Supplementary figure 4
Supplementary Data

Nbs1 is Required for RPA Phosphorylation Following Replication Fork Stall and Collapse

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Supplemental Experimental Procedures

Peptide-mediated delivery of antibodies into HeLa cells.
Chariot®–antibody complexes were formed by incubating 2-3 µg of non-immune IgG, with 4 µg of presonicated Chariot® (ChariotTM; Active Motif, Carlsbad, CA) in PBS (pH 7.4) for 30 min at 25 °C. HeLa cells were overlaid with the pre-formed complexes according to the manufacturer’s recommendations. Briefly, the delivery of Chariot®–antibody complexes was performed by incubating Chariot®–antibody complexes directly in cell medium with cells for 3 h at 37 °C.

Chariot-antibody Transformation Efficiency. To determine antibody transformation efficiency, cell-associated fluorescence of a total of 10,000 cells was determined using flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes, NJ). Chariot®–antibody treated cells were fixed and permeabilized using the cytofix/ cytoperm kit (BD Biosciences) and stained with Alexa Fluor 568 secondary antibody (Molecular Probes, Carlsbad, CA) at a 1:250 dilution. Percentage of cells with Alexa Fluor 568 fluorescence was determined using CellQuest pro software (BD Biosciences).
Flow cytometric analysis. HeLa cells were harvested at 0 hr, 18 hr, and 24 hr time points and fixed with 70% ethanol. Approximately 10^6 cells/ml were incubated with propidium iodide and RNase A, then analyzed using a FACSCalibur (BD Biosciences). Data was plotted using CellQuest software; approximately 10,000 events were analyzed for each sample. To detect H2AX phosphorylation mock- and HU-treated cells were fixed in 1% methanol-free formaldehyde solution and permeabilized in 70% ethanol at -20°C over night. Cells were washed in PBS, resuspended in 1% BSA in 0.1% Tween PBS (BSA-T-PBS) for 5 min and incubated with 2µg/ml monoclonal γH2AX antibody (Upstate, Temecula, CA) over night at 4°C. Cells were washed in BSA-T-PBS and suspended in BSA-T-PBS containing 10µg/mL goat anti-mouse secondary antibody (Alexa Fluor 647, Invitrogen). After 1h incubation wash with BSA-T-PBS, cells were suspended in propidium iodide (PI) staining solution containing 5 µg/mL PI (Sigma) and RNase A in PBS (100 mg/mL, Sigma) and incubated for 30 min in the dark. The intensity of fluorescence was measured with the BD FACSArray (BD Biosciences) and data were analyzed using WinList (Verity) software.

Figure legends

Fig. S1. Transfection efficiency determined by flow cytometry. HeLa cells were transfected with non-immune IgG isotype control, anti-RPA32S4PS8P or Nbs1 SP343 antibody-Chariot® complexes. Transfection efficiencies averaged approximately 80%. The transfection efficiencies shown are 85% and 89% for anti-RPA32S4PS8P and Nbs1 S343, respectively.
Fig. S2. Cell cycle analysis following protein transfection with Chariot® reagent. HeLa cells were mock-transfected or transfected with non-immune IgG-Chariot® complexes, fixed at the indicated times and cell cycle analysis was performed.

Fig. S3. HU toxicity in NBS-ILB1 cells and transgenes following HU treatment. Cells were treated with 5 mmol HU for 3 and 8 hours; for the 8 hour time point HU-containing medium was replaced by regular medium after 3 hours and cells were incubated for additional 5 hours. Cell survival was analyzed using the Live/Dead assay.

Fig. S4. H2AX and cell cycle analysis of cells either mock-treated or treated with HU for 3 h. Bivariate analysis was performed with a BD-FACSArray demonstrating H2AX phosphorylation predominantly in S-phase of HU-treated cells.