

SUPPLEMENTAL MATERIAL AND METHODS

Live cell imaging and laser micro-irradiation

In select live cell imaging and laser micro-irradiation experiments, cells were treated with either DMSO or 10 μ M NU7441 (Selleck Chemicals) for 30 min before micro-irradiation. Experiments were performed and analyzed as described in the Materials and Methods. Live cell imaging and laser micro-irradiation experiments with were performed in the Ku80 deficient cell line Xrs5 complemented with Ku80 or the Ku80 5A mutant.

Gel shift assay

Gel shift assays were performed using purified Ku proteins incubated with 100nM 80mer forked dsDNA in a binding buffer containing 20mM HEPES (pH 8.0), 50mM KCl, 5mM MgCl₂ and 1mM DTT and 5% glycerol. Ku heterodimers containing either Ku70 wild-type (0.6 μ M), 5A or 5D (0.6 μ M, 1.3 μ M) were incubated in a 10 μ l final reaction volume on ice for 30 min. The DNA bound Ku complex was separated via a 4% Tris-Glycine native gel, dried and analyzed by Typhoon 9410 (GE Life Sciences).

Neutral comet assay with synchronous cells.

For the comet assay in S phase, cells were irradiated with 8 Gy of gamma rays 2.5 hours after release from the second thymidine block. A nonirradiated control was included in the assay. Cells were allowed to repair for 30 min, collected by trypsinization and immediately placed on ice. The rest of the comet assay was performed according the manufacturer's protocol (CometAssay kit, Trevigen), except for changes to the lysis and gel running conditions. When performing comet assays with S phase cells, actively replicating regions and proteins impede proper lysis and hinder migration of DNA, affecting subsequent tail formations after electrophoresis. To overcome these challenges, a modified lysis protocol and electrophoresis conditions were employed. After mixing an appropriate number of cells with LM Agarose (low melt agarose), cells were spread evenly onto the wells of the slide (Trevigen). The slides were then placed at 4°C for 30 min. Proteinase K (100 μ g/ml) (Sigma) was added to the lysis solution

(Trevigen). The slides were immersed in the cold lysis solution for 1 hour at 4°C to prevent further repair and then incubated at 37°C overnight. Electrophoresis conditions employed were 21 volts for 1 hour at 4°C.

Cell cycle analysis by flow cytometry

Cell cycle distribution was evaluated by measuring the propidium iodide (PI) (1) fluorescence intensity. Cells were collected by trypsinization and centrifuged at 100 x g at 4°C for 5 min. The medium containing trypsin was removed and the cells were washed once with 1 x PBS. Cell pellets were dissolved in cold 70% ethanol and stored at 4°C overnight. Cells were centrifuged (100 x g, 5 min). The supernatant was removed and cell pellets were dissolved in 1 ml of PI staining solution (10µg/ml PI, 50µg/ml RNaseA dissolved in PBS) per 1×10^6 cells for 20 min at 37°C. Samples were measured in a flow cytometer (FC 500 MCL, Beckman Coulter) according to pre-established protocols that were optimized for each cell line. To obtain standard histograms, a minimum of 15000 events were counted and gated.

Detection of BrdU by Flow Cytometry

For thymidine double block experiments with BrdU incorporation, 25 µM BrdU was added to the medium and incubated for 30 min before fixing the cells with 70% ethanol for flow cytometric analysis and stored at 4°C overnight. The next day, cells were washed twice with 1 x PBS and cell pellets were resuspended in 1 ml of 2M HCl containing 0.5% Tween 20. Cell suspensions were incubated for 45 min at 37°C, followed by neutralization with PBS; the process was repeated twice. The pellet was then dissolved in 100 µl of 1 x PBS containing 0.1% Tween 20, 1% BSA and 1:100 dilution of anti-BrdU antibody (347580, BD Biosciences) and incubated in the dark at RT for 2 hours. After incubation, cells were washed twice with 1% BSA in PBS. The cell pellet was then dissolved in 100µl of 1 x PBS containing a 1:1000 dilution of secondary antibody (Alexa Fluor 488, Molecular Probes) and incubated in dark at RT for 1 hour. Following incubation, cells were washed twice with PBS containing 1% BSA. The pellet was resuspended

in 1 ml PBS containing the PI staining solution. The rest of the protocol for PI staining is as described above (data not shown).

SUPPLEMENTAL FIGURE LEGENDS

Sup. Fig. 1. Treatment with either **(A)** the broad specificity PI3KK inhibitor wortmannin (WMN) or **(B)** the specific DNA-PKcs inhibitor (NU7441) resulted in retention of Ku at laser-generated DSBs. The plotted graph shows the relative fluorescence intensity of YFP-tagged Ku80 in U2-OS cells at DSBs after micro-irradiation following treatment with either DMSO, 5 μ M WMN or 10 μ M NU7441.

Sup. Fig. 2. (A) DNA-PKcs phosphorylates Ku *in vitro*. Lanes 5, 6 from Fig. 1C, containing γ -³²pATP in their reaction mixture, were resolved via 7% SDS-PAGE and analyzed by PhosphorImager analysis. **(B)** The gel shift assay shows 1:1 stoichiometry of Ku binding to forked dsDNA (F-DNA).

Sup. Fig. 3. Structure of Ku80 and its putative phosphorylation sites at the bridge and pillar region. **(A)** Putative phosphorylation sites in Ku80 (2). **(B)** The relative fluorescence intensity of YFP-tagged Ku80 and putative phosphorylation sites in Ku80 (Ku80-5A) at laser-generated DSBs was measured in Ku80 deficient Xrs5 cells.

Sup. Fig. 4. (A) Coomassie stained SDS-PAGE gel showing purified Ku70/80 heterodimer, Ku80 wild-type with Ku70 wild-type 5A or 5D. Purified Ku70 mutant with 6x Histidine tag caused delayed migration in SDS-PAGE compared with non-Histidine tagged Ku70 wild-type protein. **(B)** *In vitro* kinase assay data showed significantly lower phosphorylation of Ku70 5A or 5D protein as compared to wild-type Ku70. *In vitro* kinase assay samples were separated via 7% SDS-PAGE, Coomassie stained (right panel), and analyzed by PhosphorImager (left panel).

Sup. Fig. 5. Relative fluorescence intensity of YFP-tagged Ku70 WT or 5A cells in S phase. To monitor the cell cycle phase, the Ds-Red-PCNA-expressing vector was transiently transfected

into DC1 Ku70^{-/-} MEFs stably expressing Ku70 WT or 5A. S phase of cell cycle was determined by a punctate expression pattern of PCNA during S phase, as previously described (3).

Sup. Fig. 6. Expression levels of Ku70 and Ku70 mutants in complemented DC-1 Ku70^{-/-} MEFs. DC-1 Ku70^{-/-} MEF were complemented with WT or the Ku70 mutant. The stable expression of WT or mutant Ku proteins was detected by western blotting. Ku70 WT, 5A, 8A (top panel) or 5D (lower panel) were immunoblotted with a Ku70 antibody. Anti-actin antibody was used as a loading control.

Sup. Fig. 7 Blocking Ku70 phosphorylation induced an attenuation of DSB repair in S phase. (A) Cell cycle phase distributions using PI staining during various stages of the thymidine double block method. The degree of synchrony achieved for Ku70^{-/-}, WT and 5A cells were similar. Cohorts of cells 2-2.5 h post release from the second thymidine block was used for most of the assays using S phase synchronized cells. (B) DSB repair in S phase cells was evaluated in Ku70^{-/-} cells and those complemented with Ku70 WT and 5A via neutral Comet assay. Tail moment values for 0 Gy and 8 Gy for 30 min are shown. Representative images of Comet tails are shown. Error bars denote SD (C) Box plot of the data presented in A. Error bars denote SD.

Sup. Fig. 8. Structure of Ku predicted conformational changes induced by phosphorylation of Ku70.

Sup. Fig. 9. Treatment with the neddylation inhibitor MLN4924 (MLS) did not affect Ku's dynamics at laser-generated DSBs in S phase. The plot shows relative fluorescence intensity of YFP-tagged Ku80 in U2-OS cells at DSBs in S phase after micro-irradiation following treatment with either DMSO or 3μM MLN4924 for 1 hour. S phase of cell cycle was determined by a punctate expression pattern of PCNA during S phase, as previously described (3).

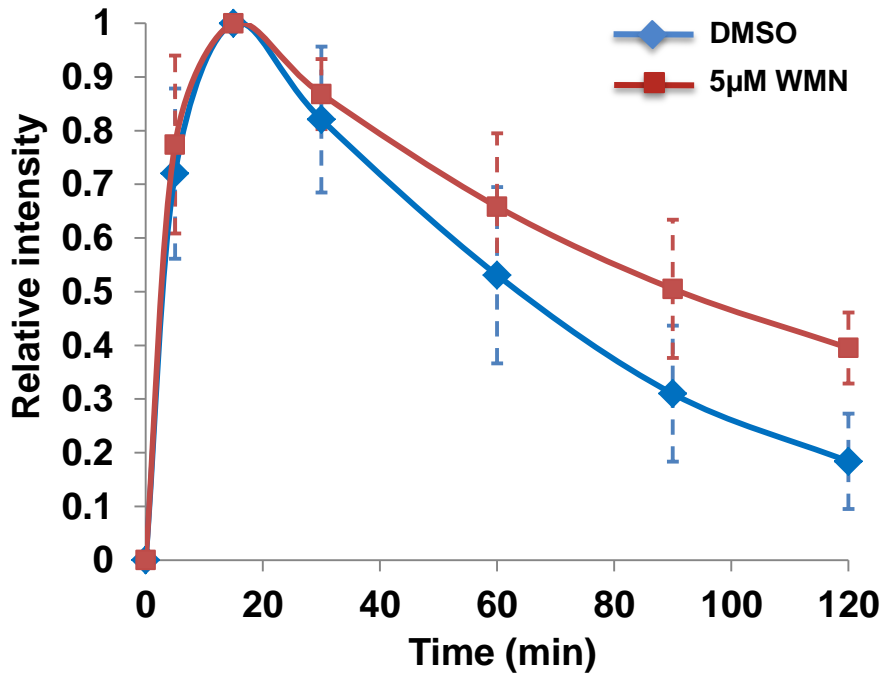
Sup. Fig. 10. Clonogenic survival assay of Ku70^{-/-}, WT and Ku70 5D.

SUPPLEMENTAL REFERENCES

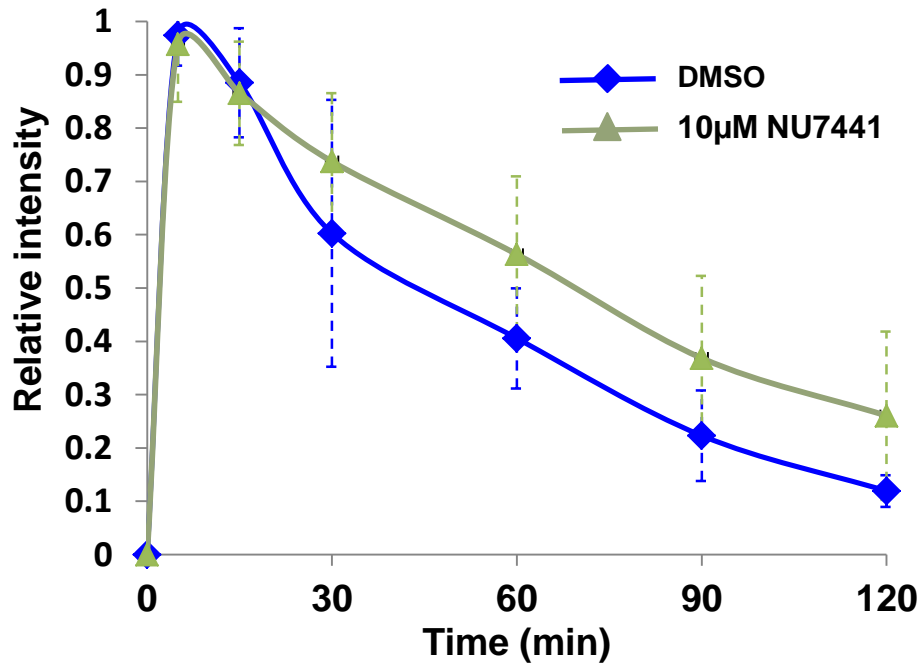
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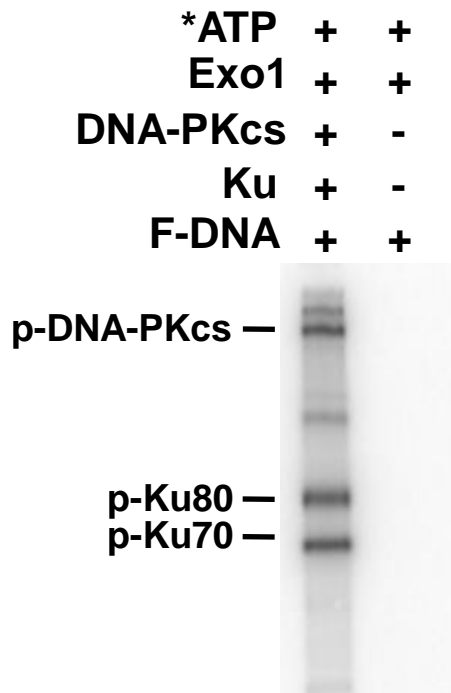
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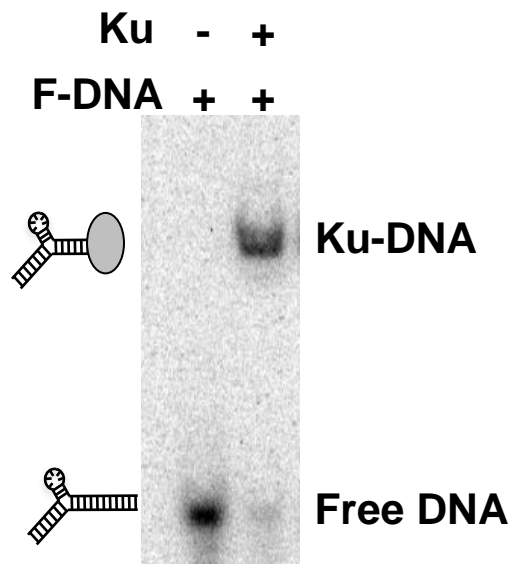


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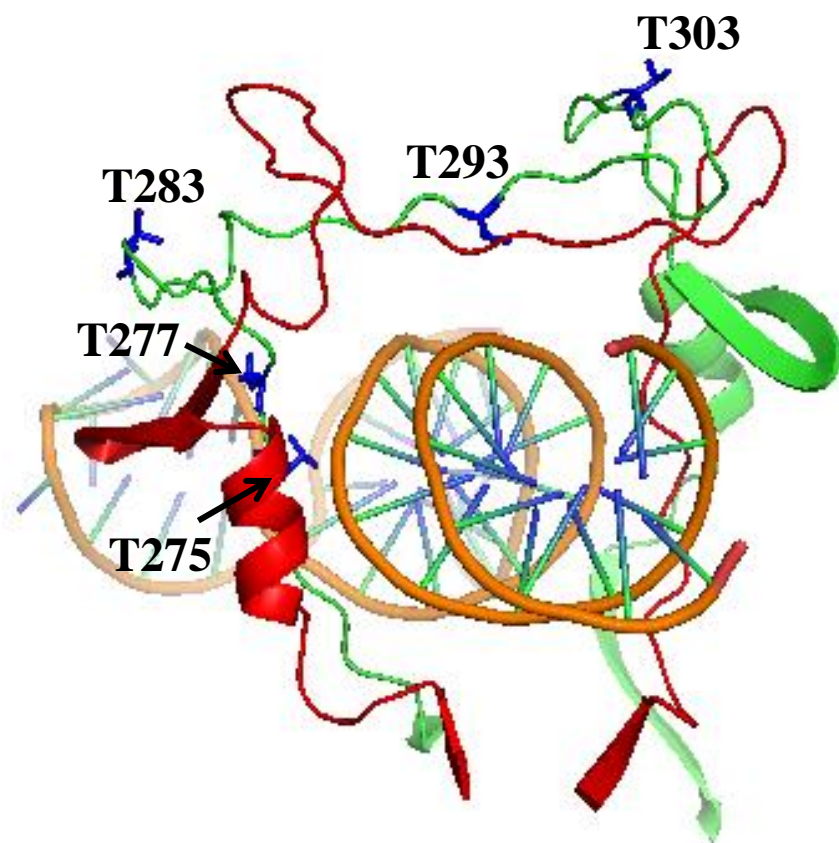
In vitro kinase assay

B

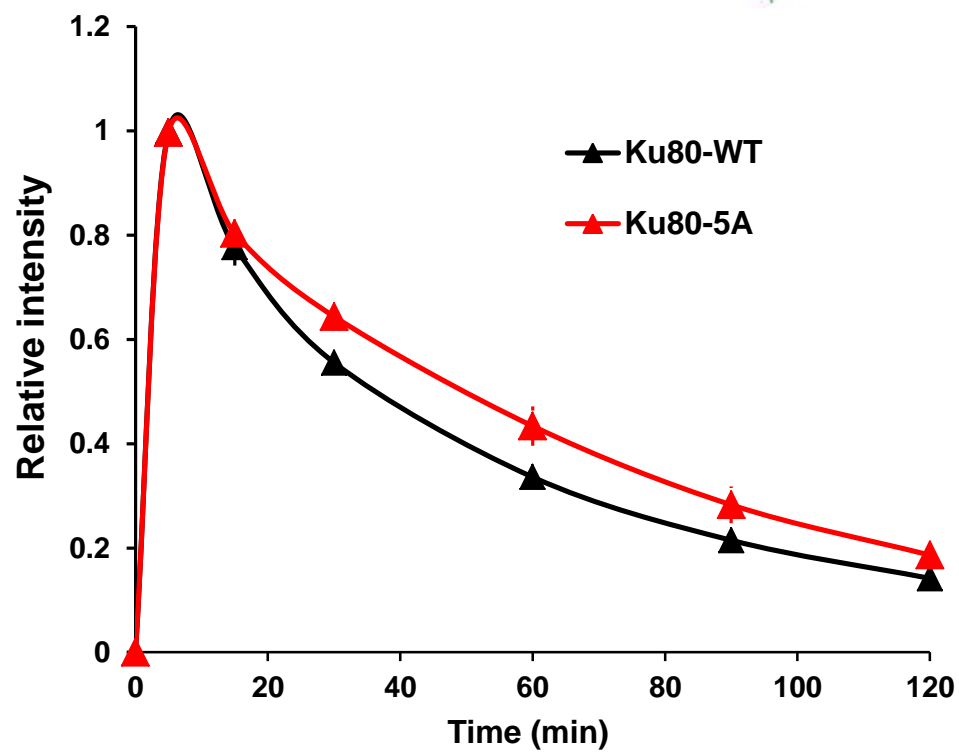


Gel shift assay

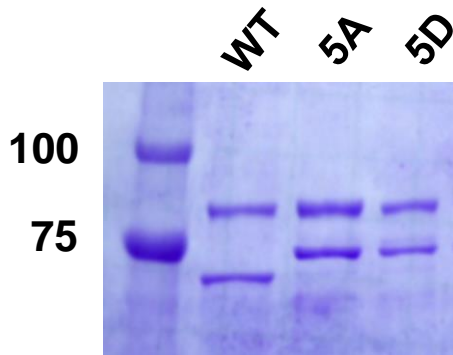
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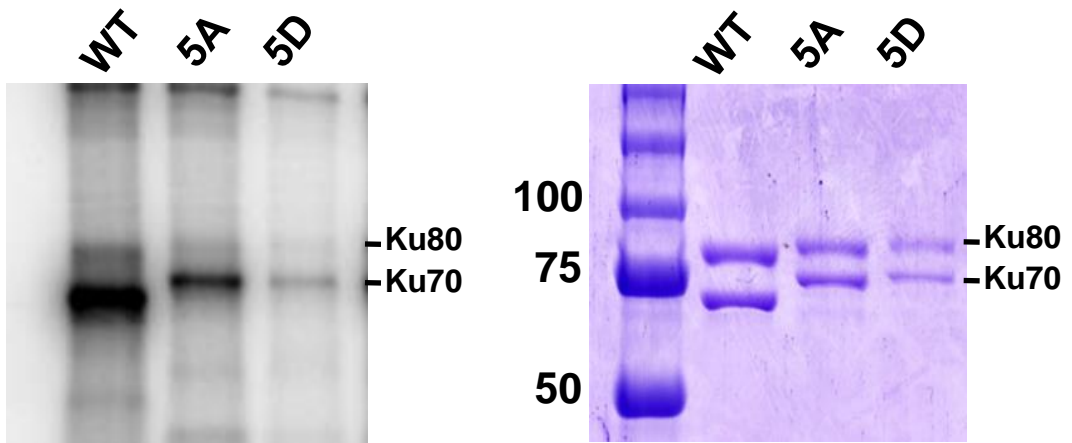
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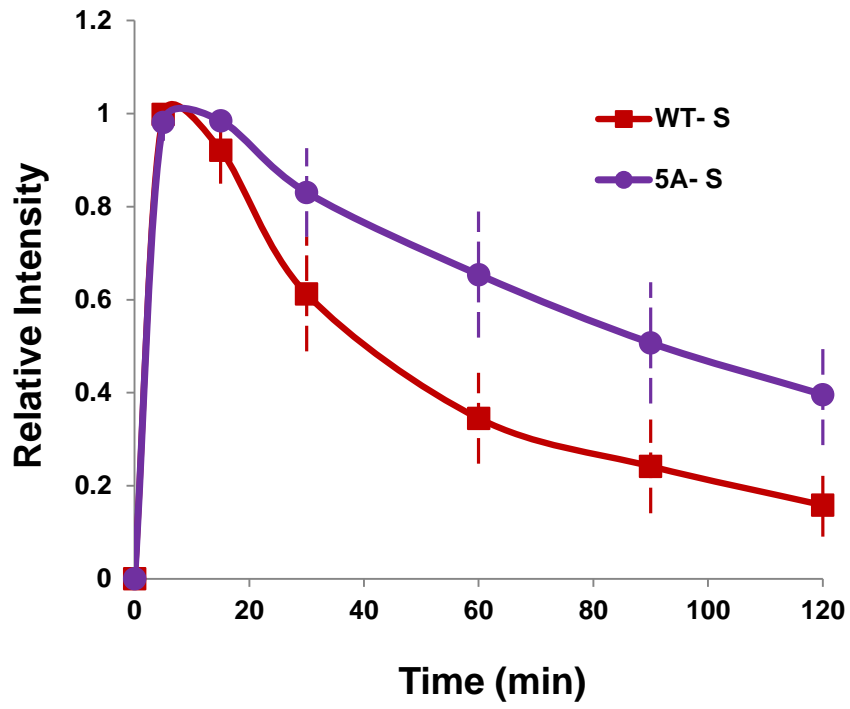
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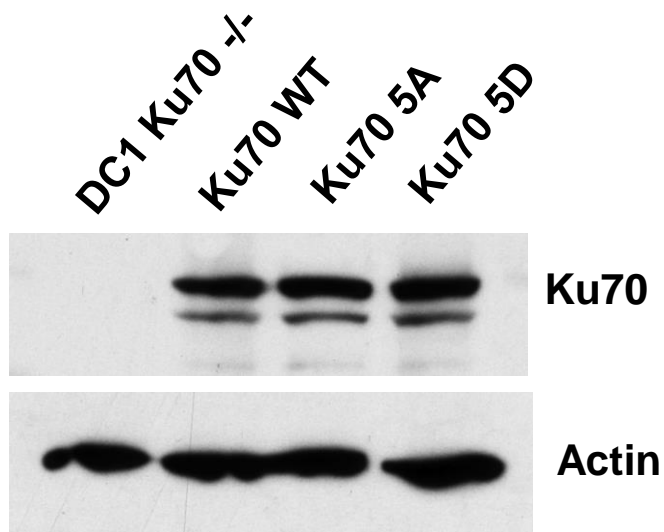
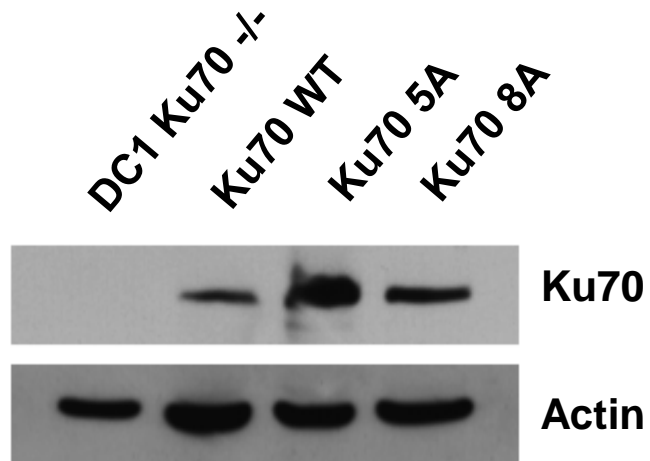
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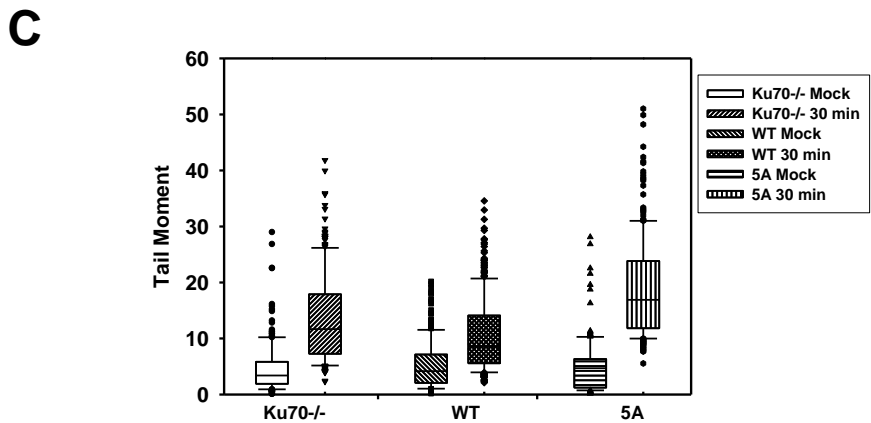
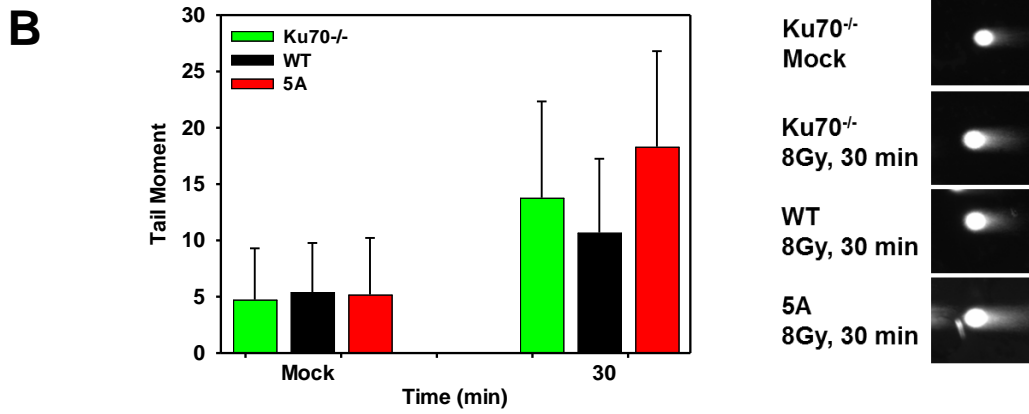
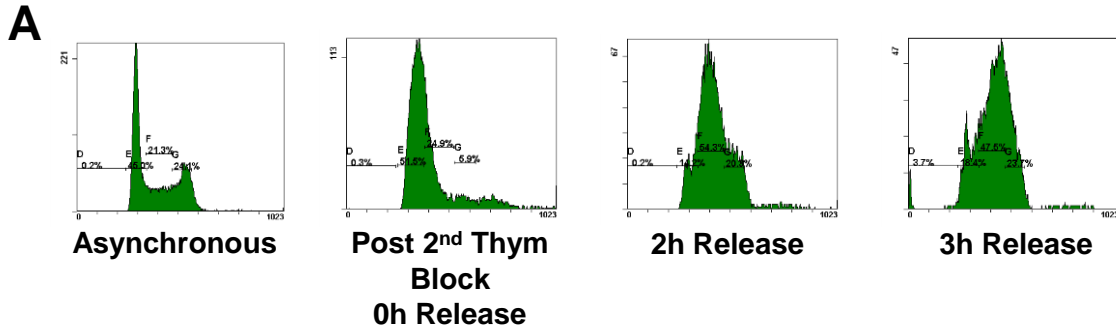
Coomassie staining

Lee_Sup. Fig 5

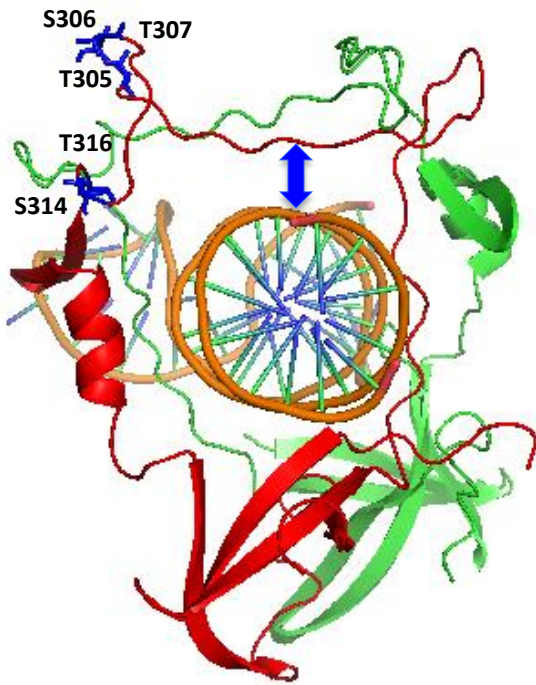


Lee_Sup. Fig 6

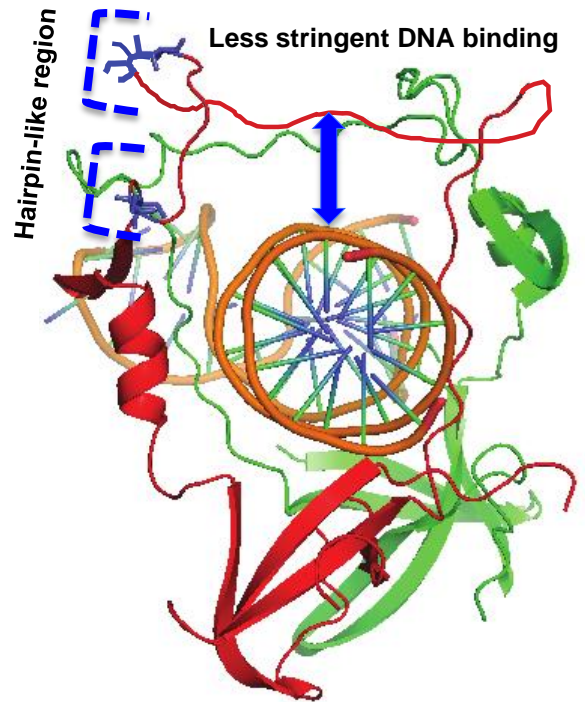




Non-phosphorylated Ku



Phosphorylated Ku



Lee_Sup. Fig 9

