Supplemental Materials and Methods

Co-immunoprecipitation (Co-IP) assay

Cells were lysed with NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing 50 mM β-glycerophosphate, 10 mM NaF and 1 mg/ml each of pepstatin A and aprotinin. Whole cell lysates obtained by centrifugation were incubated with 2μg of antibody and protein A or protein G Sepharose beads (Amersham Biosciences) for 4 h at 4°C. The immunocomplexes were then washed with NETN buffer three times and separated by SDS-PAGE. Immunoblotting was performed following standard procedures.

Immunofluorescence

To visualize ionizing radiation induced foci (IRIF), cells were cultured on coverslips and treated with 2Gy IR followed by recovery for 1 hr or as indicated. Cells were then washed in PBS, incubated in 3% paraformaldehyde for 15 min, and permeabilized in 0.5% Triton solution for 5 min at room temperature. Samples were blocked with 5% goat serum and then incubated with primary antibody for 30 min, which included anti-γH2AX (A300-081A)(1:1000 dilution), anti-BRCA2 (OP95) (1:200 dilution), anti-BRCA1(D9) (1:200 dilution), anti-53BP1(NB100-304) (1:500 dilution), anti-RPA (9H8) (1:200 dilution), anti-RAD51 (N1C2) (1:200 dilution), anti-MDC1 (05-1572) (1:1000 dilution) and anti-PALB2 (1:100 dilution). Samples were washed three times and incubated with secondary antibody for 30 min. Cells were then stained with DAPI to visualize nuclear DNA. The coverslips were mounted onto glass slides with anti-fade solution and visualized using a Nikon eclipse 80i fluorescence microscope. >200 cells were counted per experiment.

Colony Formation assay

500-2000 Cells were plated in triplicate in each well of 6 well plates. After 1 day, cells were divided into treating groups by stratified randomization and exposed to ionizing radiation or treated with the PARP inhibitor, Olaparib, and left for 10–14 days at 37°C to allow colony formation. Colonies were stained with 5% GIEMSA and counted. Results were normalized to plating efficiencies.

Dephosphorylation assay

HEK293T cells were transfected with HA-UCHL3, after 48 hr, cells are left untreated or irradiated (10Gy). After an additional 1 hr, HA-UCHL3 was immunoprecipitated by agarose conjugated with anti-HA antibody for 2 hr at 4°C. The beads were then washed three times with NETN buffer and one time with lambda phosphatase reaction buffer and incubated with lambda phosphatase (NEB) in reaction buffer (1X NEBuffer for PMP with 1 mM MnCl2) at 30°C for 20 mins. The bound proteins were eluted by 1× SDS-PAGE buffer with heating for 10 min at 95°C and subjected to immunoblotted with anti-Phospho-(Ser/Thr) ATM/ATR Substrate antibody.

Supplementary Figure Legends

Supplementary Figure 1. (A) The cell cycle profiles of the control and UCHL3 knockout cells were analyzed by flow cytometry. (B) Control or UCHL3 knockout U2OS cells were treated with IR and RAD51 foci formation was detected at different time points. Representative images are shown in the left panel. Quantification of the percentage of cells displaying foci formation is shown in the right panel. Error bars represent SEM from three independent experiments. (C) $UCHL3^{+/+}$ and $UCHL3^{-/-}$ cells were treated with IR and RAD51 foci formation was detected. Quantification of the percentage of cells displaying foci formation is shown in the right panel. Error bars represent SEM from three independent experiments. Statistical significance was determined by two-tailed student's t test, $^{**}p < 0.01$. (D) Control or UCHL3 knockout U2OS cells were treated with IR and MDC1 and PALB2 foci formation were evaluated. Representative images are shown in the left panel. Quantification of the percentage of cells displaying foci

formation is shown in the right panel. Error bars represent SEM from three independent experiments. Statistical significance was determined by by two-tailed student's t test.

Supplementary Figure 2. (A-B) Co-localization of UCHL3 with (A) RAD51 and (B) γ-H2AX at the DSB site created by I-SceI. (C) Recruitment of UCHL3 and RAD51 to I-SceI-induced DSB was evaluated by anti-RAD51 and anti-UCHL3 ChIP followed by qPCR with primers adjacent to the DNA damage site. Error bars represent SEM from three independent experiments. (D) Knocking out of UCHL3 reduced recruitment of RAD51 to I-SceI-induced DSB site. ChIP assays were performed as described in C. (C-D) Error bars represent SEM from three independent experiments.

Supplementary Figure 3. (A) RAD51 or RAD51-ssDNA nucleoprotein filaments were incubated with GST-UCHL3 *in vitro* and UCHL3/RAD51 interaction was detected by western blot. (B) Control or UCHL3-deficient U2OS cells were lysed under denaturing conditions; RAD51 was immunoprecipitated, and blots were probed with the indicated antibodies.

Supplementary Figure 4. (A) Sensitivity of $UCHL3^{+/+}$ and $UCHL3^{-/-}$ MEF cells to radiation was assessed using colony formation assay. Error bars represent SEM from three independent experiments.

Supplementary Figure 5. (A) RAD51 was immunoprecipitated from cells and the product was divided into two equal parts. One part was left untreated and the other one was deubiquitinated by UCHL3 *in vitro*. Next, the products were analyzed by western blot and the blots were probed with the indicated antibodies. (B) RAD51 immunoprecipitates generated as (A) were incubated with GST-BRCA2-BRC4 *in vitro*. The interaction between RAD51 and BRCA2-BRC4 was then examined as indicated. (C) Cell lysates from Control or UCHL3 knockout U2OS cells were subjected to immunoprecipitation with control IgG or RAD51 antibodies. The blots were probed with the indicated antibodies.

Supplementary Figure 6. (A) Conserved lysine residues in vertebrates, zebrafish and Xenopus RAD51 are highlighted. The N terminus of RAD51 is boxed in red; the core domain of RAD51 is boxed in black.

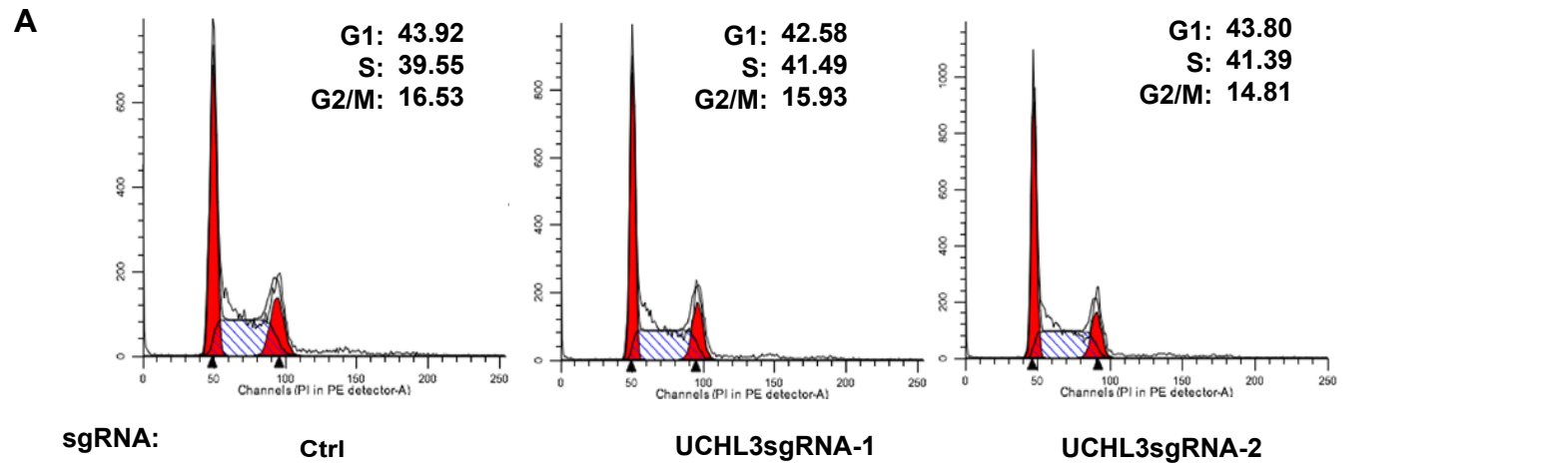
Supplementary Figure 7. (A) D loop reactions conducted with WT RAD51 and RAD51 3KR mutant proteins. ‘’-ATP’’ denotes reaction without ATP. Reactions were performed for the indicated time. (B) U2OS cells transfected with indicated constructs were lysed and cell lysates were subjected to immunoprecipitation with Myc antibody. Blots were probed with the indicated antibodies. (C) UCHL3 deficient cells transfected with the indicated constructs were treated with IR. After 1 hr, the cells were lysed and cell lysates were subjected to immunoprecipitation with Myc antibody. Blots were probed with the indicated antibodies.

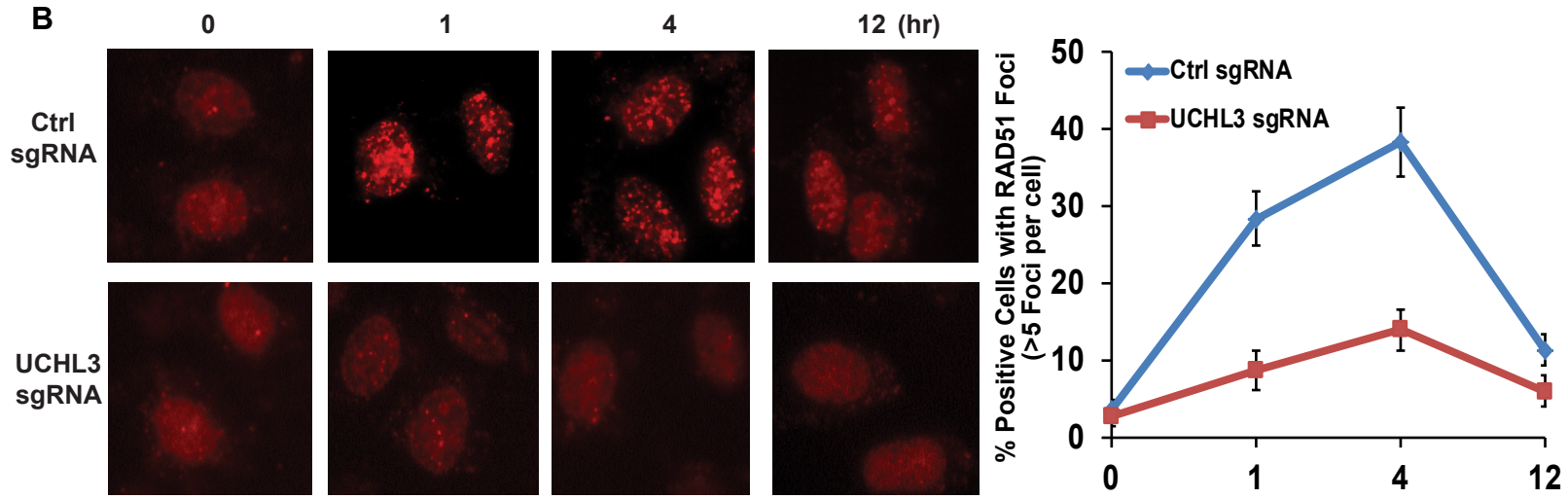
Supplementary Figure 8. (A) U2OS cells transfected with Myc-RAD51 were irradiated and stained with indicated antibodies. (B) U2OS cells stably expressing RAD51 shRNA together with shRNA-resistant WT or 3KR Myc-RAD51 were left un-irradiated, and γ-H2AX and Myc-RAD51 were detected by immunofluorescence with indicated antibodies. Spontaneous γ-H2AX foci formation is quantified in the right panel. Error bars represent SEM from three independent experiments.

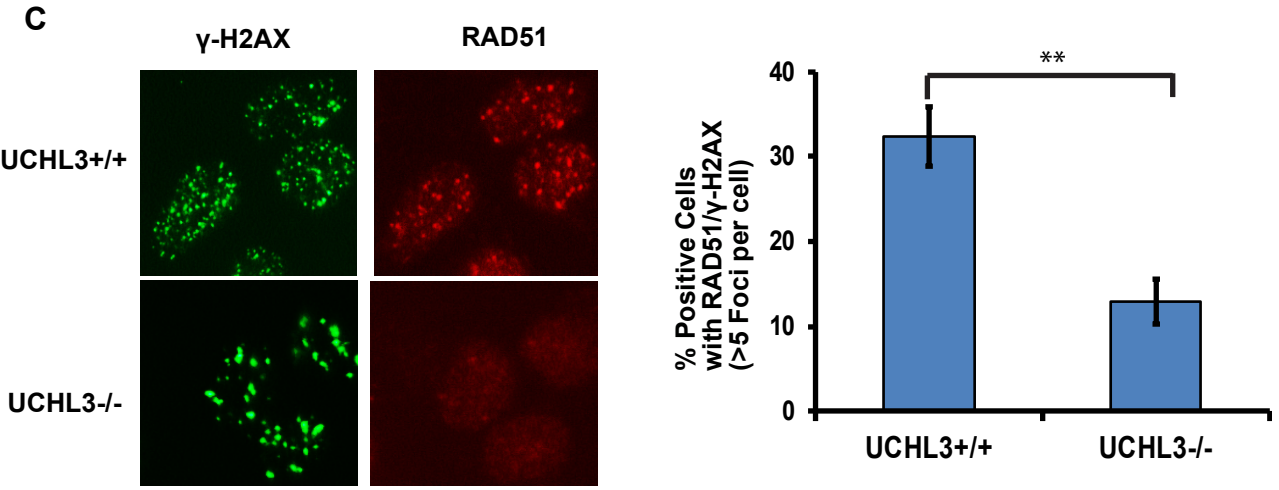
Supplementary Figure 9.(A) HEK293T cells transfected with HA-UCHL3 (WT or S75A) were left untreated or irradiated (10Gy). HA-UCHL3 was immunoprecipitated and blots were probed with the indicated antibodies. (B) U2OS cells stably expressing HA-UCHL3 WT or S75A were left un-irradiated, and γ-H2AX and RAD51 were detected by immunofluorescence.

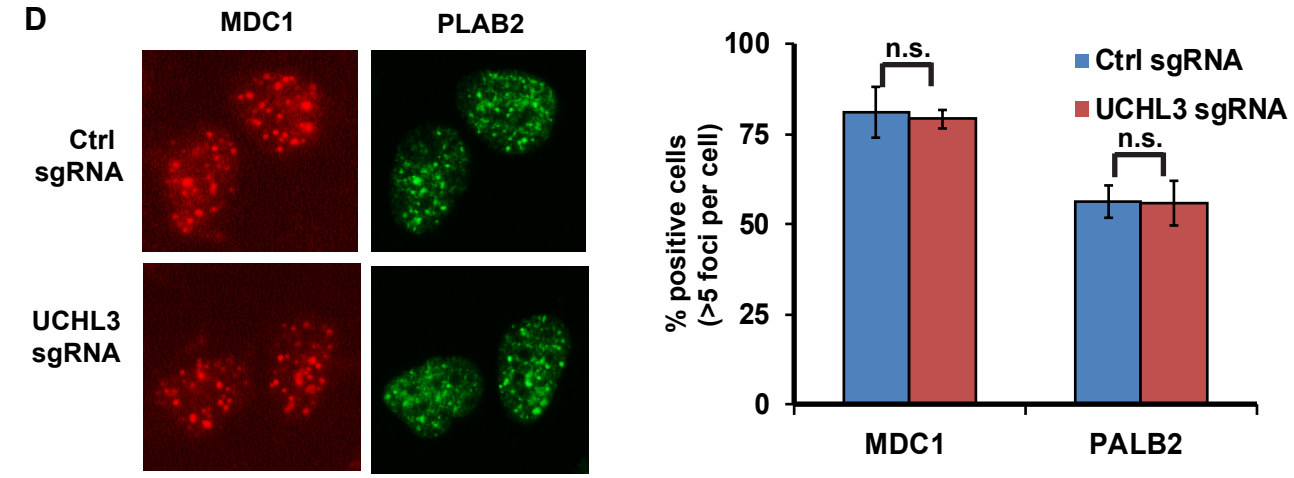
Supplementary Figure 10. (A-C) Survival assays for control and UCHL3 knockout BT549 cells stably expressing RAD51 shRNA exposed to (B) Olaparib and (C) IR. (A) UCHL3 and RAD51 expression were detected by western blot (B-C) Error bars represent SEM from three independent experiments.

Luo_289439 _Supplementary Figure 1









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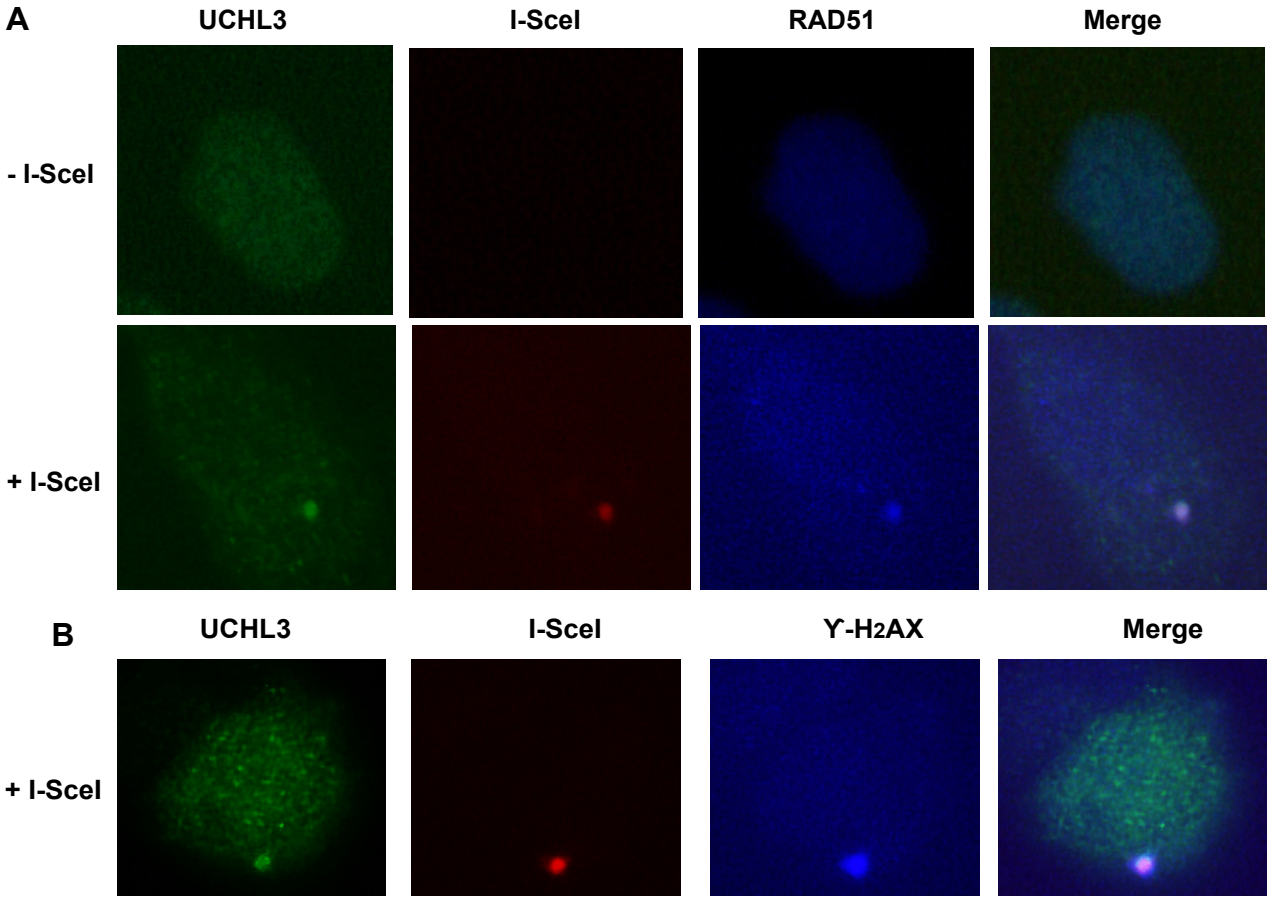

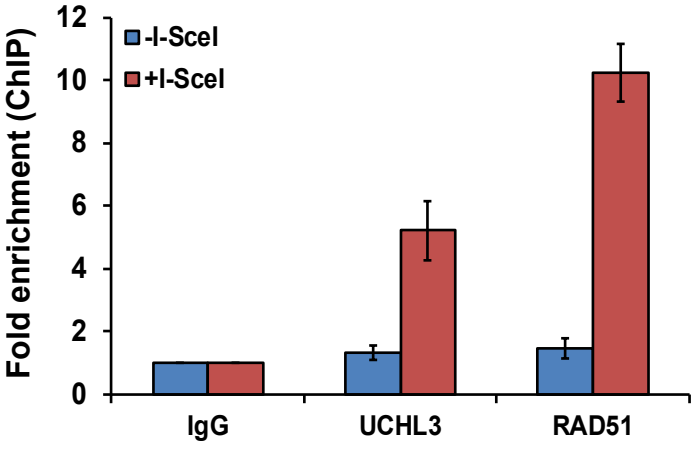

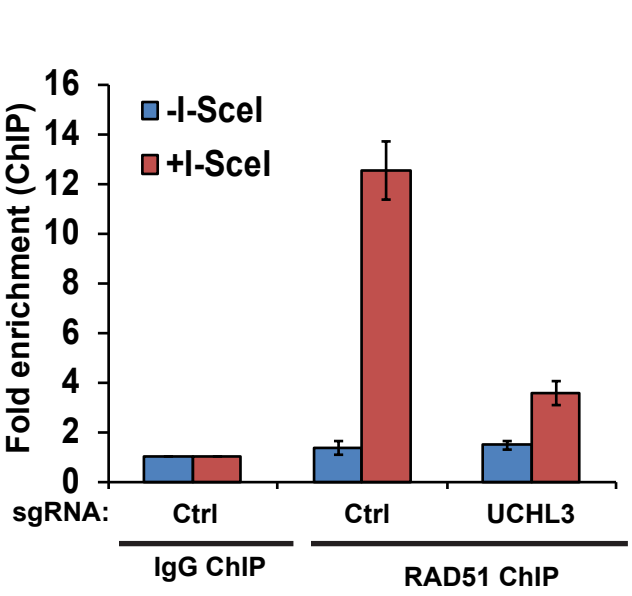

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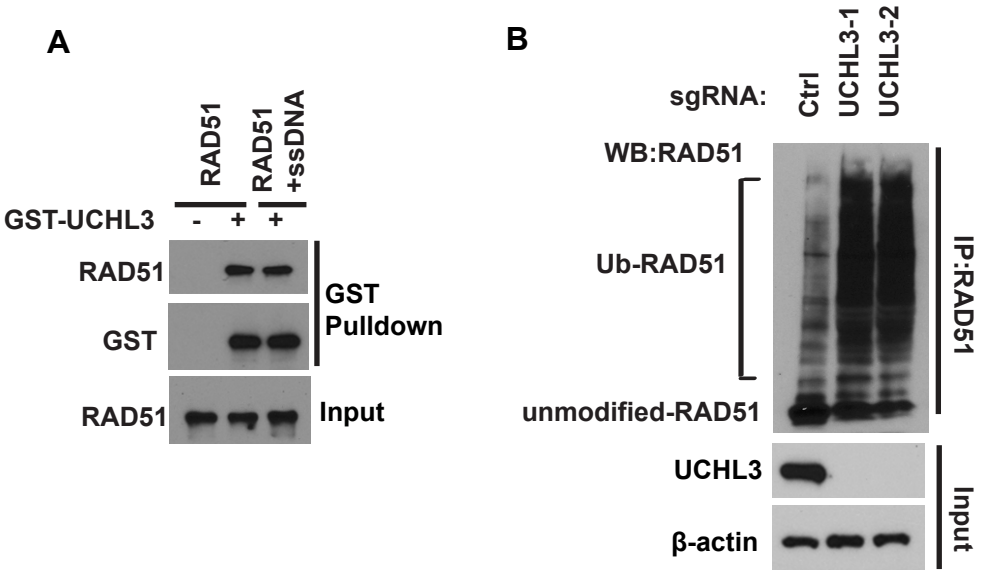

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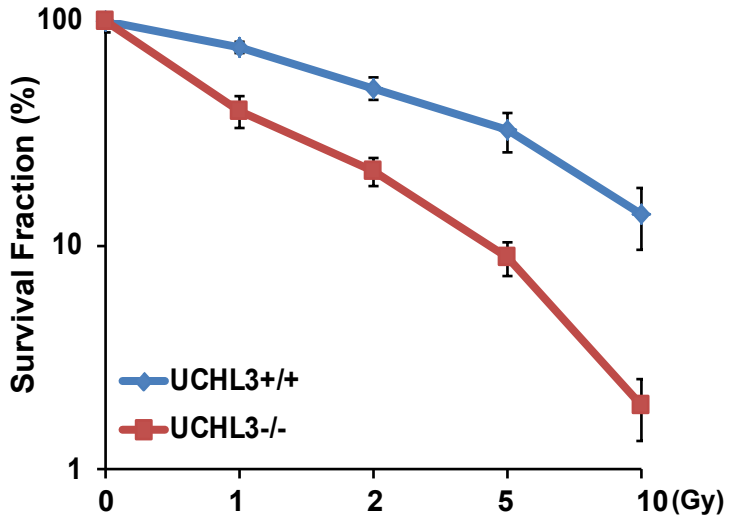

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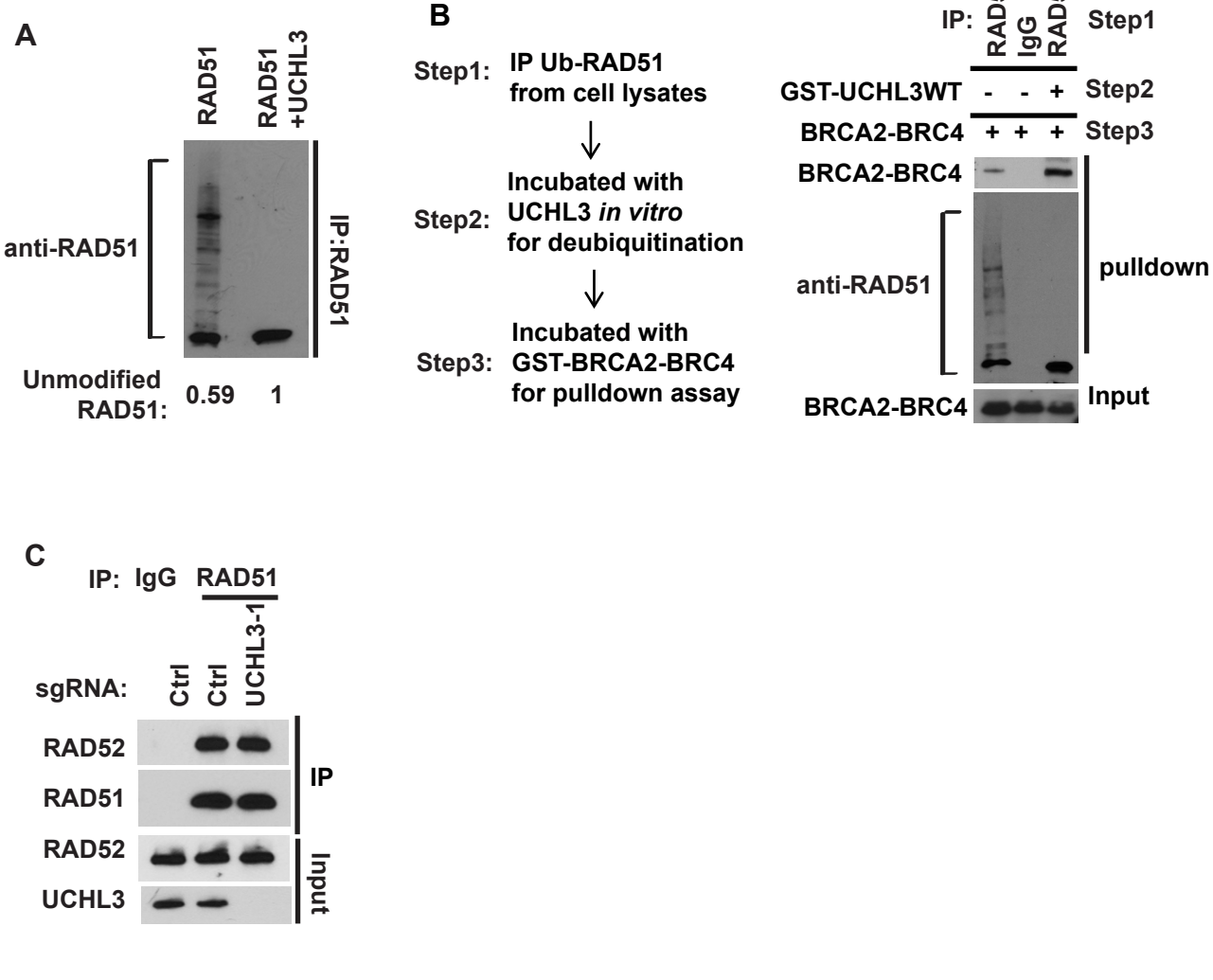

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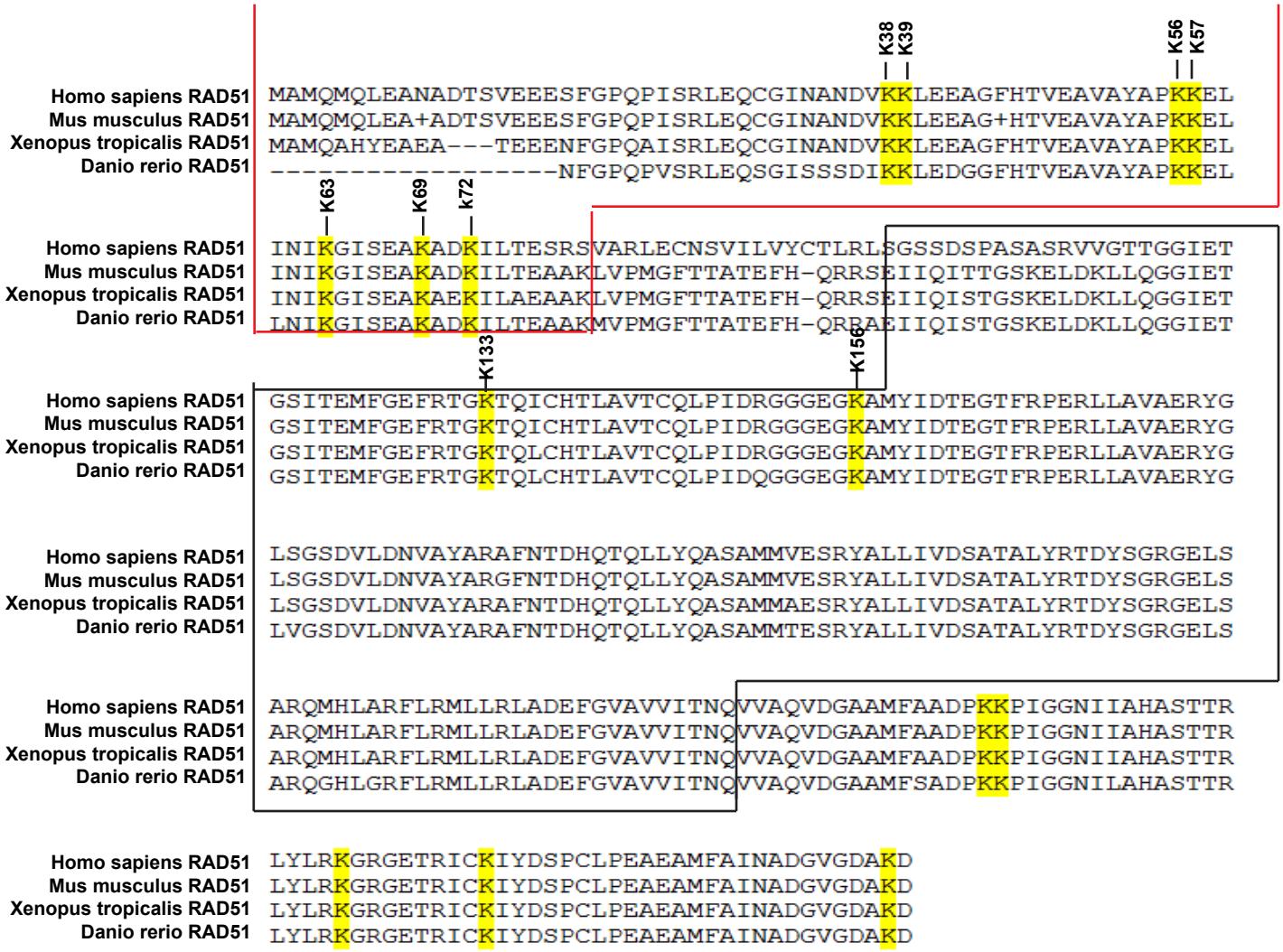

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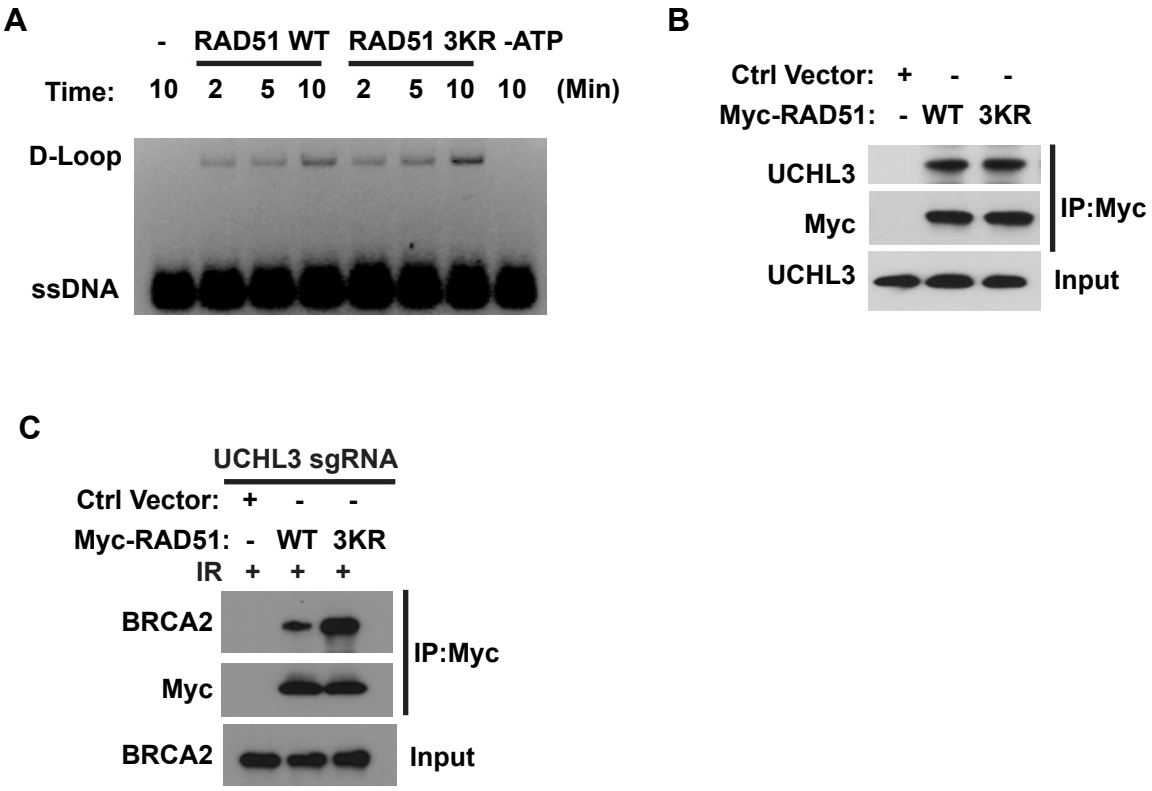

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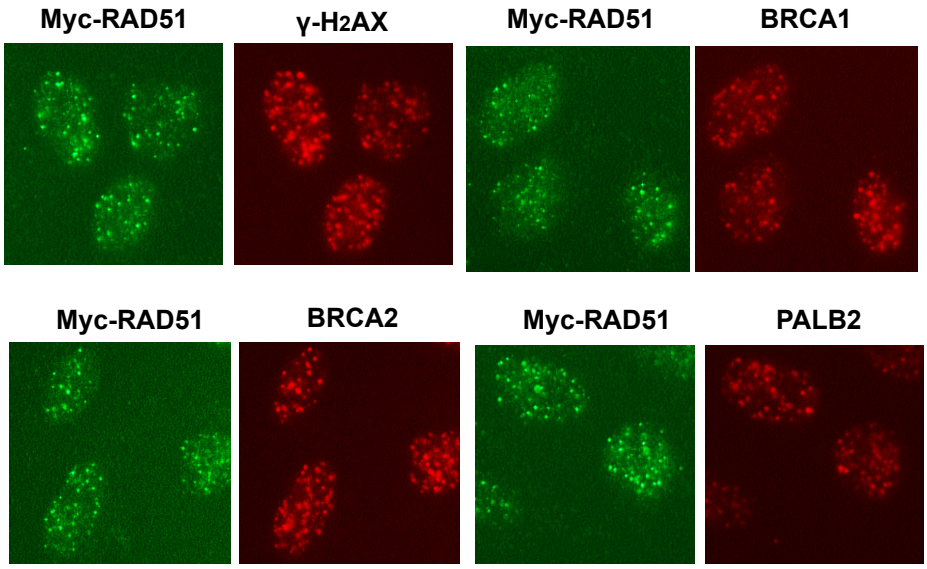

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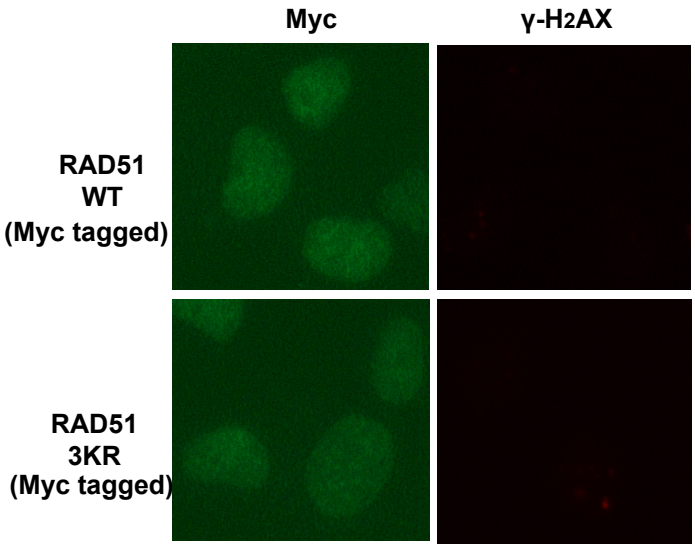

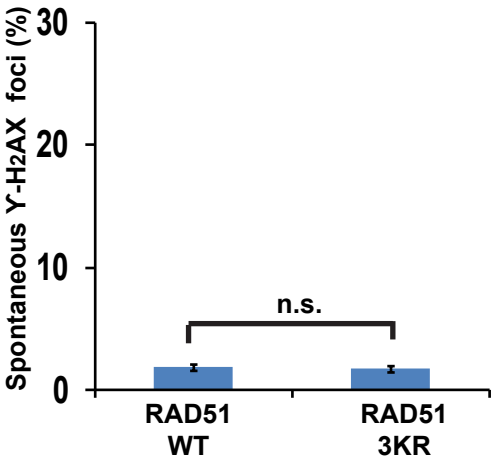

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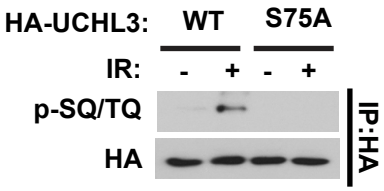

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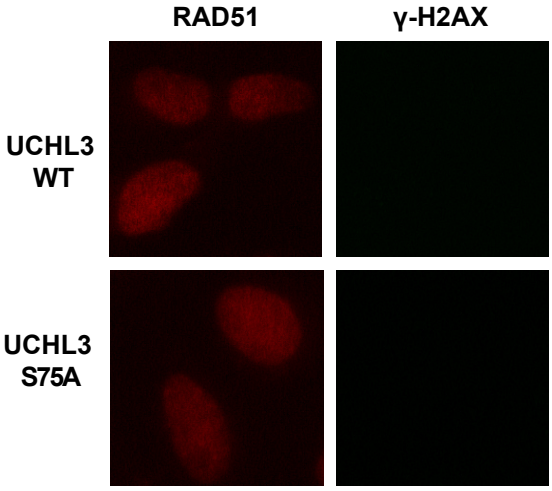

No irradiation

Luo_289439 _Supplementary Figure 10