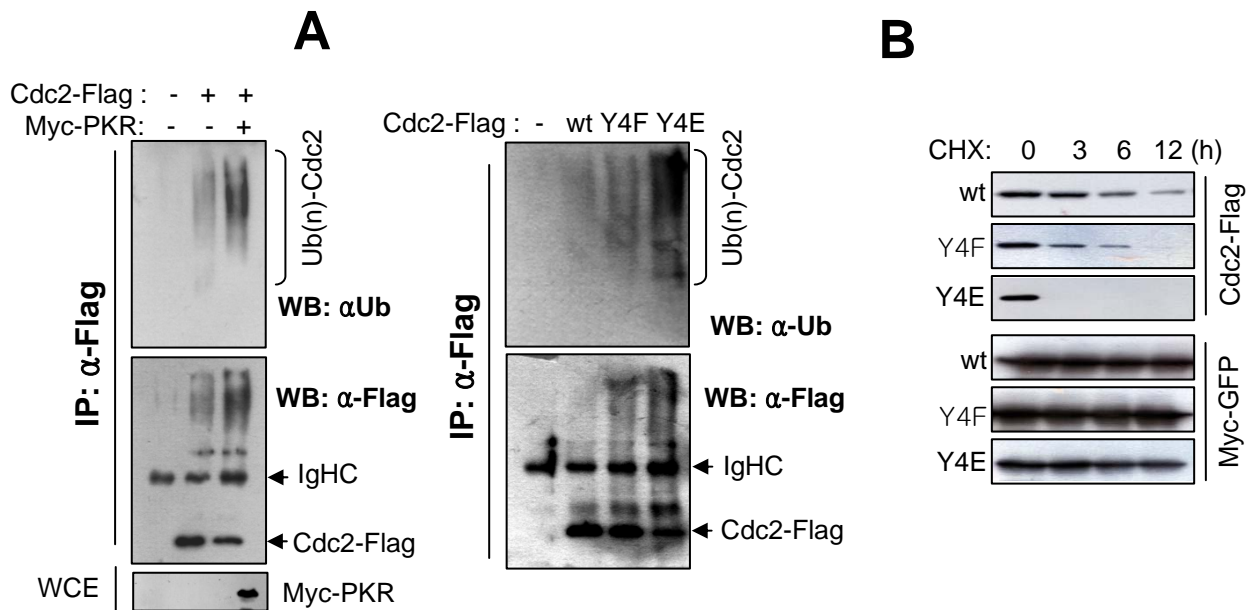


Figure S4. (A) HEK293 cells transiently co-transfected with C-terminal Flag-tagged *cdc2* (*cdc2-Flag*) and/or *Myc-PKR* plasmids together with *Myc-mock* and/or *Flag-mock* plasmids as a transfection control (left). Wild type or mutant Cdc2-Flag-expressing plasmids were transfected into the HEK293 cells (right). After 48 h, these cells were treated with 20uM MG132 for 5 h prior to harvest. Cdc2-Flag was immunoprecipitated with anti-Flag mAb. Following Cdc2 normalization after IP, Cdc2 poly-ubiquitinations were assessed by Western blot analysis with HRP-conjugated anti-ubiquitin mAb. Ten % of whole cell extracts (WCE) were also probed with anti-Myc mAb (left bottom). IgHC; immunoglobulin heavy chain. (B) HEK293 cells were co-transfected with indicated *wt* and mutant *cdc2-Flag* and *Myc-GFP* (as a transfection control) plasmids. Two days after transfection, the cells were treated with cycloheximide (CHX, 75 μ M) for the indicated time period prior to harvest. Cdc2 levels were analyzed by Western blot analysis with anti-Flag based on the concentration of Myc-GFP.

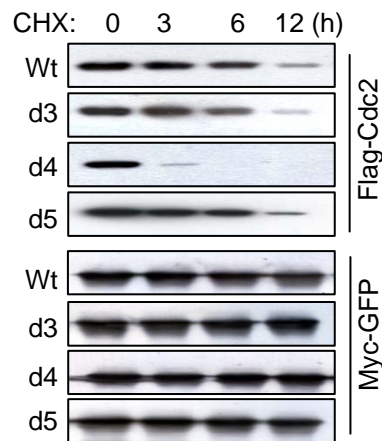


Figure S5. HEK293 cells were transfected with *wt* and mutant *Cdc2* (*Flag-cdc2*) and *Myc-GFP* control plasmids. Two days after transfection, the cells were treated with cycloheximide (CHX, 75 μ M) for the indicated time periods prior to harvest. Wild type (*wt*) and mutant *Cdc2* levels were analyzed by Western blot analysis with anti-Flag (upper panel) and anti-Myc (lower panel) mAbs, respectively.

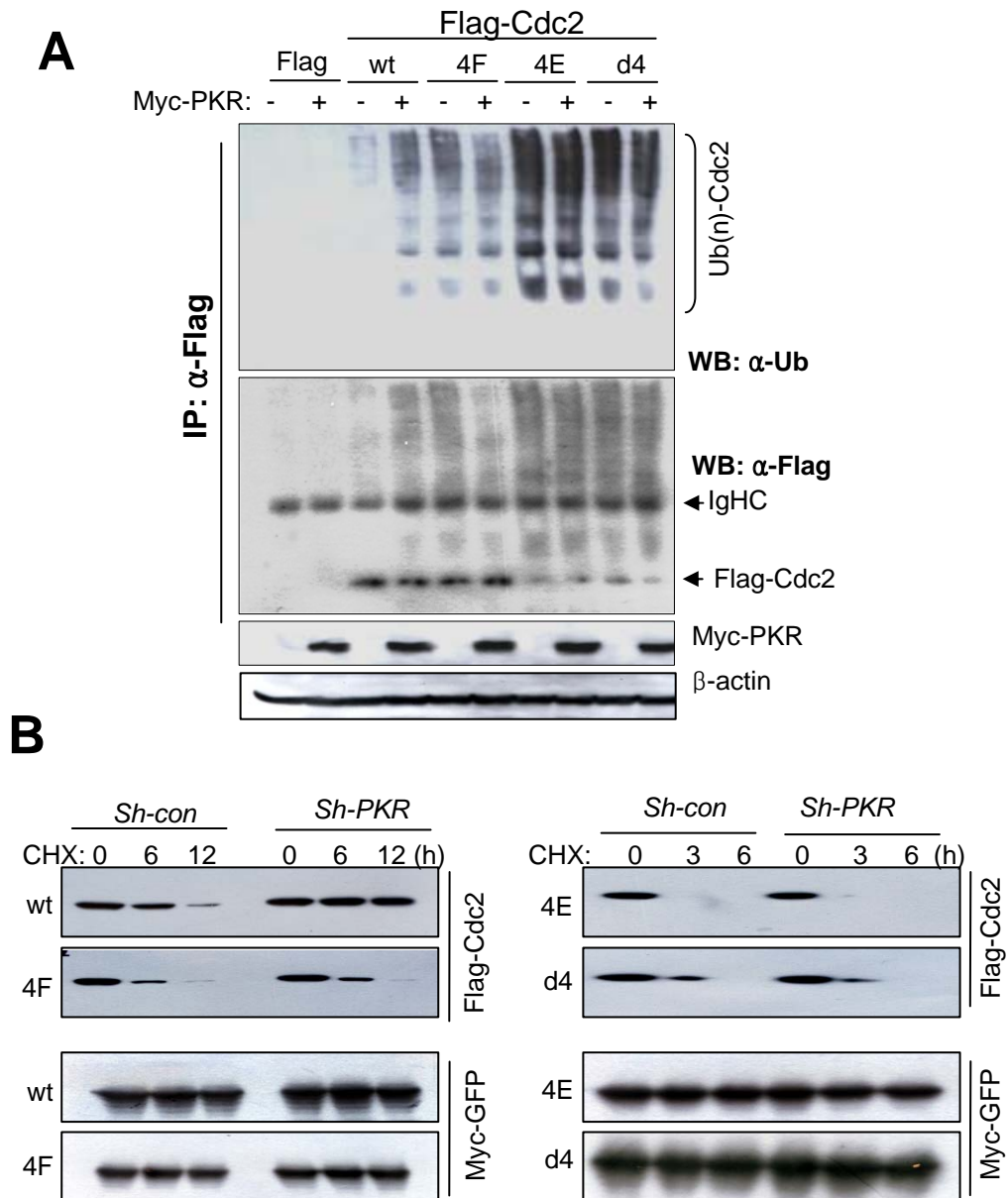


Figure S6. (A) HEK293 cells transfected with *wt* or mutant Flag-Cdc2 were cultured for 48 h, and then treated with 20 μ M MG132 for 5h prior to harvest. Following immunoprecipitation with anti-Flag mAb, Cdc2 poly-ubiquitinations were assessed by Western blot analysis with anti-Ub (upper panel) and anti-Flag (lower panel) mAbs. IgHC; immunoglobulin heavy chain. (B) *sh-con* and *sh-PKR* HEK293 cells were transfected with *wt* and mutant Cdc2 (*Flag-cdc2*) and *Myc-GFP* control plasmids. Two days after transfection, the cells were treated with cycloheximide (CHX, 75 μ M) for the indicated time periods prior to harvest. Wild type (*wt*) and mutant Cdc2 levels were analyzed by Western blot analysis with anti-Flag (upper panel) and anti-Myc (lower panel) mAbs, respectively.

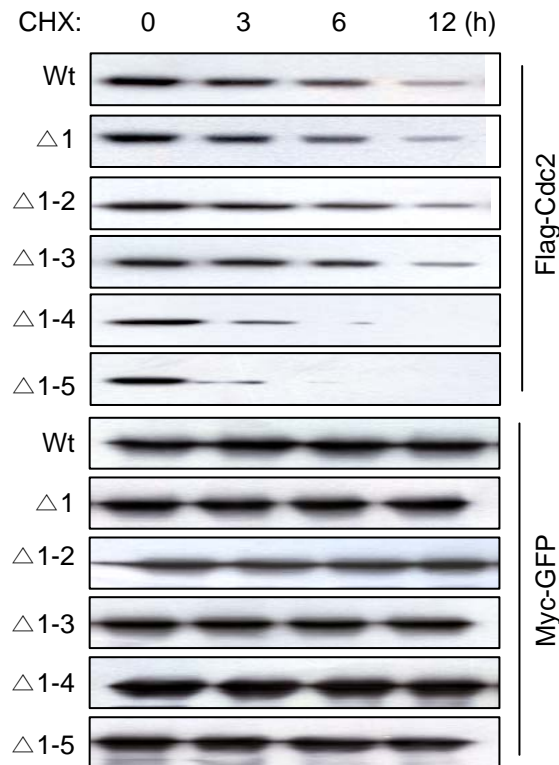


Figure S7. HEK293 cells were transfected with *wt* and mutant *Cdc2* (*Flag-cdc2*) and *Myc-GFP* control plasmids. Two days after transfection, the cells were treated with cycloheximide (CHX, 75 μ M) for the indicated time periods prior to harvest. Wild type (*wt*) and mutant *Cdc2* levels were analyzed by Western blot analysis with anti-Flag (upper panel) and anti-Myc (lower panel) mAbs, respectively.

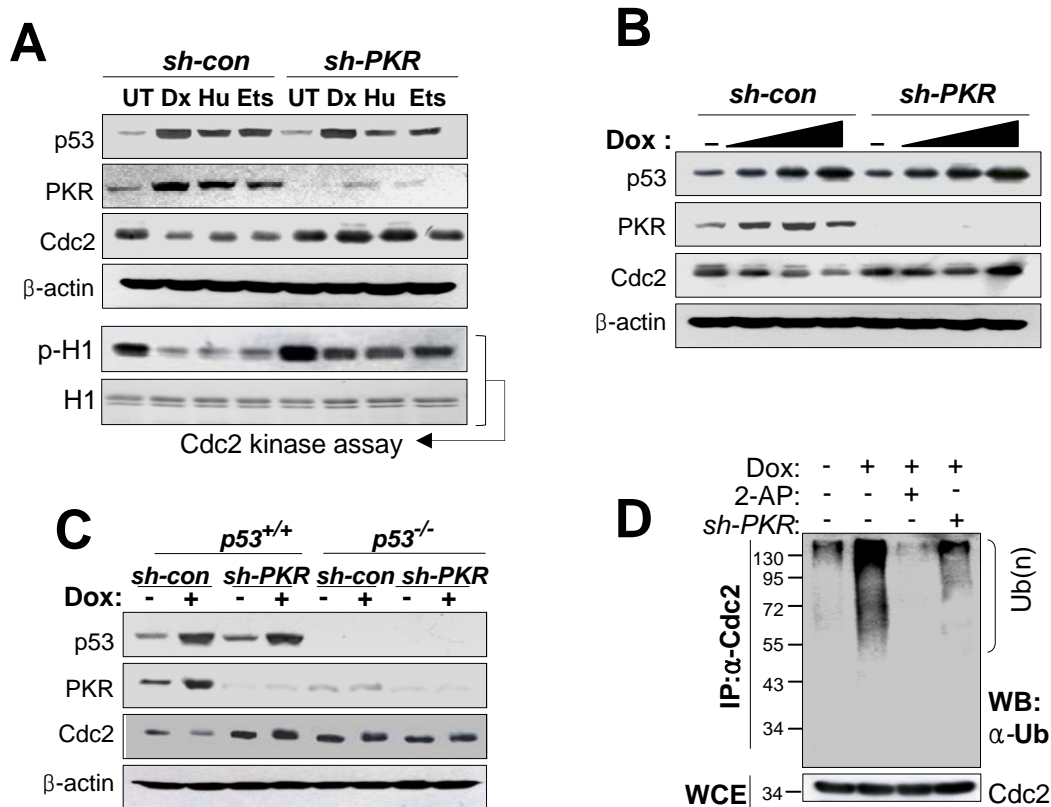


Figure S8. Genotoxic drug-induced cdc2 down-regulation is likely attributed to PKR-mediated Cdc2 ubiquitination.

(A) *sh-con* (normal) or *sh-PKR* (PKR^{KD}) HCT116 cells were treated with 0.2 μ M doxorubicin (Dox), 0.5 mM hydroxyurea (HU), or 2.5 μ M etoposide (ETS) for 24 h, and were then subjected to Western blot analysis (upper). Cdc2-associated kinase assay were performed with Cdc2 immune complexes obtained from the cells treated with genotoxins (bottom). (B) *sh-con* and *sh-PKR* HCT116 cells were treated with increasing concentrations of Dox (0, 0.1, 0.2 and 1 μ M) for 24 h, and then subjected to Western blot analysis. (C) *sh-con* or *sh-PKR* HCT116 ($p53^{+/+}$ or $p53^{-/-}$) cells were treated with Dox for 24 h, and then subjected to Western blot analysis. (D) *sh-con*(-) and *sh-PKR*(+) HCT116 cells were treated with Dox and/or 2 mM 2-AP for 24hr, and additionally treated with MG132. Cdc2 ubiquitination was assessed following Cdc2 normalization after IP.

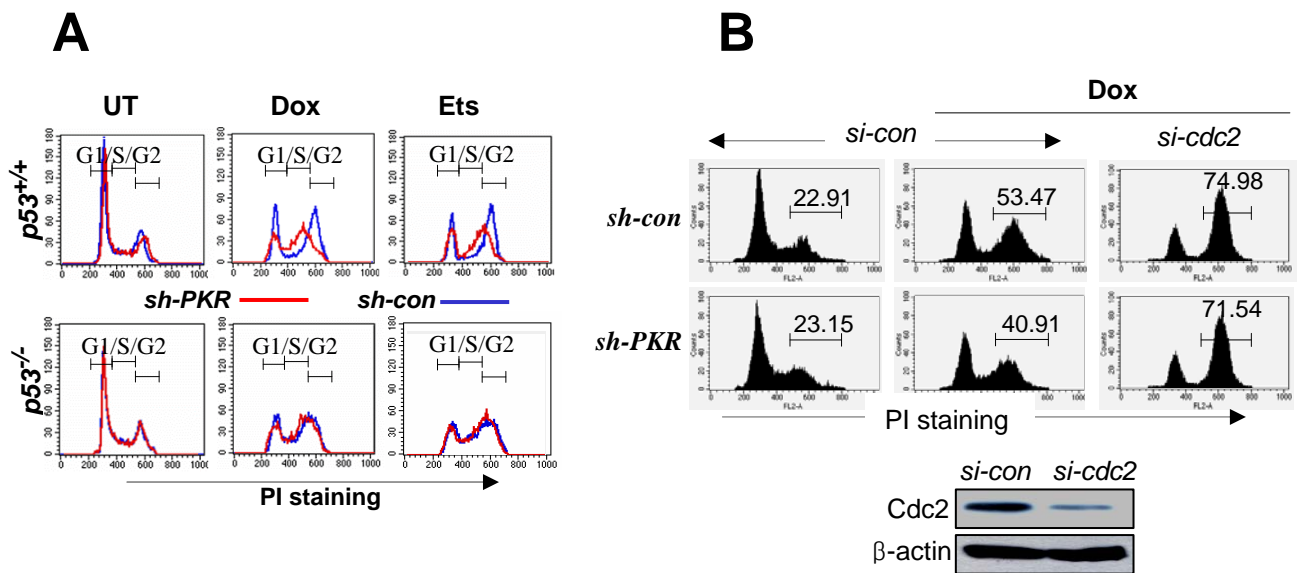


Figure S9. Genotoxic drug-induced G2 arrest is at least in part attributed to the involvement of PKR downstream of p53.

(A) *sh-con* and *sh-PKR* HCT116 (*p53*^{+/+} or *p53*^{-/-}) cells were treated with Dox and Ets for 12 h, and their cell cycles were analyzed by flow cytometry. (B) *sh-con* and *sh-PKR* HCT116 *p53*^{+/+} cells were transfected with 100 nM *Cdc2* siRNA. After 24h, cells treated with Dox for an additional 12 h were subjected to cell cycle analysis. Cells in the G2 phase are indicated. *Cdc2*-knockdown was demonstrated (bottom).

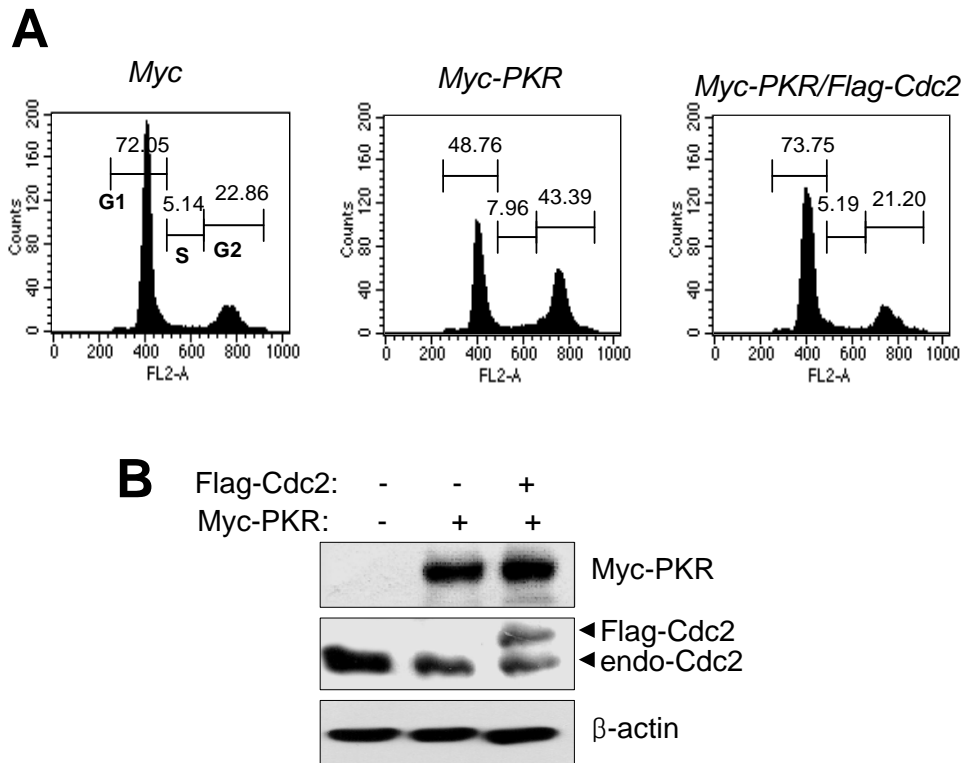


Figure S10. (A) HEK293 cells were co-transfected with 3 μ g of Myc-PKR and/or 3 μ g of Flag-Cdc2. After 48h, the cells were subjected to cell cycle analysis by flow cytometry with CellQuest software. G1, S and G2 phases are indicated on the plots. (B) PKR and Cdc2 expressions were analyzed in each cell sample by Western blot analysis.

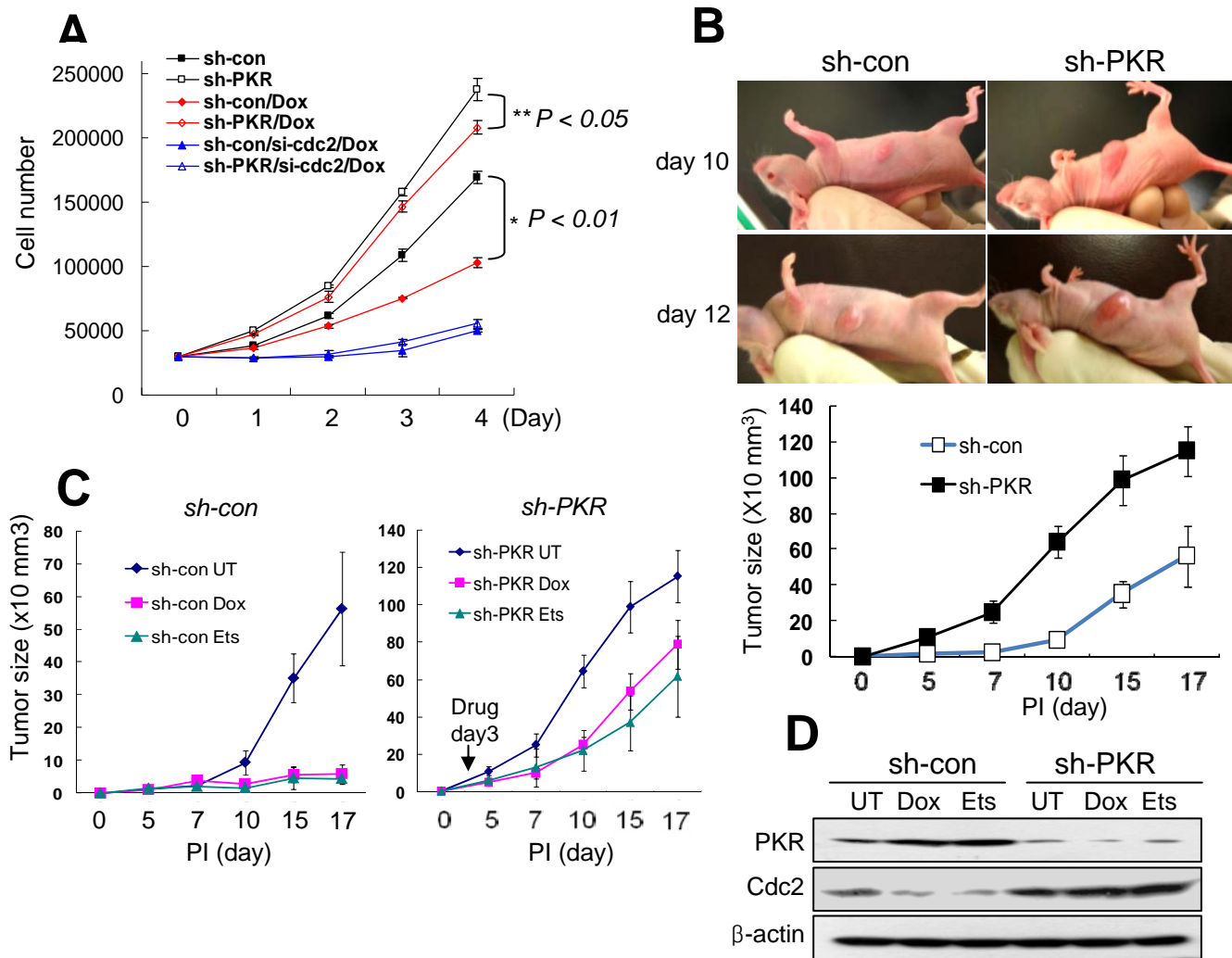


Figure S11. (A) *sh-con* and *sh-PKR* HCT116 cells were transfected with *Cdc2* siRNA and cultured in the presence of 50 nM Dox. The number of cells was counted using a CellTiter96R Assay kit (Promega). Data are represented as means \pm SEM. $n=3$. * P values; as compared with the group of untreated cells. (B) Nude mice were inoculated subcutaneously (*s.c.*) (10^7 cells/inj) with *sh-con* and *sh-PKR* HCT116 cells. Tumor-bearing mice were photographed on day 15th after injection. (left). Tumor growth was monitored for 17 days (right), and are represented as means \pm SEM ($n=4$). (C) Nude mice were inoculated subcutaneously (10^7 cells/inj) with *sh-con* and *sh-PKR* HCT116 cells. Three days later, the mice were treated *i.p.* once with Dox (2mg kg⁻¹) or Ets (25mg Kg⁻¹). Tumor growth was represented as means \pm SEM ($n=4$). (D) *Cdc2* and PKR were examined from each tumor. Cells, isolated from the PKR-normal (*sh-con*) and PKR^{KD} (*sh-PKR*) HCT116 tumors on day 17th, were untreated or treated with with 0.2 μ M Dox (Dox), or 2.5 μ M etoposide (ETS) for 24 h, and the level of PKR and *Cdc2* were examined by Western blot analysis with appropriate antibody.

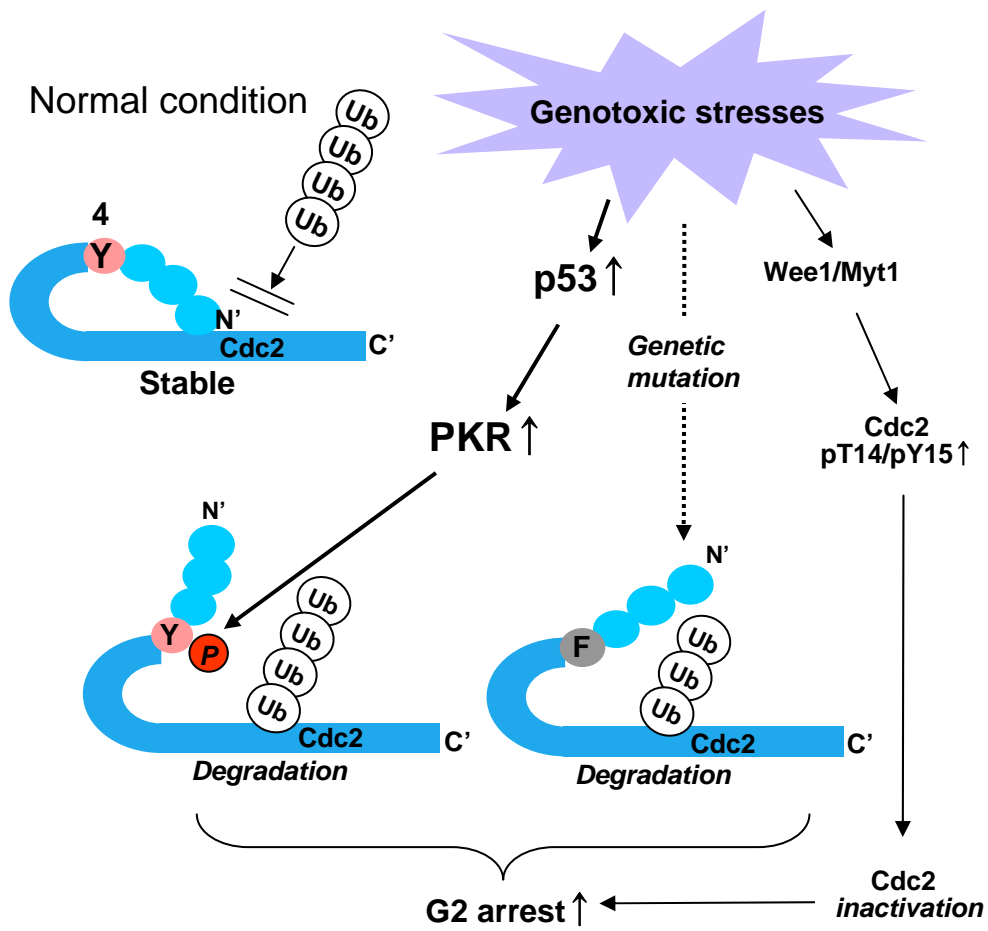


Figure S12. Schematic model of the mechanism underlying PKR-mediated Cdc2 poly-ubiquitination under genotoxic stresses.