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Supplementary Information

ATM-mediated phosphorylation of polynucleotide kinase/phosphatase (PNKP) is required for effective DNA double-strand break repair

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Fig. S1

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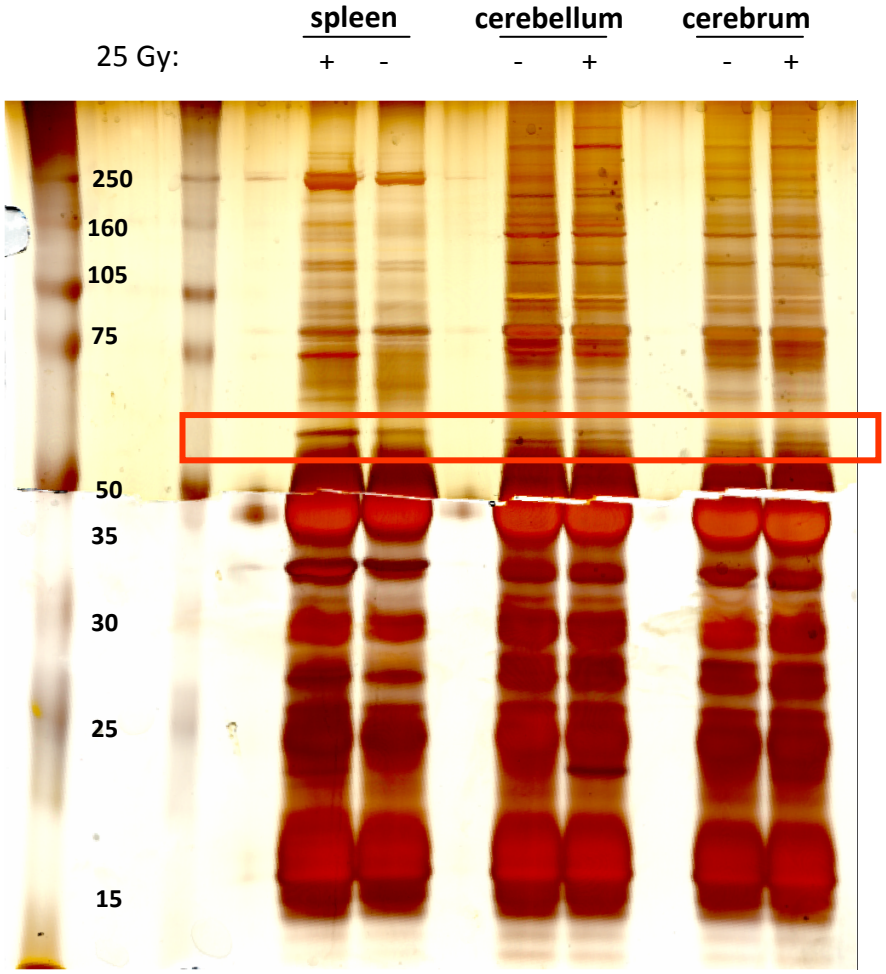
A



B

(I)

Mouse: ELSTSGSQPDAPPD

Antibody: VQKSMGSQEDDSGN

(II)

Mouse: ELSTSGSQPDAPPD

Human: ETRTPESQPDTPPG

Fig. S2

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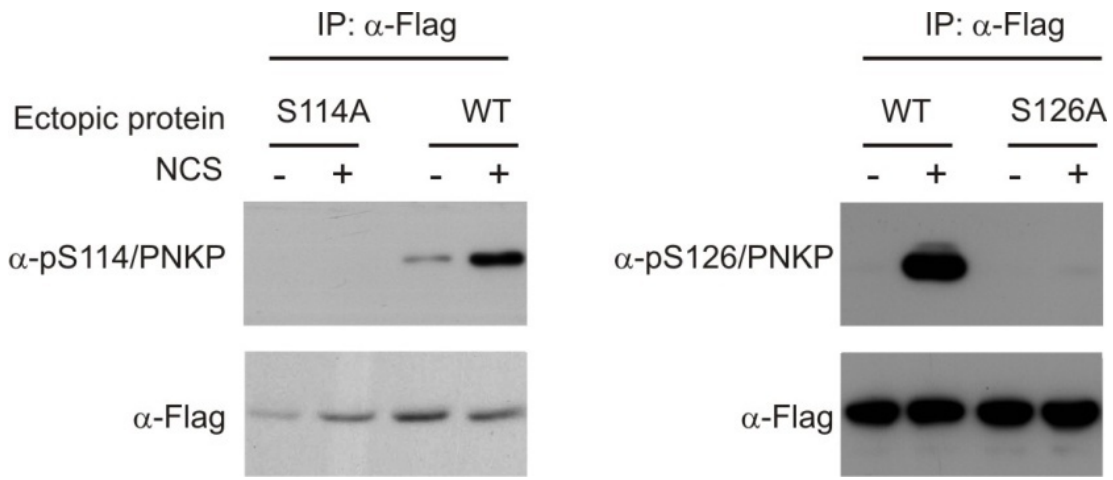

Fig. S3

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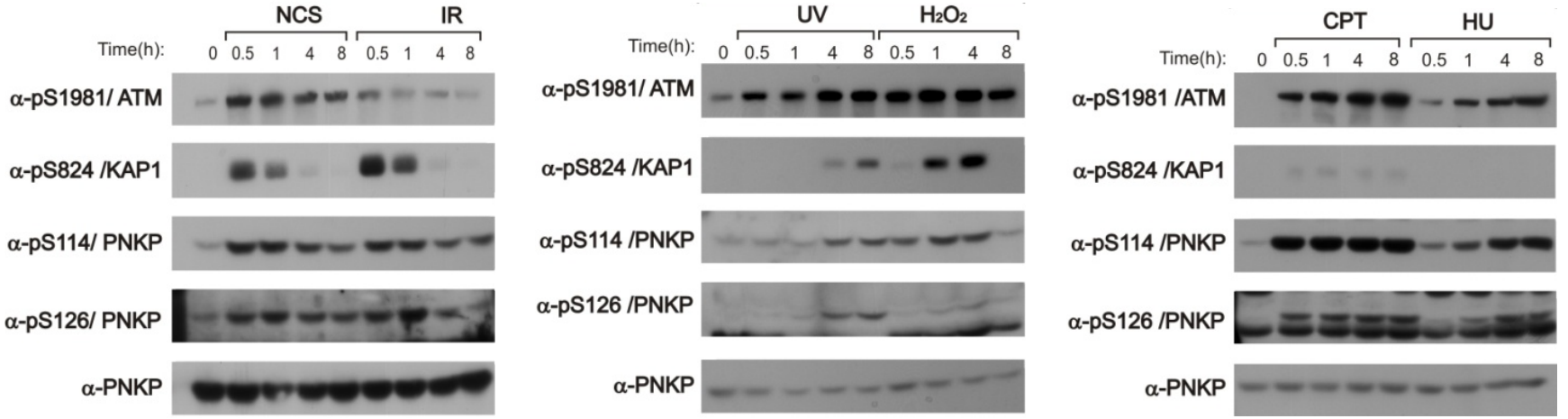

Fig. S4

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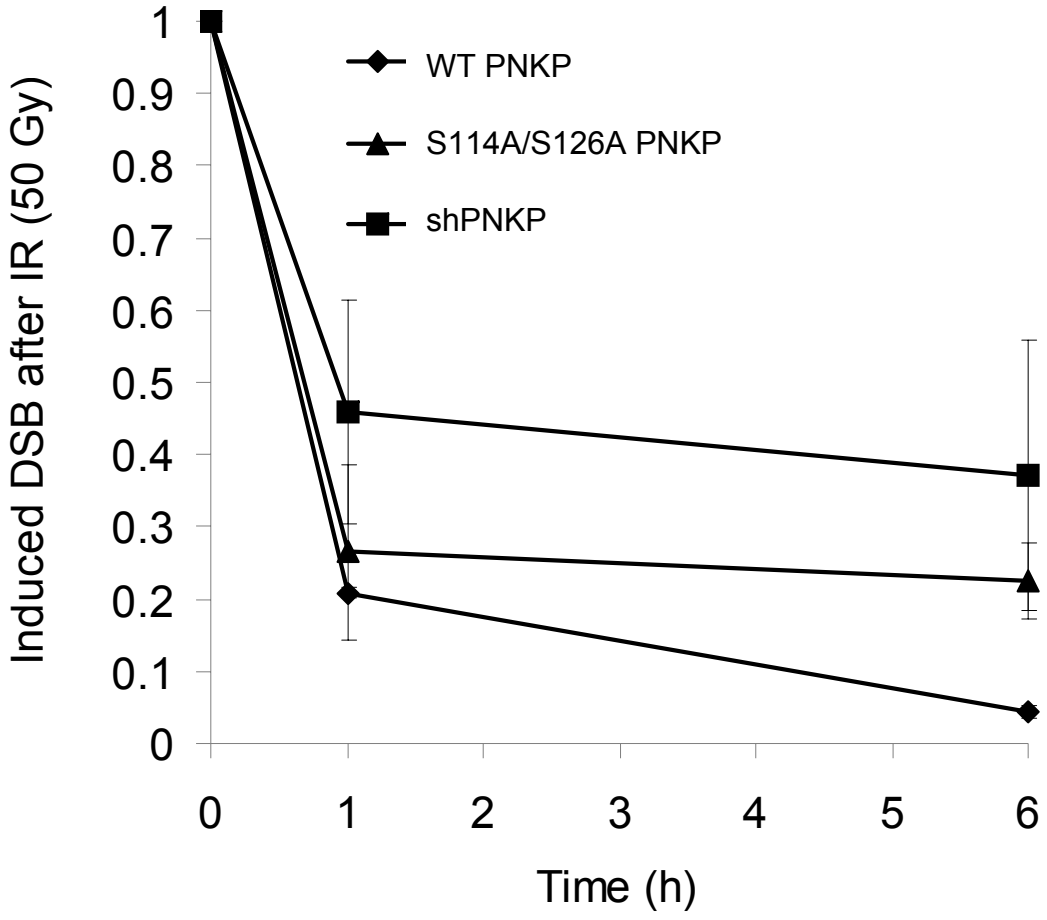

Fig. S5

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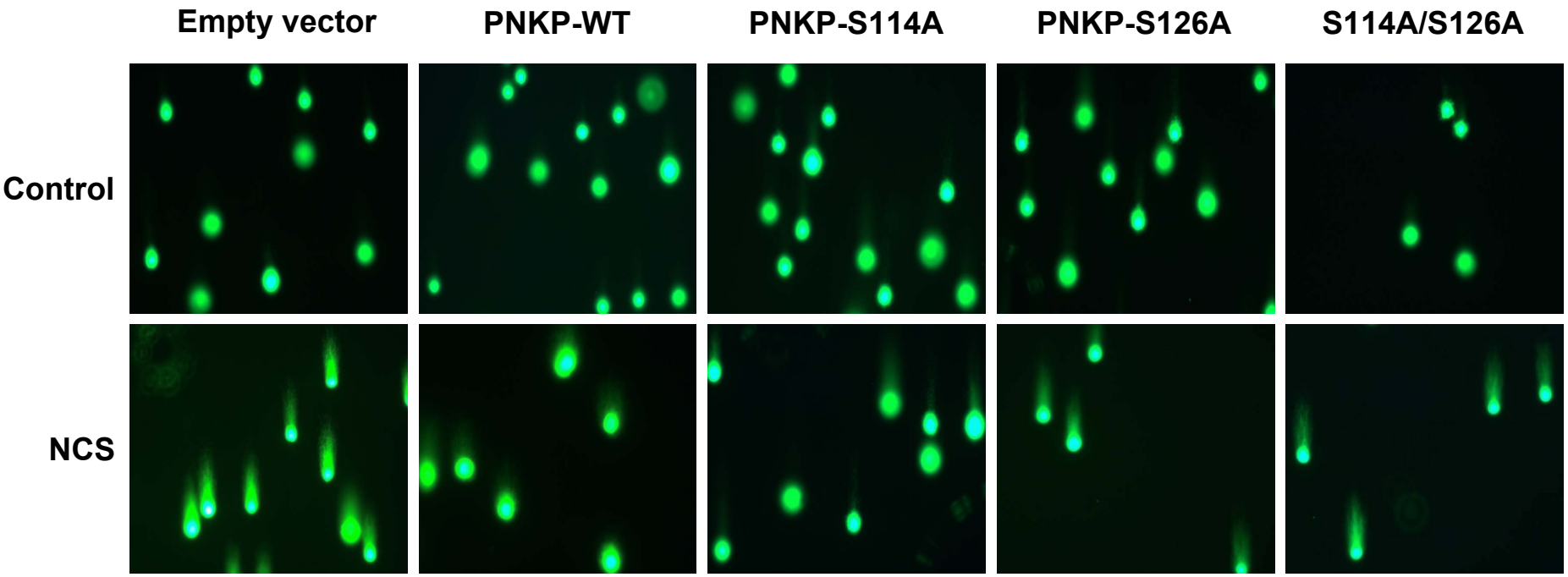

Fig. S6

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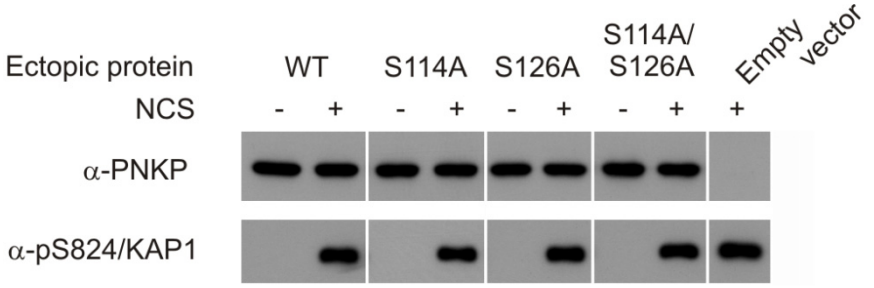
A



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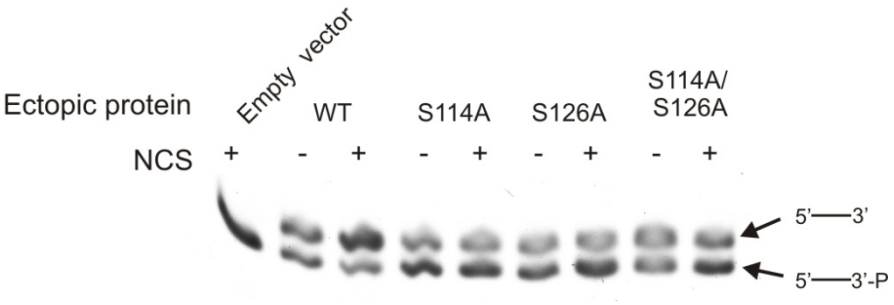

C

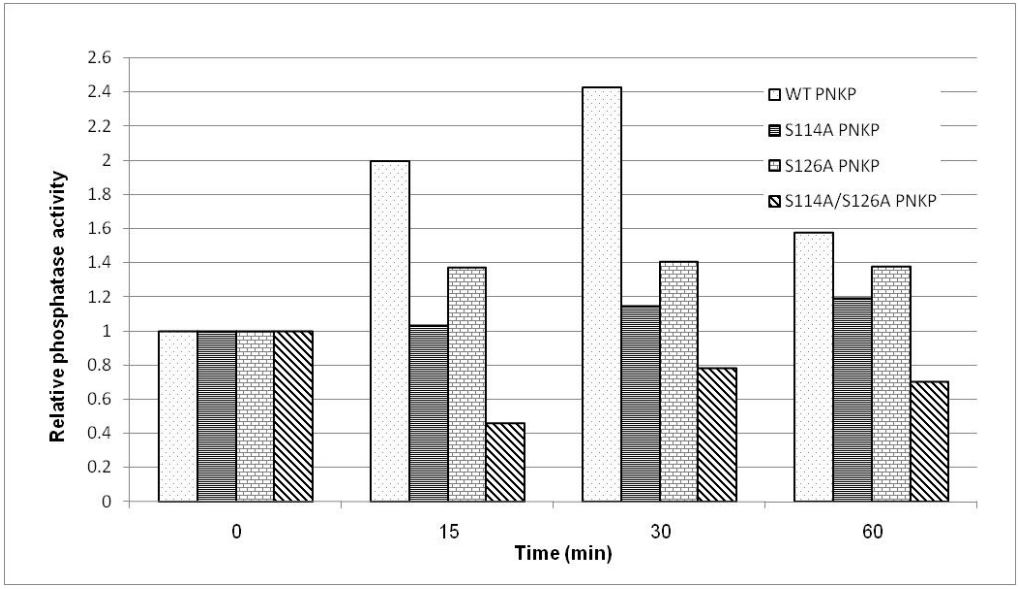

Fig. S7

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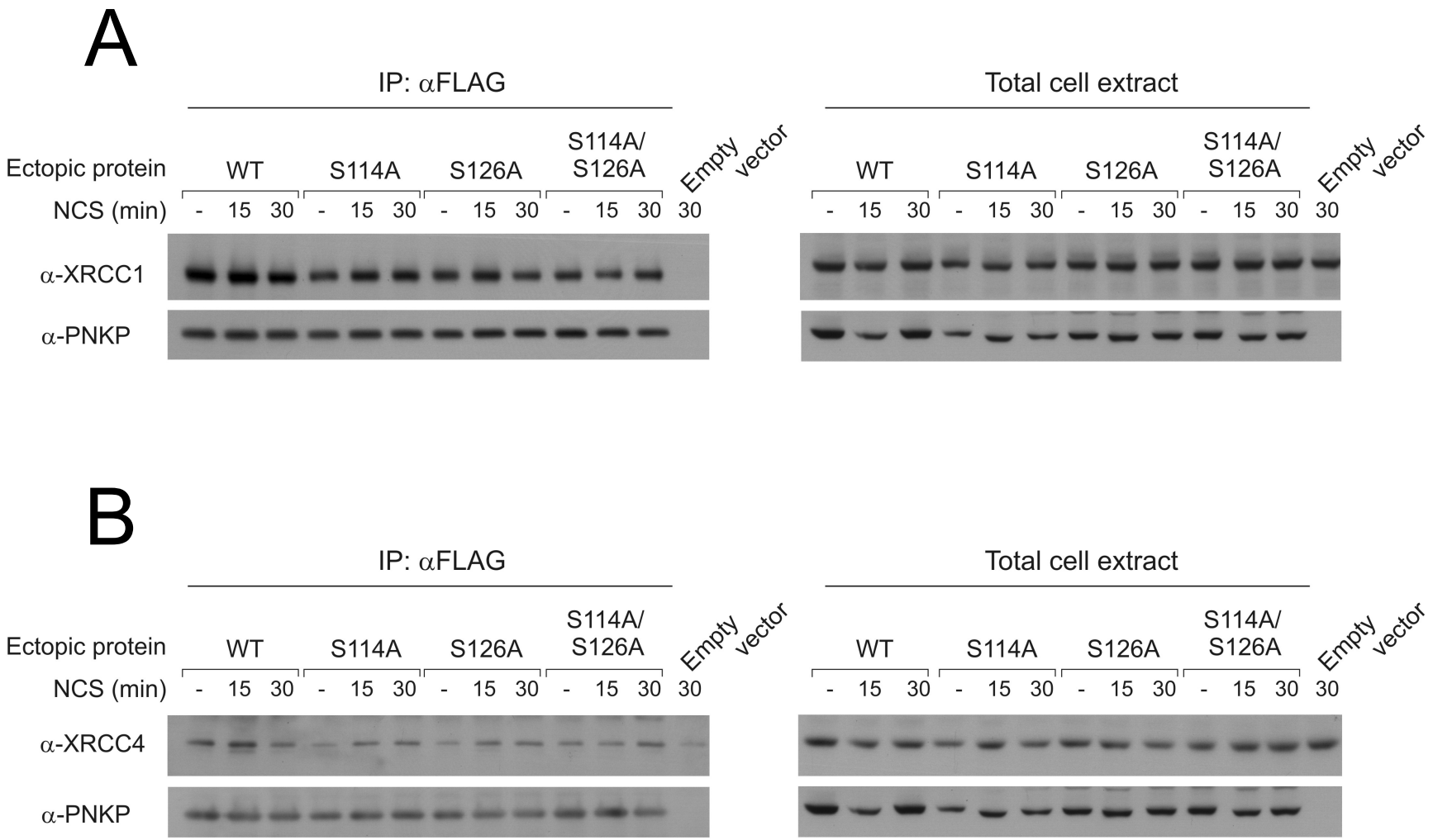

Fig. S8

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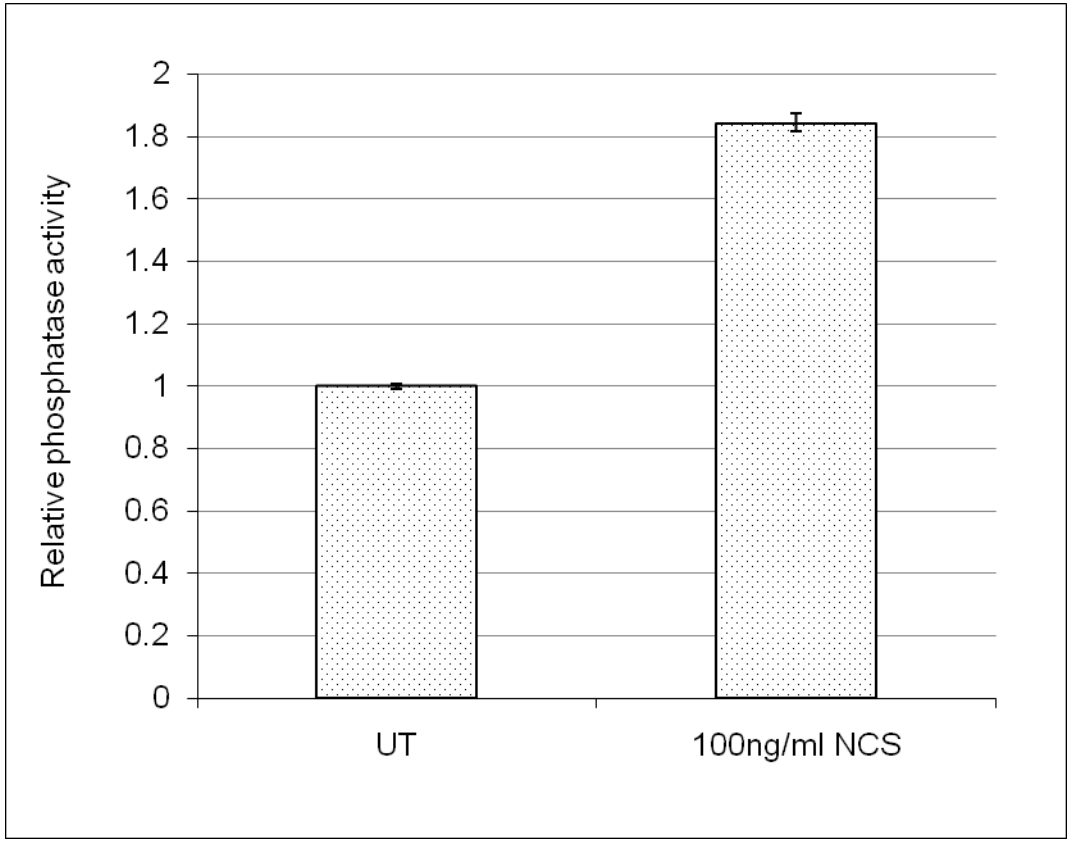

Supplementary figure legends

Fig. S1. A. Identification of mPNKP as a putative PIKK substrate. An antibody against a phosphorylated peptide (GS^pQE) was used to immunoprecipitate phosphorylated proteins from mouse tissues 1 hr after whole body irradiation with 25 Gy of IR. The proteins were separated using PAGE and bands that appeared following the treatment were identified using mass spectrometry. The band that appeared within the area marked by the red rectangle was identified as mPNKP. B-I. Alignment of the sequence of the peptide used as an immunogen to generate the antibody and the corresponding sequence in mPNKP flanking S114. B-II. Alignment of mPNKP and hPNKP sequences in the region flanking S114.

Fig. S2. Specificity of anti-phospho antibodies. HEK293 cells expressing ectopic FLAG-tagged wild-type or phospho-mutant hPNKP (S114A or S126A) were treated with 0.5 μg/ml of NCS for 30 min. PNKP was immunoprecipitated using anti-FLAG antibody and the immune complexes were blotted with the indicated antibodies.

Fig. S3. Kinetics of hPNKP phosphorylation in response to various genotoxic agents. U2OS cells were treated with NCS (200 ng/ml), IR (5 Gy), H_2O_2 (100 μM), UV radiation ($25J/m^2$), CPT (10 μM), and HU (10 mM), and harvested at the indicated time points. Cellular extracts were subjected to western blotting analysis with the indicated antibodies.

Fig. S4. Time course of DSB rejoining measured using PFGE in A549 cells depleted for hPNKP and in the same cells expressing ectopic wild-type or phospho-mutant versions (S114A/S126A) of the protein. In this method, cells are lysed in soft agarose in order to minimize DNA breakage resulting from experimental procedures. The cellular DNA is subsequently electrophoresed in agarose in alternating electric fields – a method that allows separation of large DNA fragments at a resolution that is not provided by standard gel electrophoresis. When neutral gels are used, DSBs in the DNA create DNA smears. The amount of DNA in these smears is quantified and used to assess the extent of DNA breakage. Cells were irradiated with 50 Gy of γ-irradiation. The results are expressed in percentage of the initial damage at "time 0".

Fig. S5. Direct observation of DNA damage in A549 cells with different versions of PNKP 2 hr after treatment with 200 ng/ml of NCS, using the neutral comet assay. Representative microscopic images of the assay are shown.

Fig. S6. Assay of hPNKP phosphatase activity following DNA damage induction. A. hPNKP levels in cells in which endogenous hPNKP was replaced by wild-type or phospho-mutant versions of ectopic PNKP. B. Representative results demonstrating the assay system. The cells were treated with 0.5 μg/ml of NCS for 15 min and phosphatase activity of hPNKP was assayed by incubating the substrate shown in Fig. 5A with 5 μg of cellular extract (see Methods for further details). The band with the slower migration pattern represents the dephosphorylated oligonucleotide. Note the DNA damage-induced enhancement of wild-type hPNKP activity, which is not observed in the mutant versions.

C. Bar diagram showing the relative activity of wild-type and phospho-mutant versions of hPNKP at various time points following NCS treatment (single experiment).

Fig. S7. Interaction of wild-type and phospho-mutant versions of ectopic hPNKP with endogenous XRCC1 and XRCC4 following DNA damage induction. Ectopic hPNKP was expressed in A549 cells in which endogenous PNKP had been knocked down, and the cells were treated with 0.5 μg/ml of NCS. hPNKP was immunoprecipitated at the indicated time points using an anti-FLAG antibody, and the immune complexes were blotted with the indicated antibodies. The protein amounts in the input gels represent 10% of the amount used for immunoprecipitation.

Fig. S8. dsRed-tagged hPNKP is active and its activity is enhanced by the induction of DNA damage. Phosphatase activity was assayed following treatment with 100ng/ml of NCS for 30 min.

Supplementary Methods

Cell lines

HEK293, U2OS, HeLa and A549 cells were grown in DMEM with 10% fetal bovine serum. Lymphoblastoid cells were grown in RPMI with 10% fetal bovine serum at 37°C in 5% CO_2 atmosphere.

Chemicals and Antibodies

Neocarzinostatin (NCS) was obtained from Kayaku Chemicals (Tokyo, Japan). The ATM inhibitor KU-55933 and the DNA-PK inhibitor NU-7441 (KU-57788) were obtained from KuDOS Pharmaceuticals Ltd., UK. Hydroxyurea. CPT and H_2O_2 were obtained from Sigma-Aldrich (St. Louis, MO). The monoclonal anti-53BP1 antibody was a generous gift from T. Halazonetis (Geneva University, Switzerland). Other antibodies were polyclonal anti-PNKP (A300-258A; Bethyl Laboratories, Inc., Montgomery, TX); polyclonal anti-XRCC1 (A300-065A, Bethyl Laboratories, Inc.); polyclonal anti-XRCC4 (sc-5606, Santa Cruz, CA); anti-FLAG (M5; Sigma-Aldrich, St. Louis, MO); monoclonal anti-HSC70 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-pS957-SMC1 polyclonal antibody (Novus Biologicals, Inc., Littleton, CO). The specific phospho-antibodies against pS824 of KAP-1 (A300-767A), pS114 of PNK (BL3846), and pS126 of PNK (BL4693) were produced by Bethyl Laboratories, Inc. (Montgomery, TX); the anti-ATM mouse monoclonal antibody MAT3 was produced in our laboratory in collaboration with N. Smorodinsky. Secondary antibodies: anti-mouse and anti-rabbit IgG-Alexa 488 were purchased from Molecular Probes (Leiden, Netherlands), and HRP-conjugated anti-rabbit IgG or anti-mouse IgG were obtained from Jackson

Immunoresearch Laboratories, Inc. (West Grove, PA).

Mass spectrometry

Preparation of protein bands excised from gels for mass spectrometric analysis was carried out as previously described (Moyal et al, 2011).

Pulsed-field gel electrophoresis

Monitoring of DSB repair using pulsed-field gel electrophoresis was carried out as previously described (Lundin et al, 2005) with the following modifications: 5 x 10^5 cells were seeded per flask (25 cm^2) 48 hours prior to treatment and the DNA was homogenously labeled for 24 hours before irradiation with γ-rays (50 Gy, 7.1 Gy/min) on ice (in HBSS). Treatment was followed by incubation in pre-warmed DMEM at 37°C and 5% CO_2. The cells were harvested at the indicated time points and 5 x 10^5 cells were cast into each soft agarose insert, which was subsequently incubated in Proteinase K at room temperature for 48 hr. Electrophoresis was performed for 20 hr. For detection and quantification of [^{14}C]-thymidine, the gels were vacuum dryed (Model 583 gel dryer, Bio-Rad) for 1 hr after which they were left to expose in a phosphoimager plate for 6 days.

Supplementary References

Lundin C, North M, Erixon K, Walters K, Jenssen D, Goldman AS, Helleday T (2005) Methyl methanesulfonate (MMS) produces heat-labile DNA damage but no detectable in vivo DNA double-strand breaks. *Nucleic Acids Res* **33:** 3799-3811

Moyal L, Lerenthal Y, Gana-Weisz M, Mass G, So S, Wang SY, Eppink B, Chung YM, Shalev G, Shema E, Shkedy D, Smorodinsky NI, van Vliet N, Kuster B, Mann M, Ciechanover A, Dahm-Daphi J, Kanaar R, Hu MC, Chen DJ, Oren M, Shiloh Y (2011) Requirement of ATM-dependent monoubiquitylation of histone H2B for timely repair of DNA double-strand breaks. *Mol Cell* **41:** 529-542