Supporting Information

Yuan and Chen 10.1073/pnas.1220662110

SI Materials and Methods

Antibodies. Anti–fidgetin-like 1 (FIGNL1) antibody was raised by immunizing rabbits with GST-FIGNL1 fusion proteins containing residues 1–360 of human FIGNL1 protein. Anti-KIAA0146 antibody was raised by immunizing rabbits with GST-KIAA0146 fusion proteins containing residues 1–300 of human KIAA0146 protein. Anti-RAD51 and anti-RAD51AP1 antibodies were raised by immunizing rabbits with GST-tagged full-length human RAD51 and RAD51AP1 proteins, respectively. Antimaltose-binding protein (MBP) antibody was raised by immunizing rabbits with purified full-length MBP protein. Antisera were affinity-purified using the AminoLink Plus Immobilization and Purification Kit (Pierce). Anti-γH2AX and anti-HARP antibodies were previously described (1, 2). Anti–β-actin and anti-FLAG were obtained from Sigma. The anti-myc antibody was obtained from Santa Cruz Biotechnology.

Constructs. Human FIGNL1 cDNA was obtained from the human ORFeome collection (hORFeome v5.1; GenBank accession no. BC051867). Human KIAA0146 cDNA was obtained from Harvard PlasmID. All cDNAs were subcloned into pDONR201 (Invitrogen) as entry clones and subsequently transferred to gateway-compatible destination vectors for the expression of N or C terminal-tagged fusion protein. Triple epitope of S protein, FLAG, and streptavidin-binding peptide (SFB), myc, MBP, and GST-tagged proteins were used in this study as described in the text. All point or deletion mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene) and verified by sequencing.

Cell Culture, Transfection, and shRNAs. HeLa, U2OS, and 293T cells were cultured in DMEM supplemented with 10% (vol/vol) FBS and 1% penicillin and streptomycin. Mouse embryonic fibroblasts (MEFs) were maintained in DMEM supplemented with 15% (vol/vol) FBS, 1 mM sodium pyruvate, and 1% penicillin and streptomycin. Plasmid transfection was performed using polyethylenimine reagent (Polysciences Inc). GIPZ lentiviral nonsilencing control shRNA and shRNA target sets were purchased from Open Biosystems. The ORF of GFP was deleted by site-directed mutagenesis PCR using the following primer set: forward, 5′-CTACTAGAGGATCTGCCACCATGGAGCGCGTGGAGGAGGATCACAGCAACAC-3′; reverse, 5′-GTGTTGCTGTGATCCTCCTCCACGCGCTCCATGGTGGCAGATCCTCTAGTAG-3′. The FIGNL1 targeting sequences are #276: 5′-CAGTCTGGATTGTCAATAA-3′; #515: 5′-CGGATCAAGTTCGACCCAT-3′. The breast cancer 2, early onset (BRCA2) targeting sequence is 5′-CTCTTAGCTGTCTTAAAGA-3′. The RAD51AP1 targeting sequence is 5′-AGGAGTTAAAACAAGATAA-3′. The lentiviral shRNA target set for KIAA0146 was purchased from Sigma. The KIAA0146 targeting sequences are #29: 5′-TAATTAAGGGTCTAACAAA-3′; #30: 5′-CGGATGCAAGACAGATTAT-3′. The shRNA-resistant WT and mutant FIGNL1 constructs were generated by changing 7 nt in the shRNA#515 targeting region (G1899A, T1902C, A1905G, T1908G, C1909A, A1911G, and C1914T substitutions). The shRNA-resistant WT and mutant KIAA0146 constructs were generated by changing 7 nt in the shRNA#29 targeting region (A1257C, T1260C, G1263A, T1266C, C1267T, A1269G, and A1272C substitutions). The shRNAs were packaged into lentiviruses by cotransfecting with packaging plasmids pMD2G and pSPAX2 (provided by Songyang Zhou, Baylor College of Medicine, Houston, TX) into 293T cells; 48 h after transfection, the supernatant was collected and used for infection of HeLa, DR-U2OS, or MEF cells. Infection was repeated two times, with an interval of 24 h to achieve maximal infection efficiency. Infected cells were selected with media containing puromycin (2 μg/mL).

Tandem Affinity Purification of RAD51 or FIGNL1-Associated Protein Complexes. Tandem affinity purification (TAP) was performed as previously described (2, 3). Briefly, 293T cells were transfected with plasmids encoding SFB-tagged RAD51 or SFB-tagged FIGNL1. Cell lines stably expressing tagged proteins were selected, and the expression of exogenous proteins was confirmed by immunoblotting and immunostaining. For affinity purification, a total of 20 10-cm dishes of 293T cells stably expressing tagged protein was collected and lysed with buffer of 20 mM Tris·HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and 1 μg/mL each pepstatin A and aprotinin (NETN buffer) containing 1 mM $MgCl_2$ and 250 U/mL Benzonase Nuclease (EMD Chemicals) for 25 min. Crude lysates were cleared by centrifugation, and the supernatants were incubated with 200 μL streptavidin Sepharose beads (Amersham Biosciences) for 2 h at 4 °C. The beads were washed three times with NETN and then eluted with 3 mg/mL biotin (Sigma) for 2 h at 4 °C. The eluates were incubated with 100 μL S-protein agarose beads (Novagen) for 2 h at 4 °C and then washed three times with NETN. The proteins bound to beads were eluted by boiling with SDS sample buffer, resolved by SDS/PAGE, visualized by Coommassie blue staining, and subjected to MS analysis for protein identification (performed by Taplin Biological MS Facility at Harvard University).

Immunoblotting. Cells were lysed with NETN buffer on ice for 30 min. Cleared cell lysates were then collected and boiled in 2× Laemmli buffer and separated by SDS/PAGE. Membranes were blocked in 5% (wt/vol) milk in Tris-buffered saline with Tween (TBST) buffer and then probed with antibodies as indicated.

Binding Assays. For coprecipitation assays, constructs encoding SFB-tagged and myc-tagged proteins were transiently cotransfected into 293T cells. Cells were lysed in NETN buffer on ice for 30 min, cleared by centrifugation, and incubated with S-protein beads for 2 h at 4 °C. Beads were washed, boiled in 2× Laemmli buffer, and separated on SDS/PAGE. For direct binding assays, bacterially purified MBP- and GST-tagged proteins were incubated together in NETN buffer containing Glutatione agarose beads for 2 h at 4 °C. Beads were washed, boiled in 2× Laemmli buffer, and separated on SDS/PAGE.

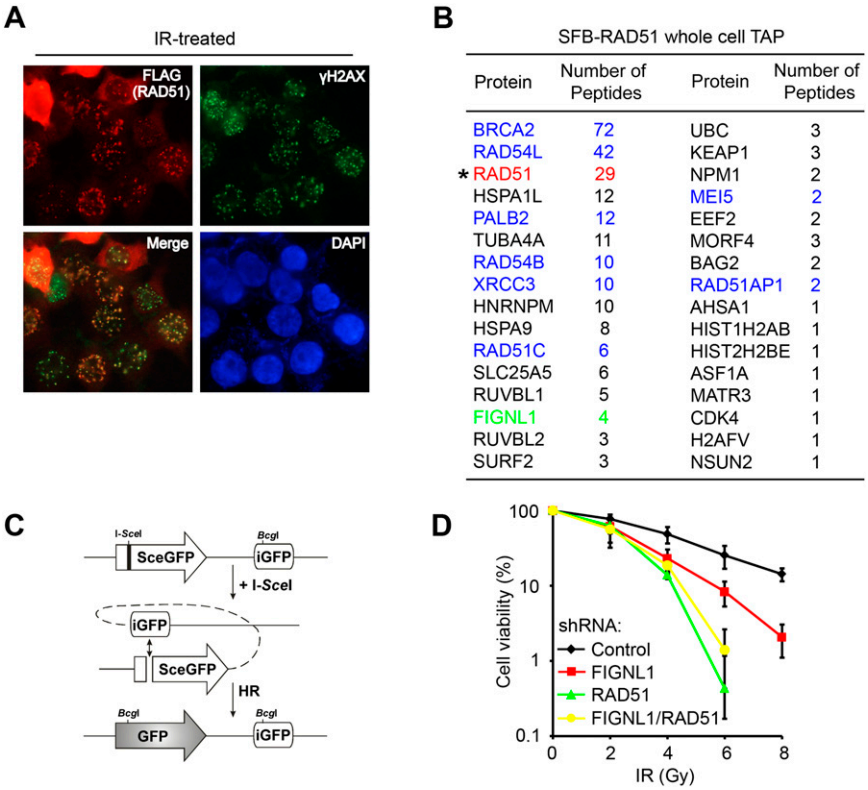
Immunostaining. Cells cultured on coverslips were washed with PBS, fixed with 3% (wt/vol) paraformaldehyde for 12 min, and extracted with 0.5% Triton solution for 5 min. Coverslips were washed with PBS and then immunostained with primary antibodies in 5% (vol/vol) goat serum for 60 min. Coverslips were washed and incubated with secondary antibodies conjugated with Rhodamine or FITC for 60 min. Cells were then stained with DAPI to visualize nuclear DNA. The coverslips were mounted onto glass slides with antifade solution and visualized using a Nikon ECLIPSE E800 fluorescence microscope with a Nikon Plan Fluor 60× oil objective lens (N.A. 1.30) at room temperature. Cells were photographed using a SPOT camera (Diagnostic Instruments) and analyzed using Photoshop software (Adobe).

Cell Survival Assays. A total of 1×10^3 cells were seeded onto 60-mm dishes in triplicates. Twenty-four hours after seeding, cells were treated with ionizing radiation (IR) or camptothecin at indicated concentrations. For camptothecin treatment, medium was replaced 24 h later, and cells were then incubated for 14 d. Resulting colonies were fixed and stained with Coommassie blue.

Numbers of colonies were counted using a Gel Doc with Quantity One software (BIORAD).

Homologous Recombination Assay. A U2OS cell clone stably expressing homologous recombination (HR) reporter direct repeat green fluorescent protein (DR-GFP) was described previously (4). This reporter consists of two differentially mutated GFP genes oriented as direct repeats. Expression of I-SceI endonuclease will generate a site-specific ds break (DSB) between the mutated GFP genes, which when repaired by gene conversion, results in a functional GFP gene. Briefly, 1×10^6 DR-GFP U2OS cells were electroporated with 20 μg pCBASce, an I-SceI expression vector described previously (5). Cells were harvested 1 d after electroporation and subjected to flow cytometry analysis to determine percentages of GFP-positive cells resulting from HR repair induced by I-SceI–induced DSBs.

1. Huen MS, et al. (2007) RNF8 transduces the DNA-damage signal via histone ubiquitylation and checkpoint protein assembly. *Cell* 131(5):901–914.
2. Yuan J, Ghosal G, Chen J (2009) The annealing helicase HARP protects stalled replication forks. *Genes Dev* 23(20):2394–2399.
3. Yuan J, Ghosal G, Chen J (2012) The HARP-like domain-containing protein AH2/ZRANB3 binds to PCNA and participates in cellular response to replication stress. *Mol Cell* 47(3):410–421.
4. Weinstock DM, Nakanishi K, Helgadottir HR, Jasin M (2006) Assaying double-strand break repair pathway choice in mammalian cells using a targeted endonuclease or the RAG recombinase. *Methods Enzymol* 409:524–540.
5. Richardson C, Moynahan ME, Jasin M (1998) Double-strand break repair by interchromosomal recombination: suppression of chromosomal translocations. *Genes Dev* 12(24):3831–3842.



Protein	Number of Peptides	Protein	Number of Peptides
BRCA2	72	UBC	3
RAD54L	42	KEAP1	3
* RAD51	29	NPM1	2
HSPA1L	12	MEI5	2
PALB2	12	EEF2	2
TUBA4A	11	MORF4	3
RAD54B	10	BAG2	2
XRCC3	10	RAD51AP1	2
HNRNPM	10	AHSA1	1
HSPA9	8	HIST1H2AB	1
RAD51C	6	HIST2H2BE	1
SLC25A5	6	ASF1A	1
RUVBL1	5	MATR3	1
FIGNL1	4	CDK4	1
RUVBL2	3	H2AFV	1
SURF2	3	NSUN2	1

Fig. S1. TAP of RAD51-containing protein complexes. (*A*) 293T cells stably expressing SFB-RAD51 were subjected to immunostaining experiments 8 h after IR treatment (10 Gy) using indicated antibodies. (*B*) TAP was performed using 293T cells stably expressing SFB-tagged RAD51. The data from MS analysis are shown. The reported HR factors are colored in blue. (*C*) Schematic representation of HR assay. The DR-GFP substrate for I-SceI–induced DSB repair is shown. (*D*) Survival curves in response to increasing doses of IR for FIGNL1 and RAD51-depleted cells are presented. Down-regulation of FIGNL1 or RAD51 using specific shRNAs was confirmed by immunoblotting (Fig. 1*D*).

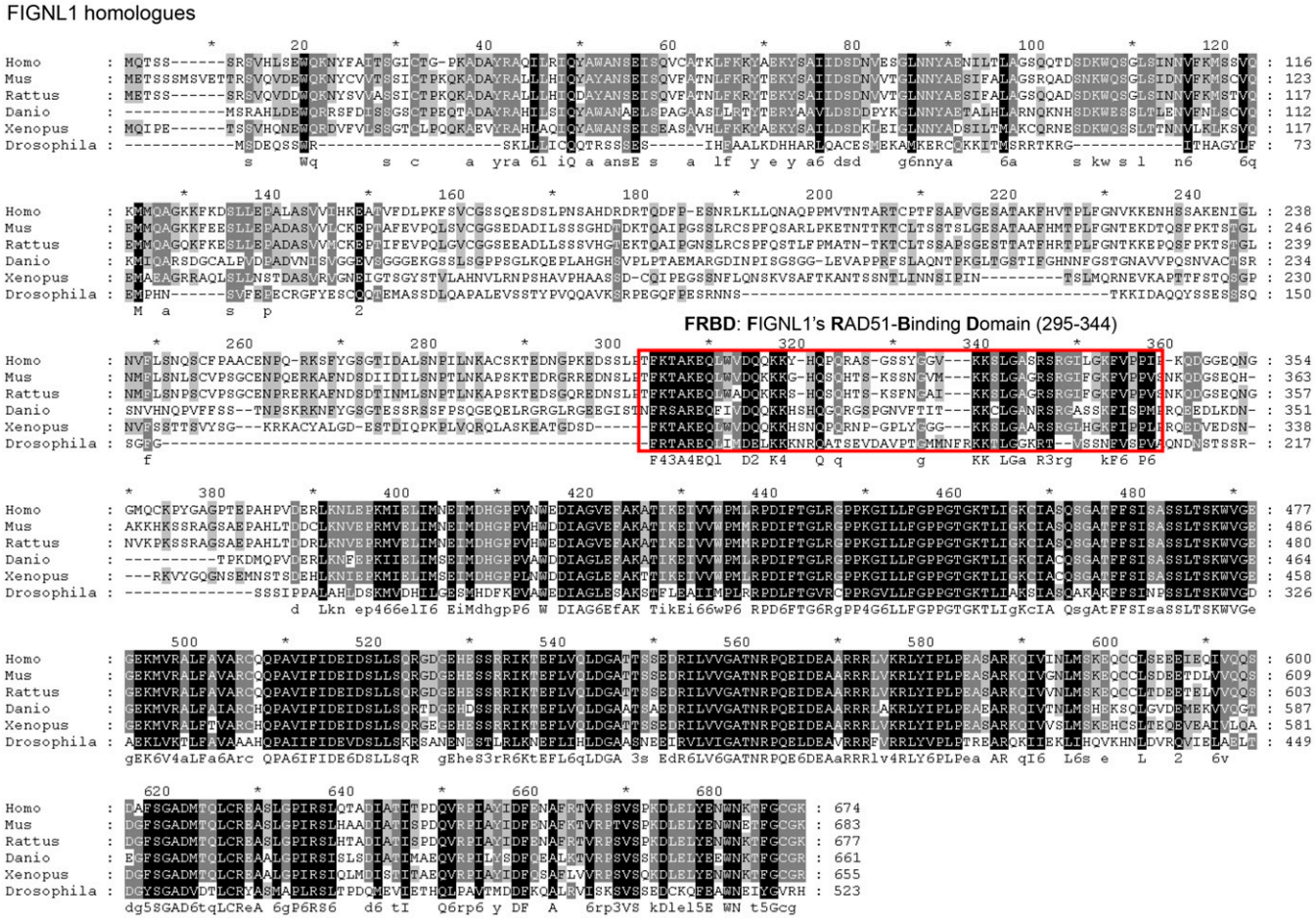


Fig. S2. Comparison of FIGNL1 amino acid sequences from different species. Black and gray shadings indicate, respectively, identical and conserved residues. The conserved FIGNL1's RAD51 binding domain (FRDB) is indicated.

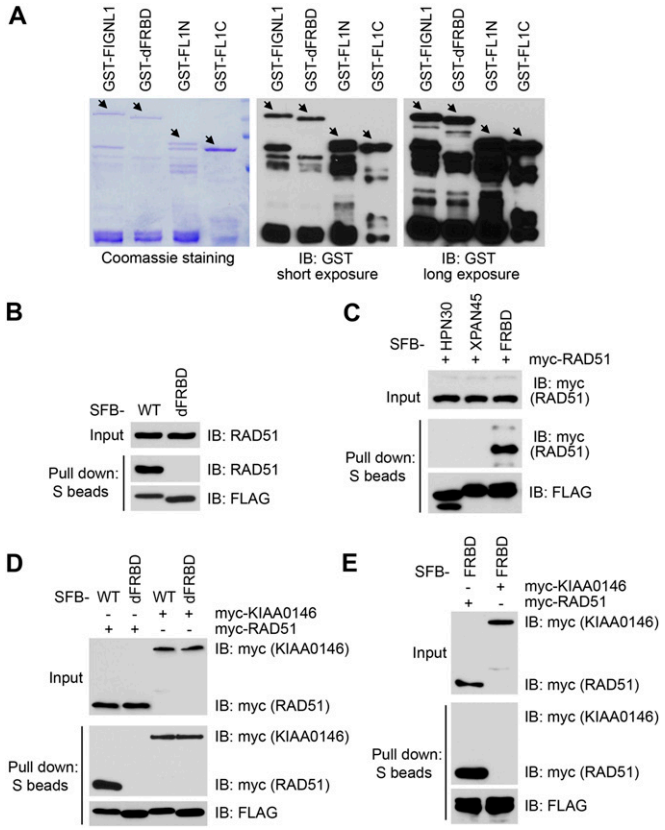


Fig. S3. FRBD domain is critical for FIGNL1 binding to RAD51. (*A*) Purification of GST-tagged FIGNL1 and FIGNL1 mutants from bacteria. Purified proteins were separated by SDS/PAGE and subjected to Coomassie staining and immunoblotting using anti-GST antibody. The main bands are indicated by arrows. The lower bands are caused by protein degradation. (*B*) FRBD is required for the binding of FIGNL1 to RAD51. (*C*) FRBD alone binds to RAD51. HPN30 (HARP RPA binding domain) and XPAN45 (XPA RPA binding domain) were used as negative controls. (*D*) The binding of KIAA0146 to FIGNL1 does not require FRBD domain. (*E*) FRBD domain strongly binds to RAD51 but not KIAA0146. In the coprecipitation assays shown in *B*–*E*, 293T cells were transfected with plasmids encoding myc- and SFB-tagged proteins as indicated. Coprecipitation was carried out using S-protein beads, and immunoblotting was performed using antibodies as indicated. IB, immunoblotting.

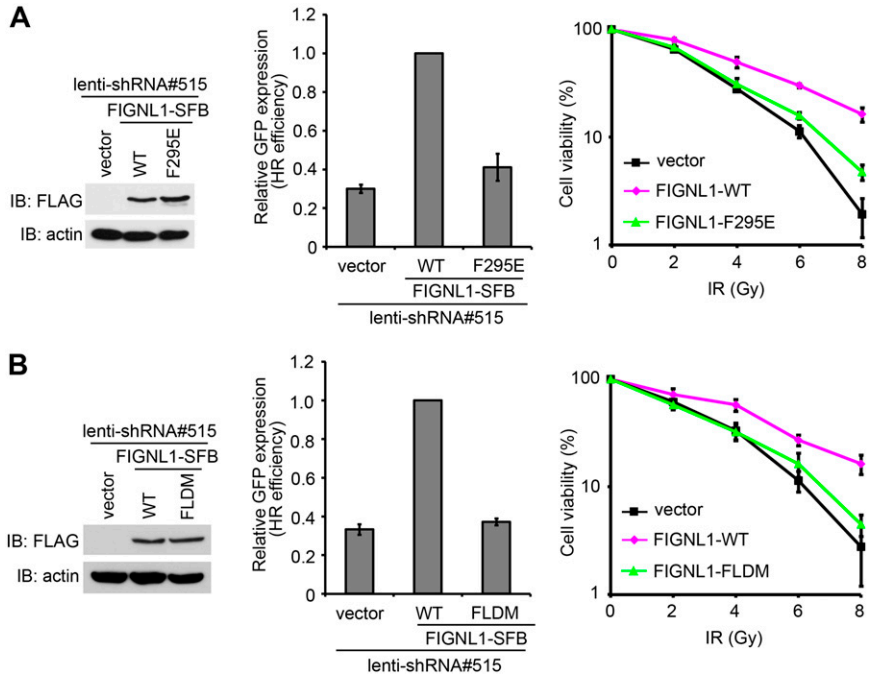


Fig. S4. F295E and ATPase mutants of FIGNL1 could not rescue HR efficiency and cellular sensitivity to IR in FIGNL1-depleted cells. (*A* and *B*) FIGNL1-depleted (shRNA#515) DR-GFP U2OS derivative cell lines stably expressing shRNA-resistant WT, F295E, or the ATPase mutant (FLDM; with both Walker A motif mutation K447A and Walker B motif mutation D500A) of FIGNL1 were generated. The empty vector was included as control. The exogenous FIGNL1 expression was confirmed by immunoblotting. Data are presented as mean ± SD from three different experiments. IB, immunoblotting.

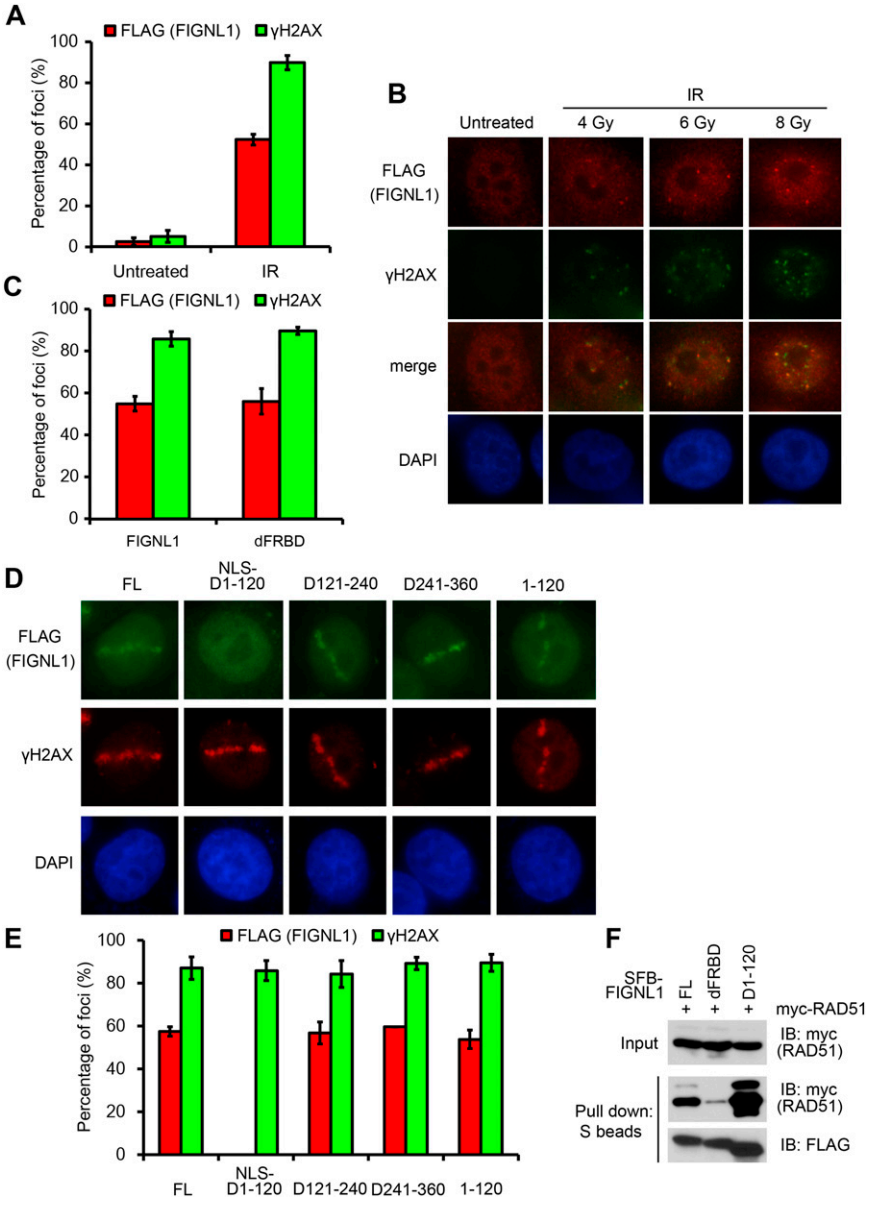


Fig. S5. FIGNL1 is recruited to sites of DNA damage. (*A*) HeLa cells were infected with lentiviral particles expressing SFB-tagged FIGNL1. (*B*) HeLa cells were infected with lentiviral SFB-tagged FIGNL1. Immunostaining experiments were performed 6 h after IR treatment (at different doses) using indicated antibodies. (*C*) FRBD domain is not required for FIGNL1 foci formation. HeLa cells were infected with lentiviral particles expressing SFB-tagged FIGNL1 or the FRBD deletion mutant (dFRBD). Immunostaining experiments in *A* and *C* were performed 6 h after IR treatment (10 Gy) using indicated antibodies. The quantification of foci-positive cells in *A* and *C* was performed by counting a total of 100 cells per sample. Cells detected with eight or more foci (γH2AX) or four or more foci (FIGNL1) are counted as foci-positive cells. Data are presented as mean ± SD from three different experiments. (*D*) The N-terminal 120 aa of FIGNL1 are required for the recruitment of FIGNL1 to sites of DNA damage. A nuclear localization signal (NLS) was added to the N terminus of mutant D1-120 (NLS-D1-120) to ensure its nuclear localization. HeLa cells were infected with lentiviral particles expressing SFB-tagged FIGNL1 or other mutants. Cells were laser-microirradiated and analyzed by immunostaining with indicated antibodies. (*E*) HeLa cells were infected with lentiviral particles expressing SFB-tagged FIGNL1 or other mutants. Immunostaining experiments were performed 6 h after IR treatment (10 Gy) using indicated antibodies. The quantification of foci-positive cells was performed by counting a total of 100 cells per sample. Cells detected with eight or more foci (γH2AX) or four or more foci (FIGNL1) are counted as foci-positive cells. Data are presented as mean ± SD from three different experiments. (*F*) The N-terminal 120 aa of FIGNL1 are not required for its binding to RAD51. 293T cells were transfected with plasmids encoding myc- and SFB-tagged proteins as indicated. Coprecipitation was carried out using S-protein beads, and immunoblotting was performed using antibodies as indicated. FL, full length; IB, immunoblotting.

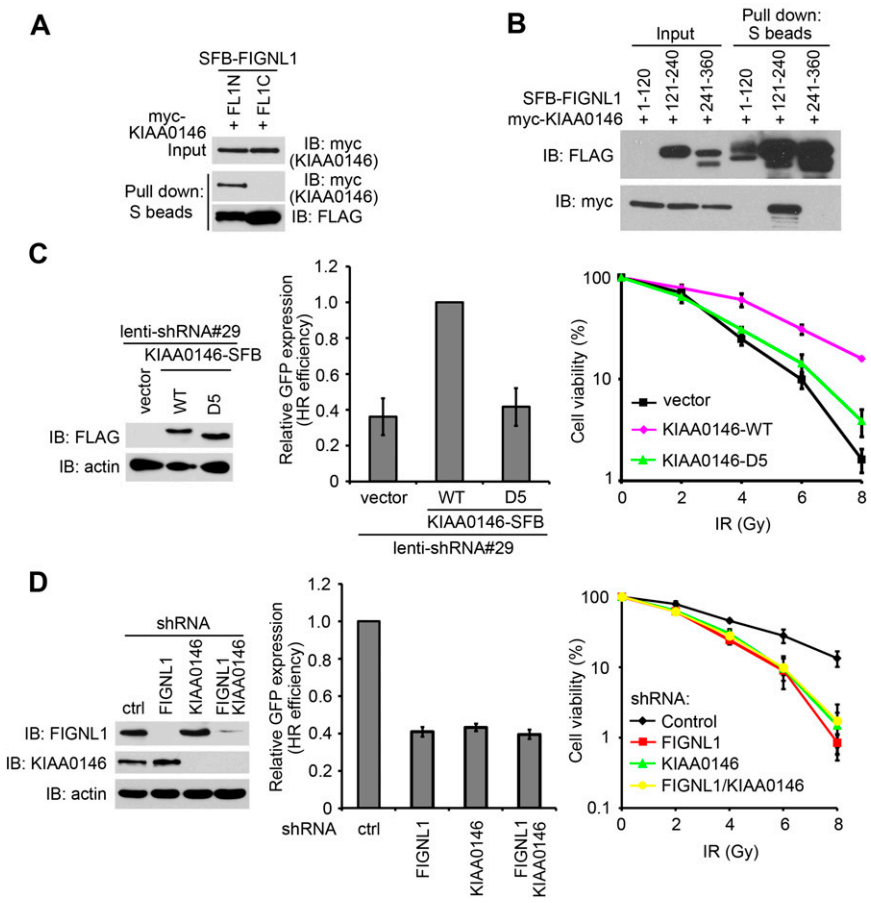


Fig. S6. The interaction between FIGNL1 and KIAA0146 is important for HR repair. (*A*) The N-terminal one-half of FIGNL1 binds to KIAA0146. (*B*) Amino acids 121–240 of FIGNL1 bind to KIAA0146. The 1–120 mutant was unstable when overexpressed in 293T cells. It could only be detected in precipitated fraction. In the coprecipitation assays shown in *A* and *B*, 293T cells were transfected with plasmids encoding myc- and SFB-tagged proteins as indicated. Coprecipitation was carried out using S-protein beads, and immunoblotting was performed using antibodies as indicated. (*C*) The C-terminal deletion mutant (D5) of KIAA0146 could not rescue HR efficiency and cellular sensitivity to IR in KIAA0146-depleted cells. KIAA0146-depleted (shRNA#29) DR-GFP U2OS derivative cell lines stably expressing shRNA-resistant WT or the D5 mutant of KIAA0146 were generated. The empty vector was included as control. The exogenous KIAA0146 expression was confirmed by immunoblotting. (*D*) FIGNL1 and KIAA0146 codepletion does not have additive effects on HR repair and cellular sensitivity to IR. Down-regulation of FIGNL1 and KIAA0146 in DR-GFP U2OS cells was confirmed by immunoblotting. In *C* and *D*, DR-GFP U2OS cells were electroporated with pCBASce plasmids. The percentage of GFP-positive cells was determined by flow cytometry 48 h after electroporation. The data were normalized to those results obtained from cells infected with control shRNA (set as 1.0). Data are presented as mean ± SD from three different experiments. Survival curves are shown for indicated cell lines in response to increasing doses of IR. Data are presented as mean ± SD from three different experiments. IB, immunoblotting.

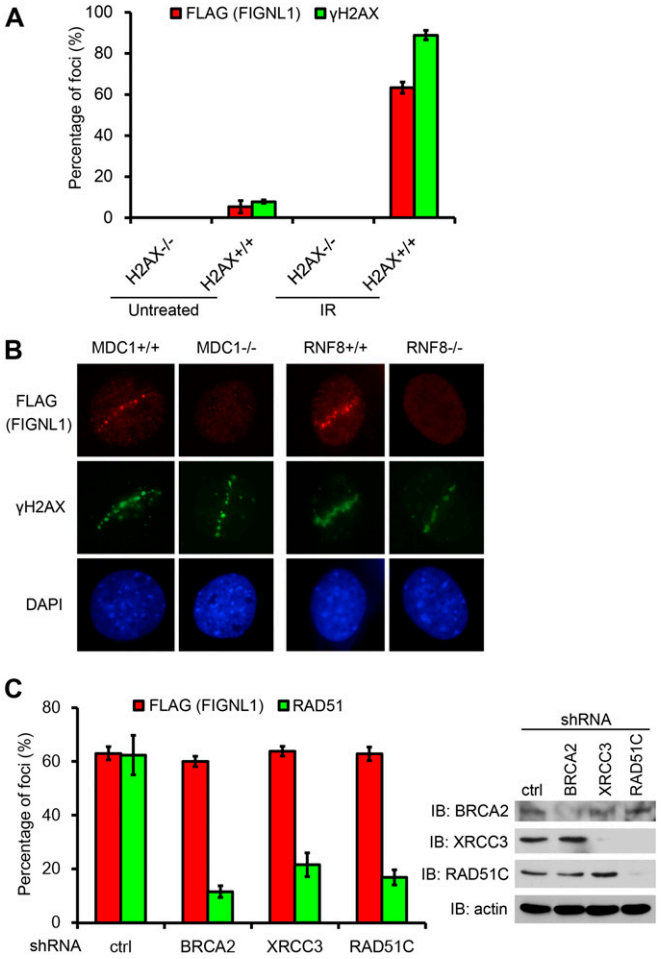


Fig. S7. FIGNL1 is retained at sites of DNA damage in an H2AX-, MDC1-, and RNF8-dependent but BRCA2-, XRCC3-, and RAD51C-independent manner. (*A*) $H2AX^{-/-}$ and $H2AX^{+/+}$ MEFs were infected with lentiviral particles expressing SFB-tagged FIGNL1. Cells were irradiated and fixed 6 h after IR. Immunostaining was carried out with indicated antibodies. The quantification of foci-positive cells was performed by counting a total of 100 cells per sample. Cells detected with eight or more foci (γH2AX) or four or more foci (FIGNL1) are counted as foci-positive cells. Data are presented as mean ± SD from three different experiments. (*B*) $MDC1^{-/-}$, $RNF8^{-/-}$, and their corresponding WT MEFs were infected with lentiviral particles expressing SFB-tagged FIGNL1. Cells were laser-microirradiated and analyzed by immunostaining with indicated antibodies. (*C*) BRCA2, XRCC3, or RAD51C depletion does not affect FIGNL1 foci formation after IR treatment. HeLa cells stably expressing SFB-tagged FIGNL1 were infected with lentiviral shRNAs as indicated. Cells were irradiated and fixed 6 h after IR. The knockdown efficiency was confirmed with immunoblotting. Immunostaining was carried out with indicated antibodies. The quantification of foci-positive cells was performed by counting a total of 100 cells per sample. Cells detected with eight or more foci (RAD51) or four or more foci (FIGNL1) are counted as foci-positive cells. Data are presented as mean ± SD from three different experiments. Ctrl, control; IB, immunoblotting.