SUPPLEMENTARY MATERIAL

Supplementary Methods

Primer sequences used:

Human PNKP in pGEX-6P-1:

5'-BamH1: CGGGATCCATGGGCGAGGTGGAGC

3'-XhoI: CCGCTCGAGTCAGCCCTCGGAGAACTG.

S114A: GCACACCAGAAGCCCAGCCAGATAC

S126A: GCACCCCTCTGGTGGCCCAAGATGAGAAG

A114D: GCACACCAGAAGACCAGCCAGATACTCCG

A126D: GCACCCCTCTGGTGGACCAAGATGAGAAGTCCG

Supplementary Figure Legends

Supplementary Figure 1: Stoichiometry of phosphorylation of PNKP *in vitro.* *(*A). One µg purified PNKP was phosphorylated *in vitro* by purified DNA-PK as described in the main text using ^{32}P-labelled γ-ATP. Samples were removed at the times indicated and analysed by SDS PAGE. The gel was stained with Coomassie blue and exposed to X-ray film. Top panel: Coommassie stained gel showing region corresponding to PNKP. Position of molecular weight markers (in kDa) is shown on the left hand side. Bottom panel: Autoradiogram of corresponding region of the gel. (B). Bands corresponding to radioactively labeled PNKP were excised from the gel and counted in a scintillation counter by Cerenkov radiation. Phosphorylation was plotted as ^{32}P incorporation with time. The stoichiometry of phosphorylation was calculated by comparison to known

amounts of γ-ATP. The stoichiometry of phosphorylation was calculated as 0.34 and 0.35 pmole of phosphate per pmole of PNKP in two separate experiments.

Supplementary Figure 2: Level of PNKP expression in human cells transiently transfected with PNKP. HeLa cells were transiently transfected with pcDNA-PNKP-V5 (HeLa-T) as described in Materials and Methods. Whole cell extracts from detergent lysed cells (NP-40 extraction, see main text for details) were generated from transfected HeLa cells (lane 1), non-transfected HeLa cells (lane 2), human lymphoblastoid cells BT/C3ABR (lane 3), or human breast cancer cells MCF7 (lane 4). Fifty µg of total protein was run on SDS PAGE and immunoblotted with an antibody to total PNKP. The positions of PNKP-V5 and endogenous PNKP are indicated. Blots were probed with an antibody to Ku80 as loading control.

Supplementary Figure 3: Identification of *in vivo* phosphorylation sites by mass spectrometry. PNKP was immunoprecipitated from HeLa cells that had been transiently transfected with V5-tagged PNKP as in Supplementary Figure 2. Phosphopeptides were analysed by mass spectrometry as described in Materials and Methods. Panels A and B show example spectra for phosphopeptides containing S114 or S126, respectively. The unambiguously identified amino acid sequence is shown at the top of each figure. The asterisk indicates the identified phosphoserine in each peptide.

Supplementary Figure 4: Characterization of phosphospecific antibodies to S114 and S126 of PNKP. (A) Purified wt-PNKP or PNKP containing serine to alanine mutations at S114 (S114A), S126 (S126A) or both together (S114-S126A) was incubated with purified DNA-PK either in the absence (-) or presence (+) of ATP as indicated. Reactions were run on SDS PAGE and immunoblots were probed with phosphospecific antibodies to S114 (Ab: pSer-114) or S126 (Ab: pSer-126) as indicated. The lower panel shows total PNKP protein. (B) ShRNA stable knock down cells stably expressing vector (vec), or shRNA resistant PNKP-WT (WT), PNKP-114A-126A (2A) or PNKP-114D-126D (2D) were unirradiated or irradiated 10 Gy. After 1 hour, PNKP was immunoprecipitated and run on SDS PAGE. Blots were probed with phosphospecific antibodies to S114 or S126 or total PNKP as indicated.

Supplementary Figure 5: Identification of appropriate doses of the ATM inhibitor KU55933 and the DNA-PK inhibitor NU7441 in combination with IR. HeLa cells were pre-treated with 0, 2, 5, 10, or 15 μM of KU55933 or NU7441 for 60 min prior to irradiation with 10 Gy. Cells were harvested after 60 min and whole cell extracts were resolved by SDS PAGE as described above. Westerns were immunoblotted for various ATM and DNA-PKcs target phosphorylation sites as indicated.

Supplementary Figure 6: Characterization of cell lines stably expressing shRNA resistant PNKP. (A) A549 cells stably expressing an shRNA to PNKP (clone 13) were transfected with vector alone (v), or vector expressing shRNA resistant forms of PNKP-WT (WT), PNKP-114A-126A (2A) or PNKP-114D-126D (2D) and stable cell lines

generated. Whole cell NETN extracts were made and 50 µg total protein was analysed by SDS PAGE and western blot with an antibody to PNKP. The asterisk (*) indicates a non-specific band that shows equal loading.

(B) Cells expressing shRNA resistant wild type PNKP (wt), empty vector, or PNKP bearing alanine or aspartic acid mutations at serines 114 and 126 (2A and 2D respectively) were generated in PNKP shRNA knock down cells as described above. Cells were analysed for PNKP expression by immunofluorescence essentially as described previously (1) except that a FITC-conjugated antibody to HA (Roche, 2 µg/mL) was used.

Supplementary Figure 7: PNKP does not co-immunoprecipitate with ATM or DNA-PKcs. C13 PNKP knock down cells stably re-expressing vector (vec, lanes 2 and 3), shRNA resistant wt HA-PNK (lanes 4 and 5), HA-PNK-114A-126A (2A, lanes 6 and 7), or HA-PNK-114D-126D (2D, lanes 8 and 9) were unirradiated (-) or irradiated 10 Gy (+) and harvested after 60 mins. Extracts were treated with benzonase (10 units/mg protein) to reduce protein-nucleic acid interactions, and HA-PNKP was immunoprecipitated and resolved by SDS PAGE as described above. Blots were probed for HA (for PNKP), XRCC4, DNA Ligase IV, DNA-PKcs Ku80, and ATM as indicated. Lane 1 contained 50 µg whole cell extract from untransfected HeLa cells. XRCC4 and DNA ligase IV bands were quantitated using a Fuji imager, normalized to immunoprecipitated PNKP and expressed as fold induction after IR. Fold induction was 3.1, 2.7 and 1.8 for wt, 2A and 2D mutants, respectively. The lower 3 panels show 50

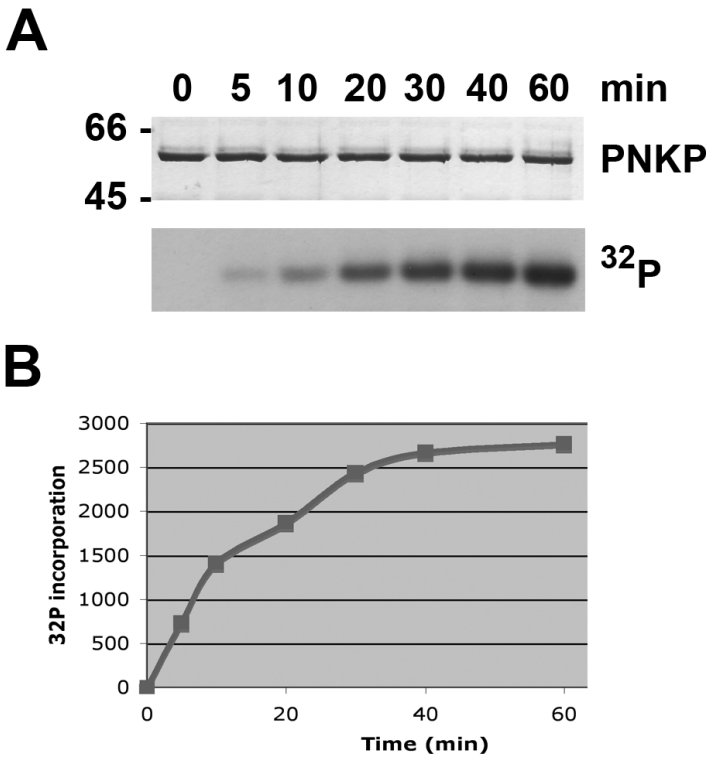
μg whole cell extract/input for each protein. Results are representative of four separate experiments.

Supplementary Reference:

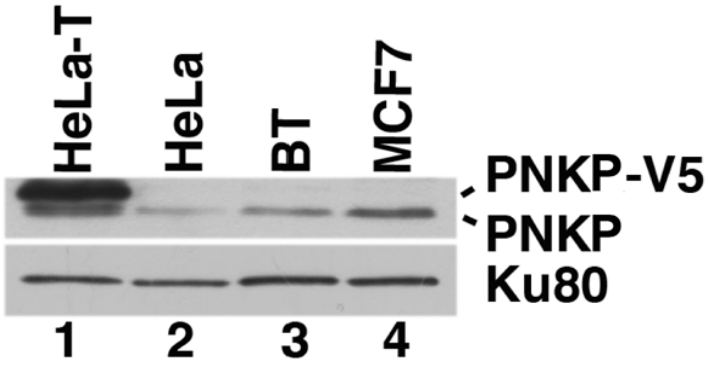
1. Douglas, P., Zhong, J., Ye, R., Moorhead, G.B., Xu, X. and Lees-Miller, S.P. (2010) Protein phosphatase 6 interacts with the DNA-dependent protein kinase catalytic subunit and dephosphorylates gamma-H2AX. *Mol Cell Biol*, **30**, 1368-1381.

Supplementary Figures

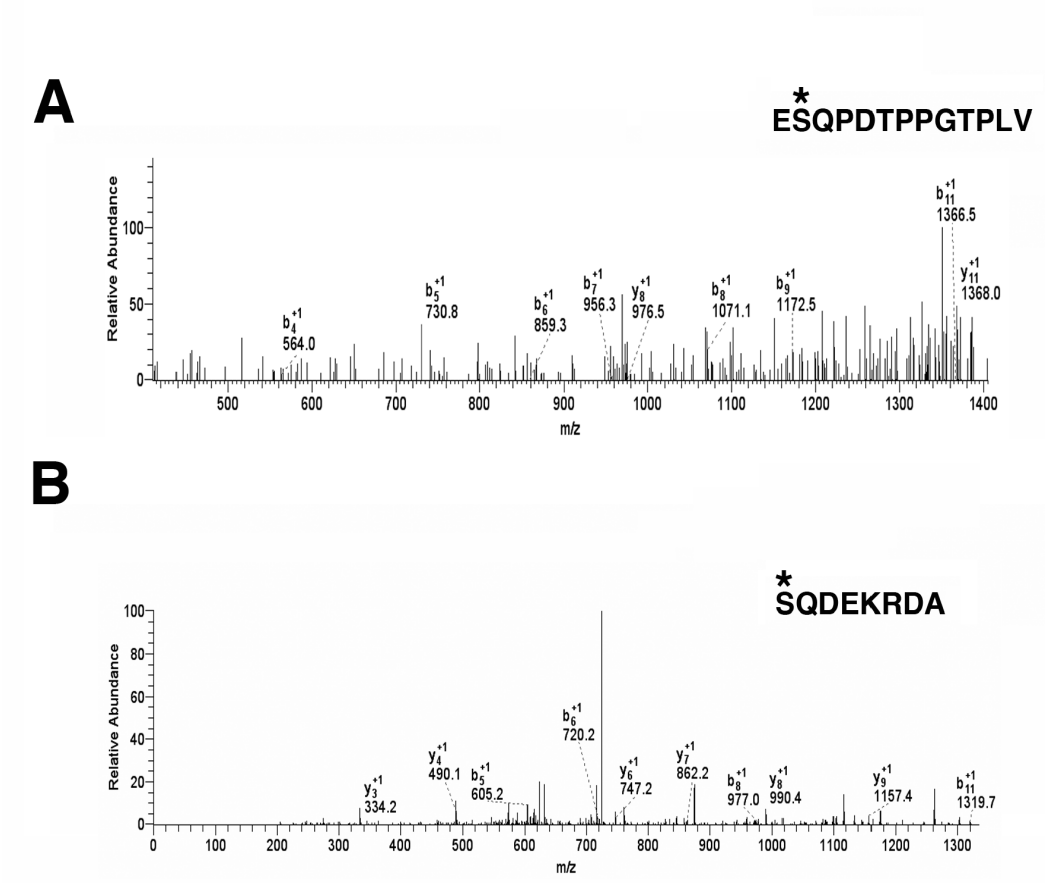
Supplementary Figure 1



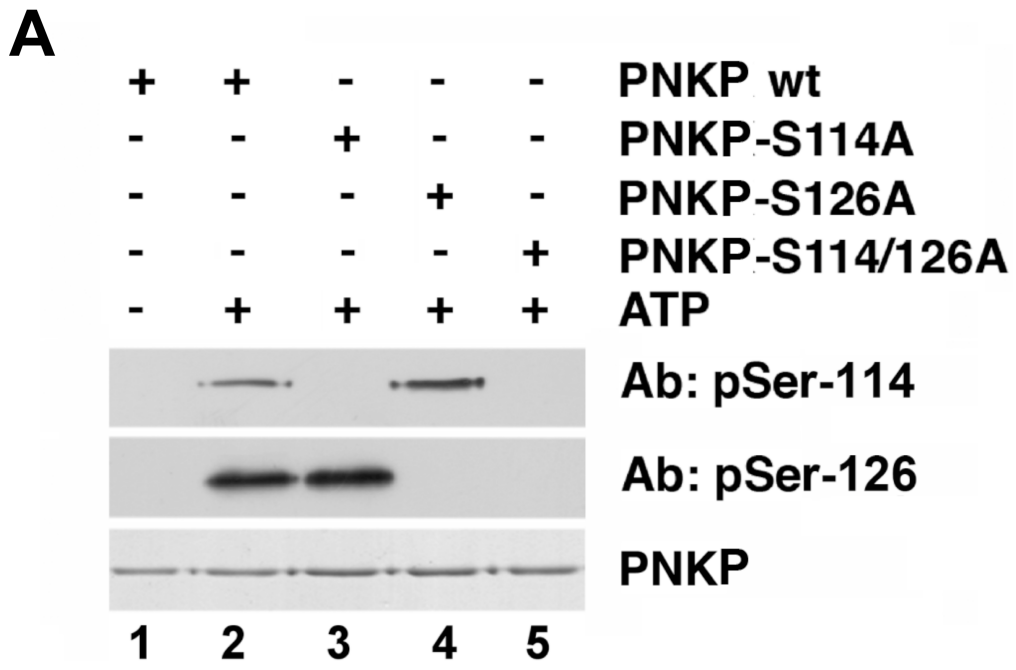
Supplementary Figure 2

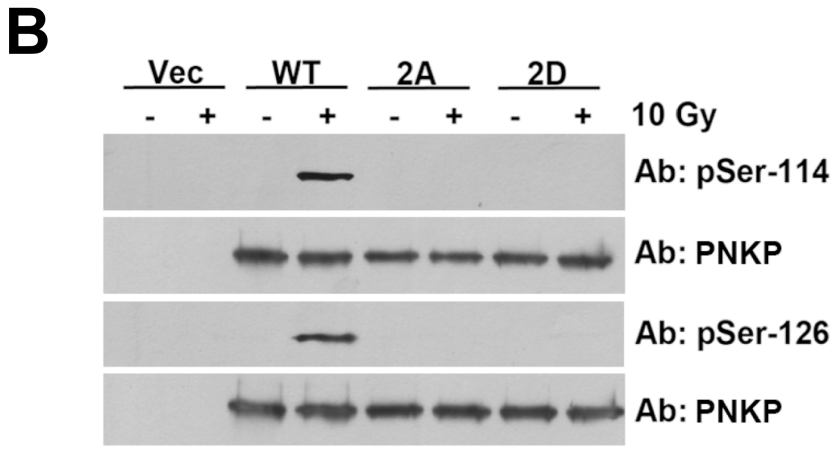


Supplementary Figure 3

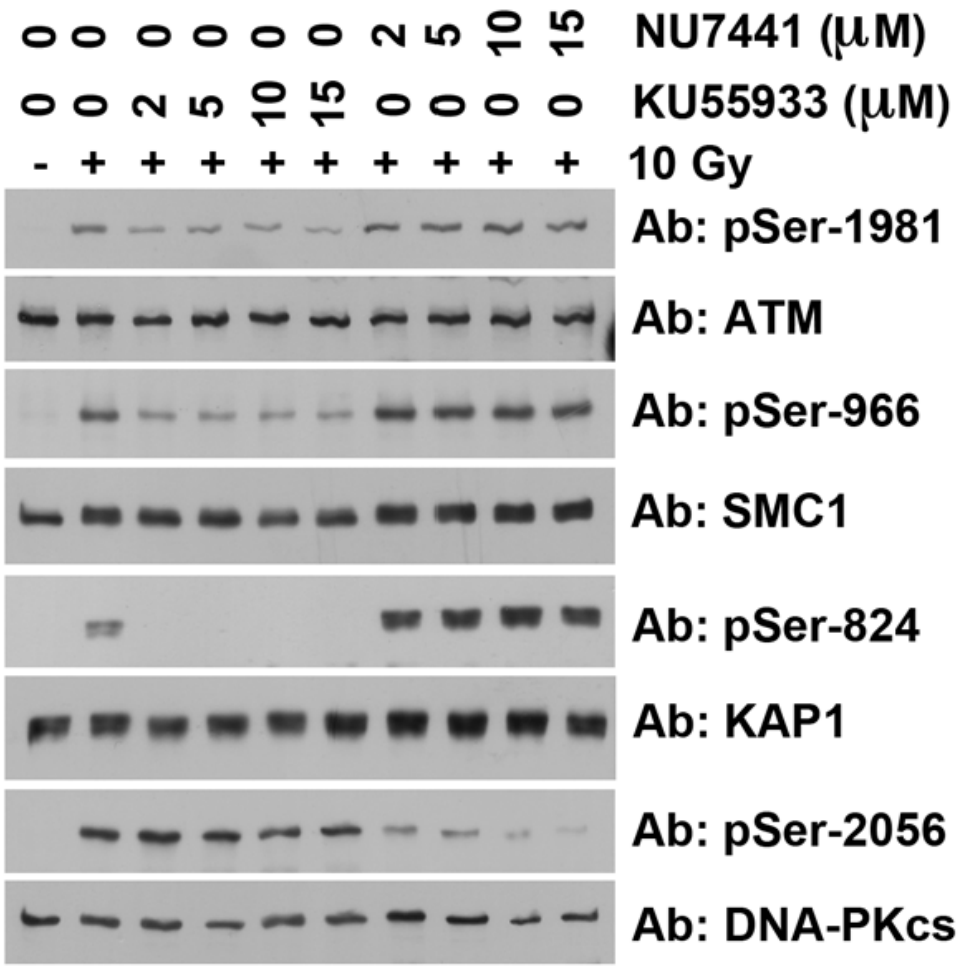


Supplementary Figure 4

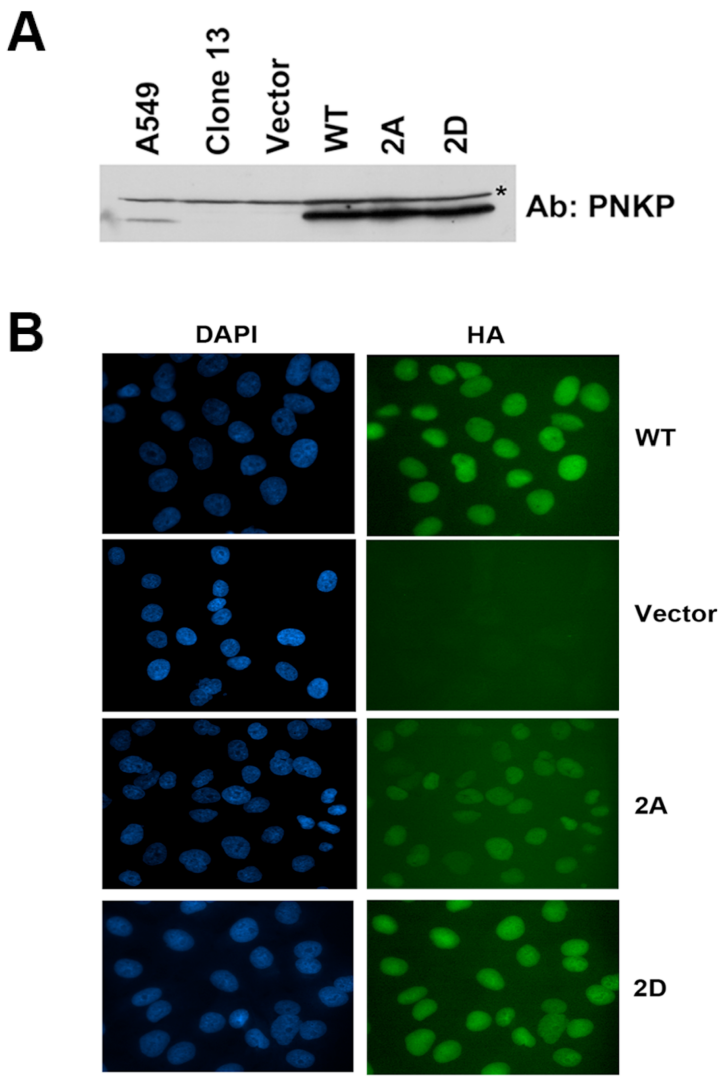




Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7