Supplementary Data

DNA-PKcs deficiency leads to persistence of oxidatively-induced clustered DNA lesions in human tumor cells

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Supplemental Experimental Procedures

siRNA Transfection and IC86621 Drug treatment

Here, we performed *Prkdc* gene silencing using siRNA transfection based on our preliminary work [1] and according to the protocols of siRNA supplier (Santa Cruz Biotechnology, Santa Cruz, CA) with some modifications. DNA-PKcs siRNA (h) is a pool of 2 target-specific 20-25 nt siRNAs designed to knock down efficiently *Prkdc* (DNA-PKcs) gene expression. To generate MCF-7 cells with silenced DNA-PKcs expression, we followed a series of preliminary experiments in order to optimize the transfection conditions. For the mock transfection, control siRNA (Santa Cruz Biotechnology) was used that consisted of a scrambled sequence conjugated to fluorescein and does not lead to any specific degradation of cellular mRNA. The optimal concentration of siRNAs was found to be 0.1 μM, and siRNA-transfection was repeated in the same way 3 days after the initial transfection (2^{nd} transfection). Our conditions resulted in a significant (~85%) reduction in the level of DNA-PKcs relative to controls, and these cells were used for all experiments [1]. In addition, the growth rate for the siRNA-treated MCF-7 cells was

only ~5% lower than that of control cells [1]. IC86621 (Sigma) is a novel chemical inhibitor of DNA-PKcs kinase activity. This experimental drug (1-(2-hydroxy-4-morpholin-4-yl-phenyl)-ethanone) has been shown to be highly specific for DNA-PKcs in comparison to other DNA-PKcs inhibitors such as wortmannin (Figure S1) [2]. In particular, IC86621 has little effect on related members of the phosphotidyl inositol kinase family such as ATM and ATR. Biochemical and genetic studies of IC86621 have demonstrated its function as a specific inhibitor of DNA-PK kinase activity [3, 4]. The drug was solubilized at a concentration of 50 mM in dimethyl sulfoxide (DMSO) as a stock solution and was stored at -20^{o}C in the dark. Aliquots were thawed just before each experiment and diluted to final concentration of 100 μM in cell medium. The final DMSO concentration was 0.2% in control and drug treated cells. MCF-7 cells were treated with 100 μM IC86621 in the growth medium for 24 h. Preliminary experiments using different IC86621 concentrations (50-200 μm) showed that this specific optimum concentration (100 μM) was not cytotoxic for the cells (determined by measuring trypan blue dye uptake), it did not retard cell growth rate and it was very efficient in blocking DSB repair after 24 hrs measured using pulsed field gel electrophoresis (data not shown). Control (medium contained only the solvent, 0.2% DMSO) and drug-treated cells were irradiated using the ^{137}Cs source as above at a dose rate of 0.55 Gy/min. Following irradiation, the growth medium was replaced with fresh medium containing 100 μM IC86621 incubated up to 24 hrs at 37^{o}C to allow repair. For incubations longer then 24 hrs, the medium containing the drug is replaced with fresh medium without IC86621 [3]. Cells were either processed for immunofluorescence or for damage measurement using pulsed field gel electrophoresis.

Detection of γ-H2AX and DNA-PKcs using Immunofluorescence

For the detection of DNA-PKcs or γ-H2AX cells were plated onto sterile glass coverslips, placed into 12 well plates and grown to ~70% confluence. The plates were then γ-irradiated using 5 Gy, returned at 37^{o}C, harvested at different post-irradiation repair time points up to 120 hrs and processed. During sample collection, medium was aspirated, and the cells washed once with PBS for 5 minutes. Cells were then fixed with 2% paraformaldehyde in PBS for 10 minutes at room temperature, and then washed twice with PBS for 5 minutes each. Coverslips were then transferred to a new 12 well plate and were permeabilized with 100% methanol at -20^{o}C and kept in these conditions until they were processed further. Once all time points were collected, samples were drained of methanol and washed twice with PBS for 5 minutes each. Primary antibody incubation was carried out in 2% BSA in PBS. For immunodetection, cells in coverslips were exposed to either a 1:500 dilution of mouse monoclonal γ-H2AX primary antibody (serine 139, Upstate, VA), 1:250 dilution DNA-PKcs rabbit polyclonal (Santa-Cruz, CA) or 1:250 rabbit polyclonal DNA-PKcs phosphorylated-T2609 (Abcam, MA) for 6 hours at room temperature. After three washes with PBS (10 min each), the cells were incubated with goat anti-mouse IgG FITC conjugate (Zymed, CA) or goat anti-rabbit IgG FITC conjugate (Zymed, CA) at a dilution of 1:500 in PBS containing 5% goat serum, for one hour at room temperature; washed with PBS for 30 min with 6 buffer changes and were counterstained with 3 μg/ml propidium iodide (Sigma, MO) in PBS for 5 min. Individual coverslips were mounted on slides and washed in 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma, St. Louis, MO) antifade medium. Digital images (exposure time 2s) were then taken using a fluorescent microscope (Olympus BX 40, at 40X objective). For the γ-H2AX quantitative analysis, mean total γ-H2AX fluorescence intensity was measured using Adobe 7.0 software and 150 nuclei were used for each time point to calculate the average values while the foci number was measured using

Quantity One software (Biorad). The accurate scoring of foci was achieved by calibrating the methodology to correspond to 1-3 foci for non-irradiated samples and approximately 20 foci for 1 Gy.

Single DNA lesion Detection using alkaline Single Cell Gel Electrophoresis (SCGE)

With alkaline SCGE, we detect all radiation induced SSBs and alkali labile sites which are converted to breaks during alkaline electrophoresis resulting to an increase in the tail moment (TMI). In addition though to normal and oxidized abasic sites, several oxidized bases including 5,6-dihydroxy-5,6-dihydrothymine, 5-formyluracil and oxazolone may be converted into strand breaks under the alkaline conditions of the single cell gel electrophoresis analysis [5, 6]. ~15,000 cells (drug-treated and controls) after H_2O_2 treatment and at three post-irradiation repair times points (0, 3 and 12 hrs) were harvested by trypsinization, washed with PBS, centrifuged and embedded in low melting agarose plugs at the final agarose concentration of 0.85%. The formation of these DNA plugs allows direct comparison with PFGE. Plugs were applied to a 3x5 inch microscope slide fully frosted with 1% normal melting point agarose (Molecular Biology Grade, Bio-Rad, Hercules, CA) dissolved in sterile 1X PBS. Next, an 18cm x 18cm cover-slip was applied and allowed to solidify at 4^0C for 10 min. Slides were then placed in 50ml lysis buffer (10 mM Tris-HCl, 2.5 M NaCl, 100 mM EDTA containing 1% Triton X-100, 1% sarcosyl, pH 10) for 1 hr at 4^0C. Slides were washed extensively in ice-cold alkaline denaturation buffer containing 300mM NaOH, 1mM Na_2EDTA (pH >13) and further incubated in the same buffer at 4^oC for 40 min. For the detection of single base lesions using repair enzymes Fpg and Endo III, following post-irradiation treatment and lysis as described above, cells were washed and acclimated to the appropriate repair enzyme reaction buffer (20 ml) i.e.,

Fpg (10 mM Tris-HCl, 10 mM $MgCl_2$ pH 7.0) and Endo-III (20 mM Tris-HCl, 1 mM EDTA pH 8.0) for 2 hrs with three buffer changes at 4^0C in the dark. For single base lesion measurement and after H_2O_2 treatment, two slides (with two sample areas each) were used, the slide 'with enzyme' (+) and the slide 'without enzyme' (-). The (-) slide followed exactly the same treatment except addition of the enzyme in the enzyme reaction buffer. The appropriate repair enzyme quantity offering maximum cleavage (1U for both enzymes) was determined from titration studies using 5 Gy-irradiated cells (data not shown). The enzyme was diluted in reaction buffer to form the 'enzyme solution' and applied on the sample area then a cover slip was applied on top, and the assembly was left on ice for 30 min and then placed at 37^0C for 1h. Following repair enzyme treatment, slides were immersed in ice-cold alkaline denaturation buffer for 1h with three buffer changes at 4^0C to stop the reaction. In all cases, the slides were then placed side by side in a horizontal electrophoresis unit. Alkaline electrophoresis was then performed in the same buffer for 20 min at 0.7 V/cm (25V, 300 mA). To remove excess alkali, the slides were washed three times for 10 min each with neutralization buffer (0.4M Tris-HCl, pH 7.5). They were then dehydrated in 100% ice cold ethanol (10 min) and left to dry in dark to allow storage of slides until analysis. To visualize and analyze the DNA damage, the slides were rehydrated with deionized water (10 min) and stained with 1x SybrGreen. Individual cells, or "comets", were viewed using an Olympus BX40 fluorescent microscope. SSBs were estimated using Tail Moment (TM: the product of tail length and % DNA in tail.). Percent DNA in tail was directly related to amount of stain fluorescent in tail. The comet analysis software package CometScore (Tritek Corp., VA) was used to score approximately 100 comets in each individual experiment and sample. Special emphasis was given to the minimization of light-exposure of

cells during the duration of processing post-irradiation. As shown in Figure 3A, treatment with Fpg or EndoIII results in an increase of TM (above the dotted lines which correspond to SSBs).

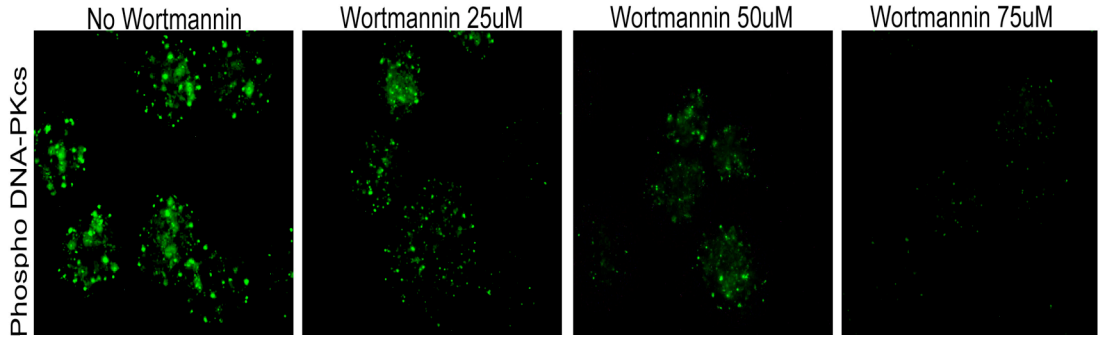

Figure S1. Detection of phosphorylated-(Thr2609) DNA-PKcs foci in wortmannin treated MCF-7 cells and after exposure to 5 Gy of γ-irradiation and repair for 24 hrs. MCF-7 cells were incubated with different wortmannin concentrations (0-75 μM) or only 0.2 % DMSO (control cells) for 24 hours. Drug treated cells or control cells were irradiated with 5 Gy of ©-rays and were incubated for 24 h at 37^0C. Cells were fixed for immunofluorescence assay before irradiation, and after 24 h following irradiation. Both control cells and drug treated irradiated cells are immunostained with rabbit polyclonal antibody against phosphor-(Thr2609) DNA-PKcs antibody (green foci). Results show no persistence of phosphorylated-DNA-PKcs foci in wortmannin treated irradiated cells compared to DMSO treated irradiated cells. The results shown here are representative of two independent experiments. These results suggest that wortmannin inhibits very efficiently the kinase activity of all members of the phosphatidylinositol-3 kinase related kinase (PIKK) family such as ATM, ATR and DNA-PKcs.

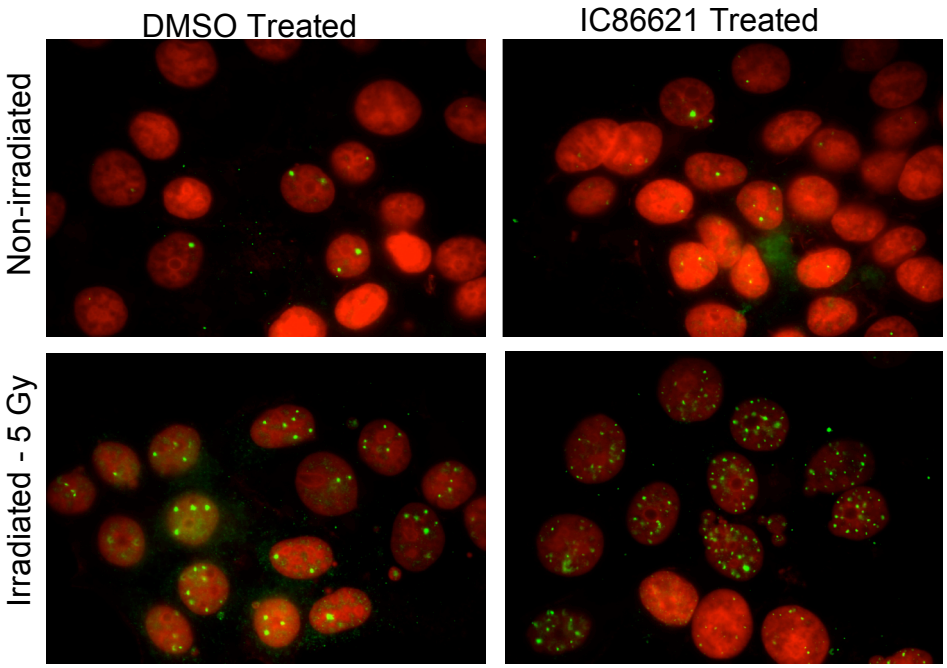

Figure S2. Persistence of phospho-(Thr2609) DNA-PKcs foci in 100 μM IC86621 treated MCF-7 cells after exposure to 5 Gy of γ-irradiation and repair for 24 hrs shown in a cell population. MCF-7 cells were incubated with either 100 μM of IC86621 or only 0.2% DMSO (control cells) for 24 hours. IC86621 treated cells or control cells were irradiated or sham-irradiated with 5 Gy of γ-rays and were incubated for 24 h at 37^0C. Cells were fixed for immunofluorescence assay before irradiation, and after 24 h following irradiation. Both control cells and IC86621treated irradiated cells are immunostained with rabbit polyclonal antibody against phosphor-(Thr2609) DNA-PKcs antibody (green foci). Propidium iodide is used to stain nucleus (red) in all cells. Photographs of the same cells were taken with FITC and propidium iodide filters and later were merged using Adobe Photoshop 7.0 software. Results show persistence of phosphor-DNA-PKcs foci in IC86621 treated irradiated cells compared to DMSO treated irradiated cells. The results

shown here are representative of two independent experiments. These results suggest that IC86621 drug is not efficient in inhibiting the phosphorylation of DNA-PKcs probably from ATM or ATR.

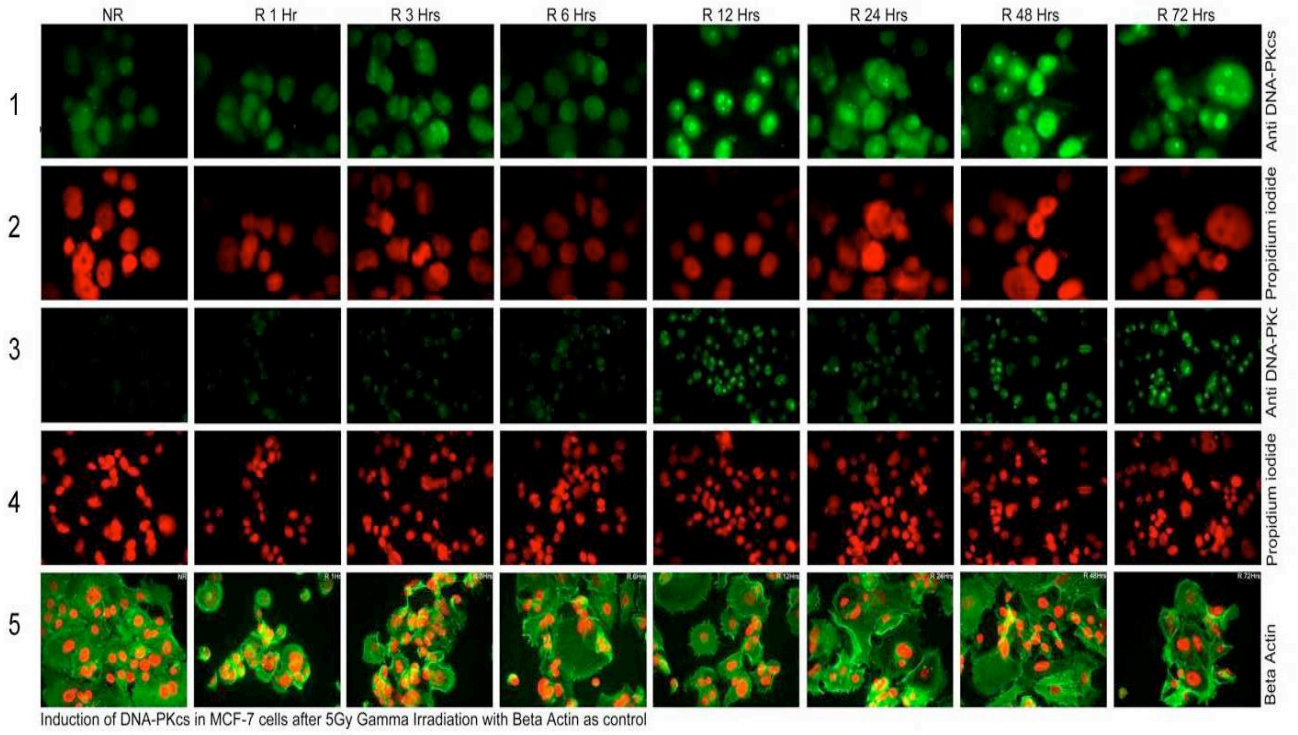

Figure S3. DNA-PKcs induction in IC86621-treated MCF-7 cells after exposure to 5 Gy of γ-rays and repair. Drug-treated MCF-7 cells grown on coverslips were exposed to 5 Gy γ-rays and were placed in 37^0C for repair. Cells were fixed at 1, 3, 6, 12, 24, 48 and 72 h post irradiation. Both sham-irradiated and irradiated MCF-7 cells were immunostained against DNA-PKcs (Rows, 1 and 3) antibody (green foci) or mouse monoclonal against beta actin antibody (Row 5). Propidium iodide is used to stain nucleus (red) in all cells. Photographs of the same cells were taken using FITC and propidium iodide filters. Row 1, FITC pictures taken at 100X; Row 2, same cells as in row 1, propidium iodide stained nuclei.; Row 3, FITC pictures taken at 20X; Row 4, same cells as in row 3, propidium iodide stained nuclei; Row 5, immunostained for beta actin (green) and nuclear stain with propidium iodide (red). Photographs of the same cells were taken using FITC and propidium iodide filters and, later were merged using Adobe Photoshop 7.0 software.

References

[1] Peddi, P.; Francisco, D. C.; Cecil, A.; Hair, J. M.; Panayiotidis, M. I.; Georgakilas, A. G. Deficient processing of clustered DNA damage in human breast cancer cells MCF-7 with silenced DNA-PKcs expression. *Cancer Lett.* 269:174-183; 2008.

[2] Sarkaria, J. N.; Tibbetts, R. S.; Busby, E. C.; Kennedy, A. P.; Hill, D. E.; Abraham, R. T. Inhibition of Phosphoinositide 3-Kinase Related Kinases by the Radiosensitizing Agent Wortmannin. *Cancer Res* 58:4375-4382; 1998.

[3] Allen, C.; Halbrook, J.; Nickoloff, J. A. Interactive competition between homologous recombination and non-homologous end joining. *Mol Cancer Res* 1:913-920; 2003.

[4] Kashishian, A.; Douangpanya, H.; Clark, D.; Schlachter, S. T.; Eary, C. T.; Schiro, J. G.; Huang, H.; Burgess, L. E.; Kesicki, E. A.; Halbrook, J. DNA-dependent protein kinase inhibitors as drug candidates for the treatment of cancer. *Mol Cancer Ther* 2:1257-1264; 2003.

[5] Pouget, J. P.; Frelon, S.; Ravanat, J. L.; Testard, I.; Odin, F.; Cadet, J. Formation of modified DNA bases in cells exposed either to gamma radiation or to high-LET particles. *Radiat. Res.* 157:589-595; 2002.

[6] Douki, T.; Ravanat, J. L.; Pouget, J. P.; Testard, I.; Cadet, J. Minor contribution of direct ionization to DNA base damage inducedby heavy ions. *Int. J. Radiat. Biol.* 82:119-127; 2006.