Cottarel et al., http://www.jcb.org/cgi/content/full/jcb.201203128/DC1



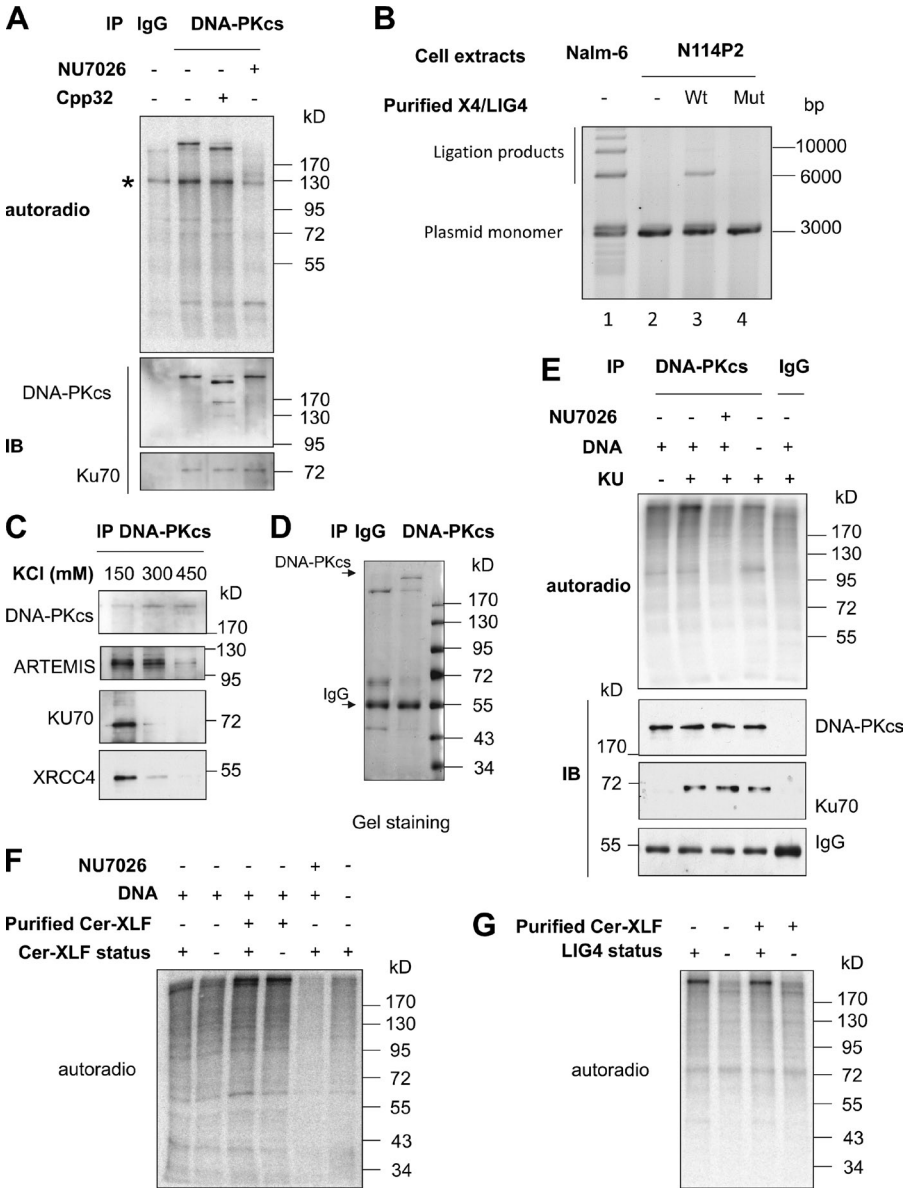


Figure S1. **Set up and test of DNA-PKcs autophosphorylation in vitro with cell extracts or purified proteins.** (A) Validation of DNA-PKcs immunoprecipitation. After incubation of Nalm6 cell extracts, DNA and γ-[$^{32}$P]ATP under standard conditions, or with NU7026 as indicated, samples were mixed with control IgG or anti–DNA-PKcs magnetic beads. Immunoprecipitation proceeded for 4 h at 4°C on a wheel followed by washing in PBS and 0.1% Triton X-100. Where indicated, purified Cpp32 (active human caspase 3; EMD Millipore) was added or not added as indicated followed by incubation in caspase 3 buffer according to the manufacturer at 37°C for 60 min, denaturation, and separation on 8% SDS-PAGE gel. The protein profile was obtained after digestion with caspase 3 was revealed with a mixture of three anti–DNA-PKcs antibodies (Abcam) and showed the expected bands (p240, p150, and p120) as previously published (Song et al., 1996). The asterisk indicates a protein coimmunoprecipitated with DNA-PKcs. (B) Complementation of NHEJ defect in vitro with purified components. End-joining assay catalyzed under standard reaction conditions with extracts from LIG4-proficient (Nalm6) or -deficient (N114P2) cells in the presence or absence of wild-type or catalytically dead X4LIG4 purified complex. DNA ligation products were separated by agarose gel electrophoresis followed by SYBR green staining. (C–E) Purification of DNA-PKcs on beads. (C) DNA-PKcs was immunoprecipitated from Nalm6 extracts followed by three washes for 10 min under rotation at 4°C in washing buffer (50 mM Hepes, pH 7.5, and 0.05% Tween 20) supplemented with KCl to the indicated final concentration. (D). Control or anti–DNA-PKcs beads obtained as in C and washed with 450 mM KCl were denatured. Protein samples were loaded on 8% SDS-PAGE gel and stained with InstantBlue. (E) Control or anti–DNA-PKcs beads obtained as in C and washed with 450 mM KCl were incubated for 15 min at 30°C under standard DNA-PKcs activation conditions with the indicated components and γ-[$^{32}$P]ATP followed by three washes in PBS and 0.1% Triton X-100, denaturation, loading on 8% SDS-PAGE, electrotransfer on membrane, and Western blotting with antibodies as indicated. (F and G) Effect of Cer-XLF on DNA-PKcs autophosphorylation in cell extracts. (F) BuC and BuS extracts (Cer-XLF status + or −, respectively) were incubated under standard DNA-PKcs activation conditions with γ-[$^{32}$P]ATP and added components as specified. Incubation was followed by immunoprecipitation with control IgG2a or anti–DNA-PKcs antibodies. Reaction samples were heat denatured, separated on SDS-PAGE gels, and electrotransferred onto membranes. (G) Nalm6 control and N114P2 *LIG4*-null extracts were incubated under standard DNA-PKcs activation conditions with γ-[$^{32}$P]ATP and added components as specified. Then, reaction samples were processed as in F. autoradio, autoradiography; IB, immunoblot; IP, immunoprecipitation; Mut, mutant; Wt, wild type.

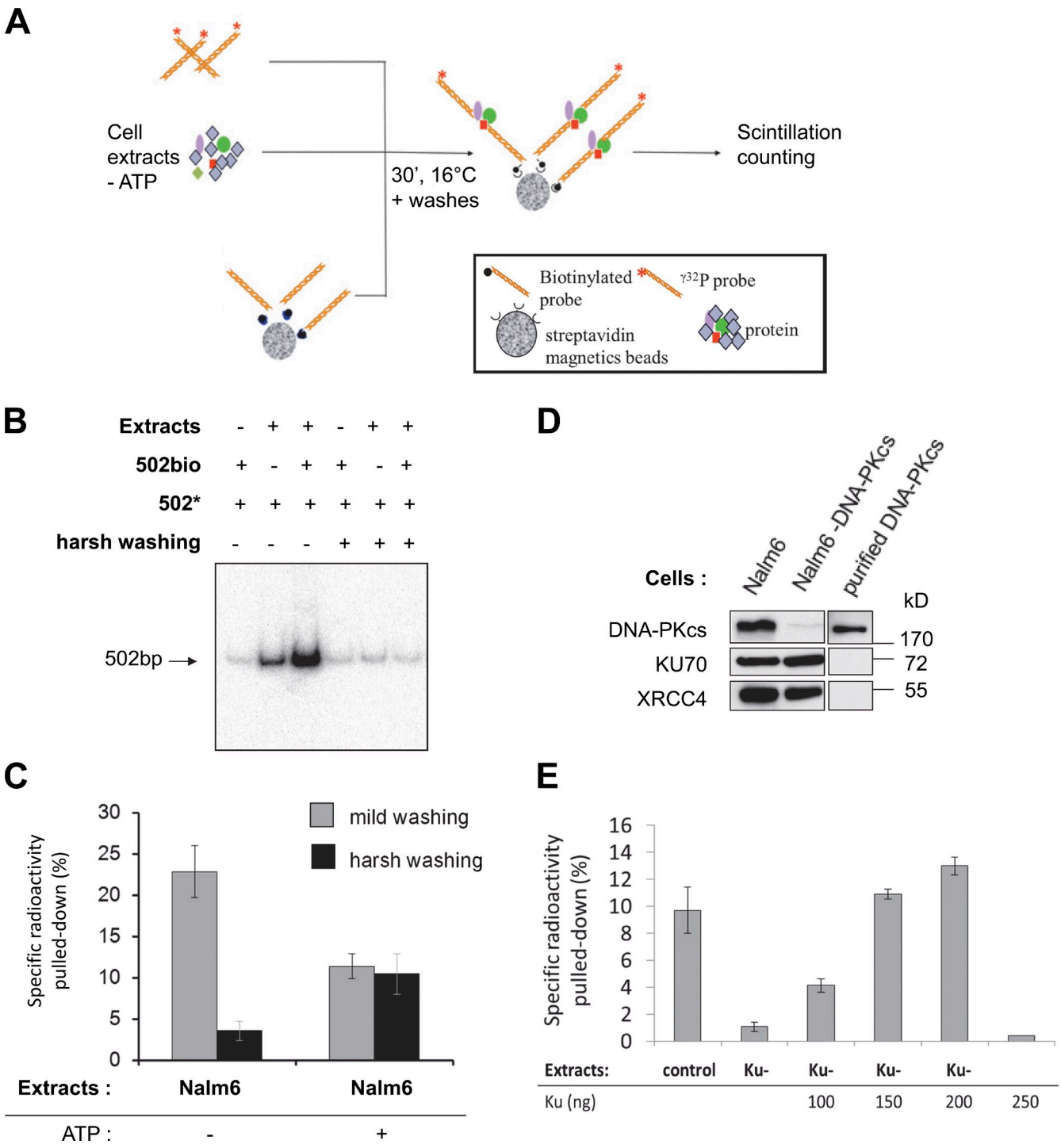


Figure S2. **Analysis of DNA end synapsis in vitro.** (A) Scheme of the end synapsis assay. NHEJ-competent extracts from human cells were incubated with 502-bp PCR DNA fragments, either radiolabeled (502*) or biotinylated at one end (502bio), and bound to streptavidin-coated magnetic beads. Then, the beads were washed in mild salt buffer to limit nonspecific association, and the radioactivity associated with the beads was measured by scintillation counting. (B) Gel electrophoresis analysis of the DNA pulled down. NHEJ-competent extracts from human cells were incubated with 502-bp PCR DNA fragments, either radiolabeled (502*) or biotinylated at one end (502bio), and bound to streptavidin-coated magnetic beads. Then, the beads were washed under mild (0.5× PBS) or harsh (0.5 M NaCl and 1% Triton X-100) conditions, and after deproteinization with proteinase K for 1 h at 50°C and extraction with phenol-chloroform, products were analyzed by electrophoresis on 5% polyacrylamide native gel followed by autoradiography with a phosphorimager (Storm System; Molecular Dynamics). Components present in the reaction were designated with plus signs. (C) Quantification of the specific radioactivity pulled down under synapsis conditions in vitro with extracts of Nalm6 cells under conditions ± ATP and mild or harsh washing as specified. (D) Western blotting analysis of Nalm6 protein extracts after immunodepletion as indicated and of the DNA-PKcs purified fraction in parallel. Protein samples were denatured and separated on 6% SDS-PAGE gel followed by electrotransfer on membrane and blotting with the antibodies as indicated. (E) Extracts from MRC5 cells expressing a conditional inducible shRNA against KU70, treated or not treated with doxycyclin for 7 d (Ku-deficient or Ku-proficient extracts, respectively; Cheng et al., 2011), and supplemented or not supplemented with the indicated amount of purified Ku heterodimer were assayed in the standard end synapsis reaction. Mean of three experiments ± SEM, except for Ku alone.

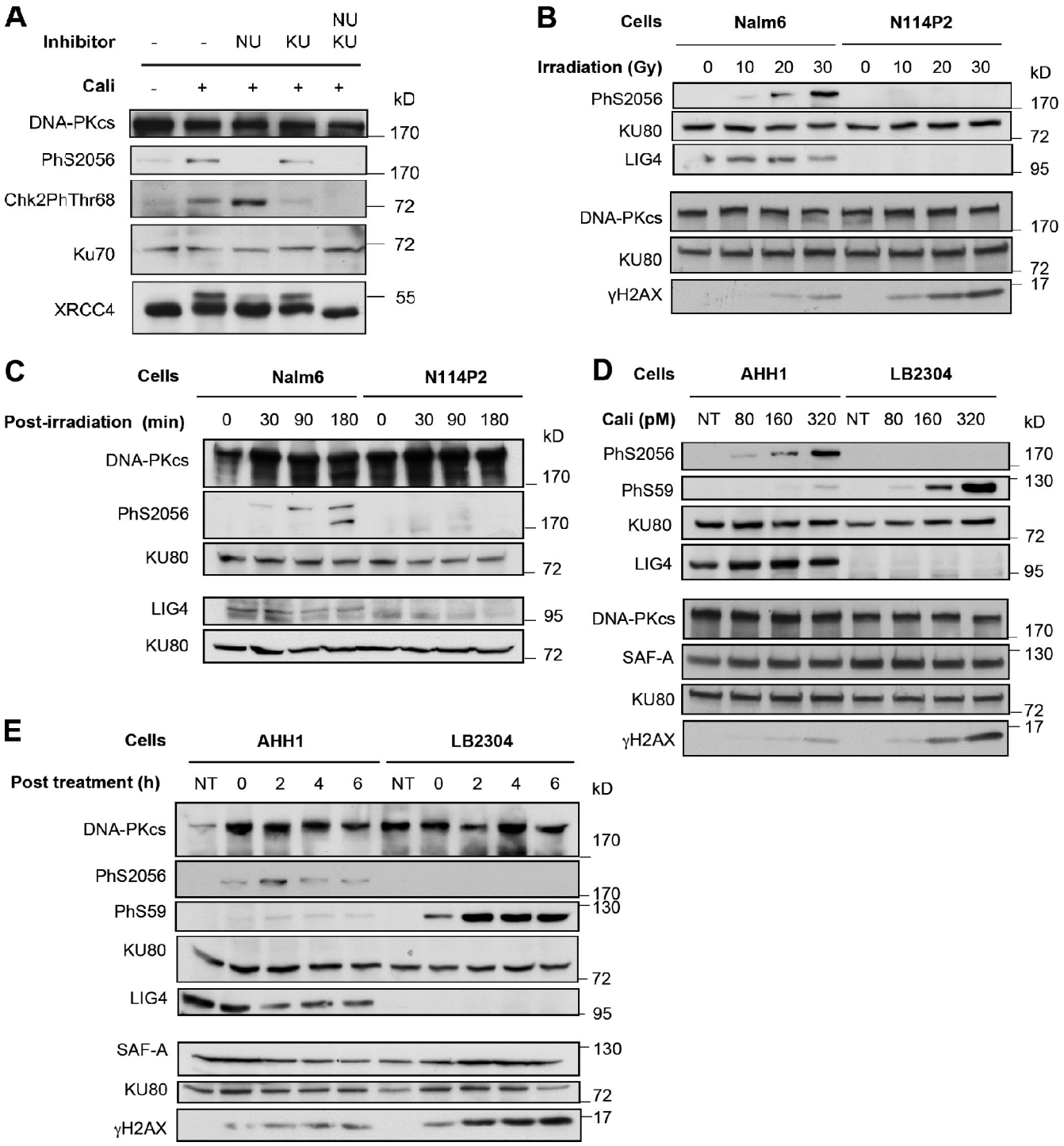


Figure S3. **Validation of the anti-PhS2056 antibody and its use in cells extracts.** (A) Western blotting on the whole-cell extracts of Nalm6 cells after treatment for 1 h with 160 pM Cali in the presence or absence of inhibitor against DNA-PKcs (NU7026, 40 μM) and/or ATM (KU55933, 20 μM) as indicated. (B and C) Defect in DNA-PKcs autophosphorylation in *LIG4*-null cells irradiated with x ray. Western blotting on the whole-cell extracts of Nalm6 control cells or N114P2 *LIG4*-null cells after x-ray irradiation with the indicated doses followed by incubation for 1 h under normal growth conditions (B) or x-ray irradiation with 30 Gy followed by incubation for the specified time (C). (D and E) DSB-stimulated DNA-PKcs autophosphorylation in other LIG4 mutant cells. Western blotting on the whole-cell extracts of AHH1 control and LB2304 *LIG4* mutant lymphoblastoid cells after treatment with increasing doses of Cali as indicated for 1 h (D) or with 160 pM of Cali for 30 min followed by incubation in fresh medium under normal growth conditions for the specified time (E).

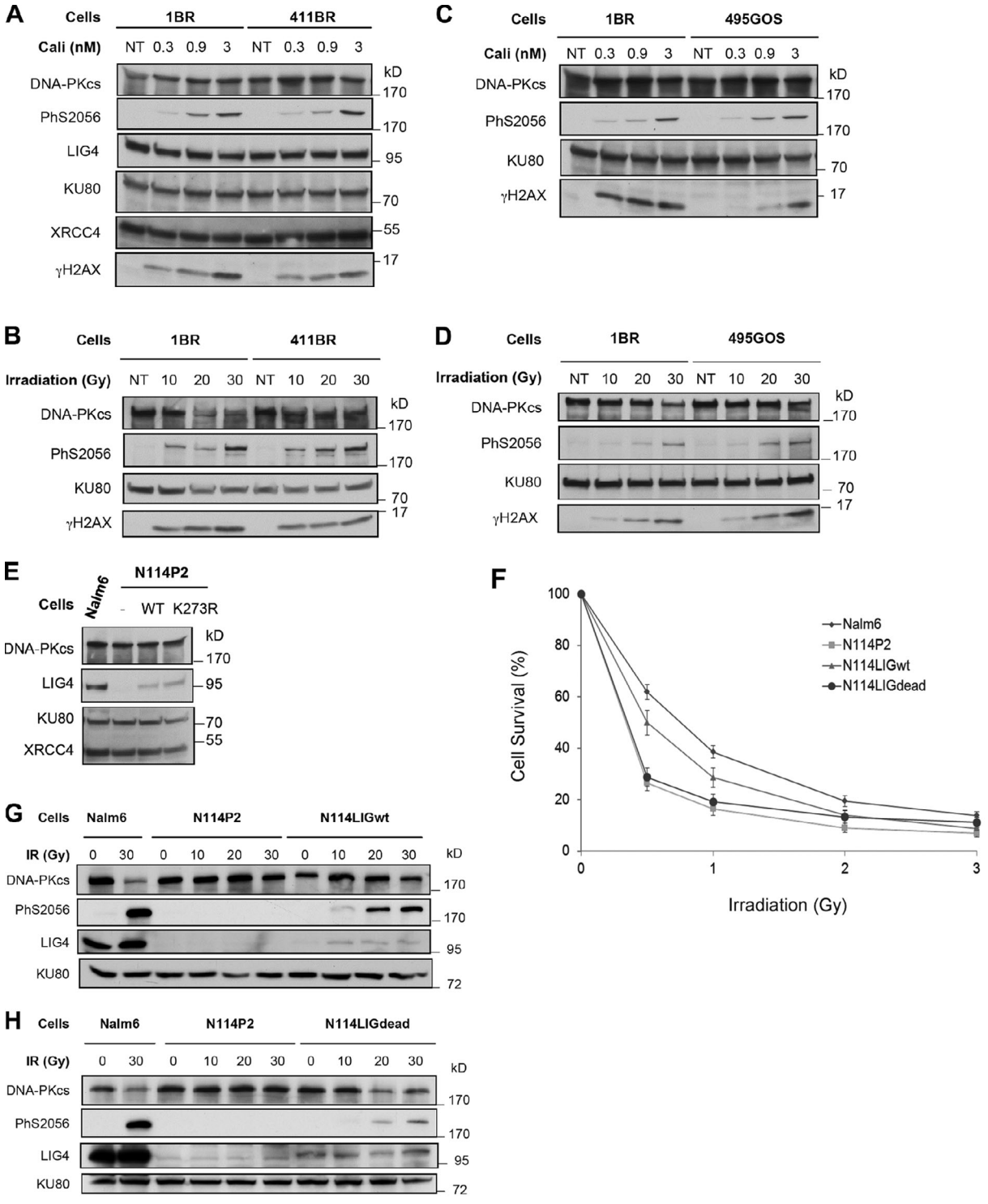


Figure S4. **LIG4 catalytic activity is not required for DNA-PKcs autophosphorylation in cells.** (A–D) DSB-stimulated DNA-PKcs autophosphorylation in ligase-dead cells. Western blotting on the whole-cell extracts of 1BR control cells or 411BR or 495GOS *LIG4* mutant cells after treatment for 1 h with increasing doses of Cali as indicated (A and C) or x-ray irradiation with the indicated doses followed by 3-h incubation under normal growth conditions (B and D). (E–H) Characterization of DNA-PKcs autophosphorylation in N114P2 cells expressing wild-type (N114LIGwt) or catalytic-dead (N114LIGdead) LIG4 protein. (E) Western blotting on the whole-cell extracts of Nalm6 control cells, N114P2 *LIG4*-null cells, or N114P2 cells expressing wild-type (WT) or a catalytic-dead (K273R) LIG4 protein. (F) Cell survival after x-ray irradiation of cells as indicated. Each value is the mean of six experiments. Error bars correspond to SD. IC50 values in Gy calculated for each cell type (Prism 4 program [GraphPad Software]) are Nalm6, 0.71; N114P2, 0.26; N114P2LIGwt, 0.5; and N114P2LIGdead, 0.26. (G and H) Cells were irradiated with increasing x-ray doses followed by 1-h incubation under normal growth conditions. Whole-cell extracts were heat denatured, separated on SDS-PAGE gels, and electrotransferred onto membranes that were blotted with antibodies as indicated.

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