Supplemental Information

The Mre11-Nbs1 interface is essential for viability and tumor suppression

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Figure S1. TALEN targeting for *Nbs1*^{mid} mutant mouse lines, Related to Figure1.
(A) Binding sites of two recombinant TALE nucleases (TALEN) targeting Mre11-interacting domain 2 of mouse Nbs1 gene. (B) Examples of genomic sequences of *Nbs1*^{mid} mutant mouse lines. Binding sites of two recombinant TALEN are indicated in blue color. XmnI restriction enzyme site that was used for initial diagnosis of gene editing by TALEN is indicated in red color. The sequence of Mre11 interacting domain 2 is indicated in green color.

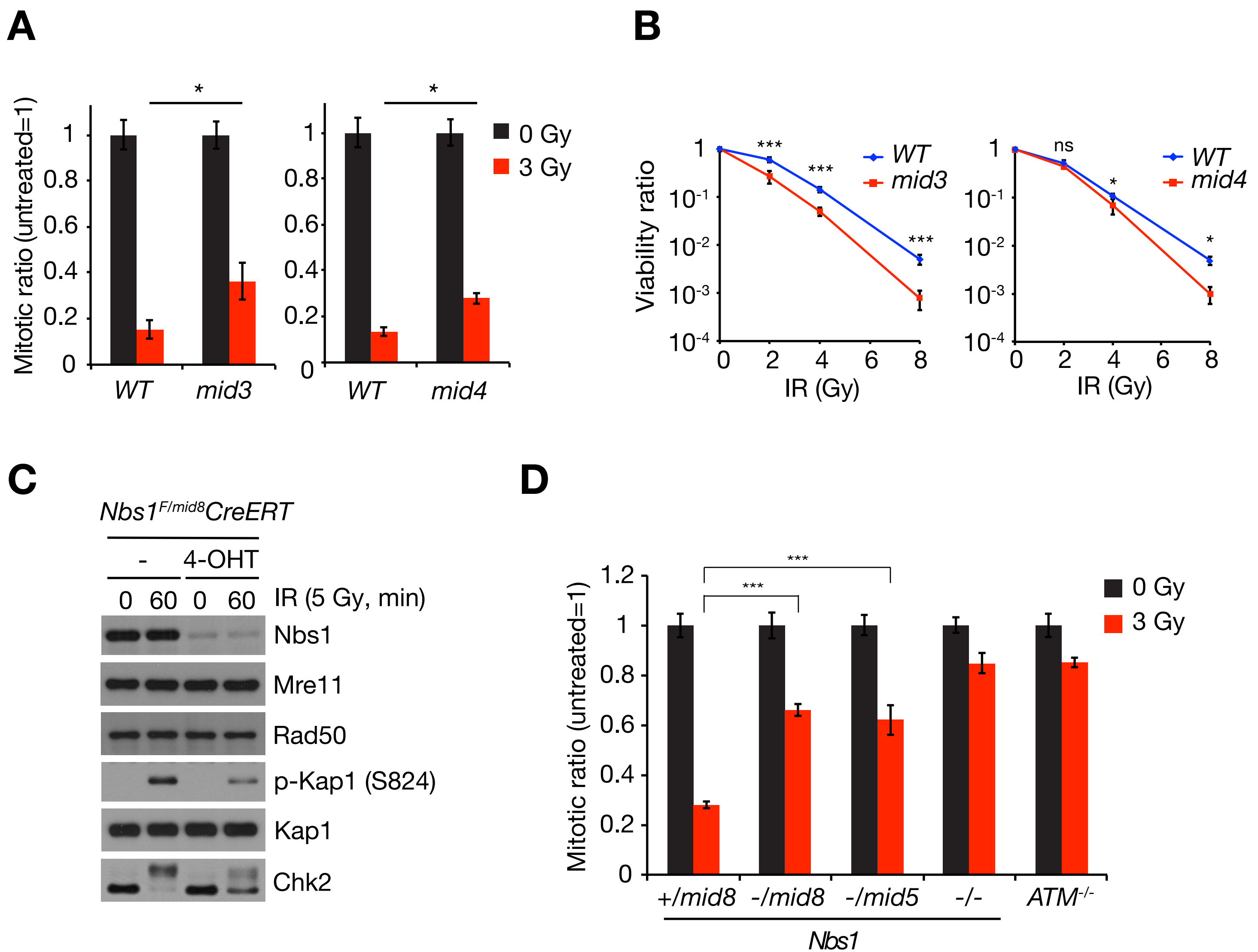

Figure S2. G2/M checkpoint and IR sensitivity of *Nbs1^{mid}* mutants cells, Related to Figure 2. (A) Analysis of G2/M checkpoint in *Nbs1^{mid3}* and *Nbs1^{mid4}* SV40-MEFs. Mitotic cells were detected by measuring mitosis-specific phosphorylation of histone H3 (Ser10). *P-value* was determined by unpaired t-test (**p<0.05*, mean ± s.d., Two or three independent experiments in triplicate). (B) Colony formation assay to determine the DNA damage sensitivity. *Nbs1^{mid3}* and *Nbs1^{mid4}* SV40-MEFs treated by different dose of IR were grown for 10 days and survived colonies were counted. *P-value* was determined by unpaired t-test (**p<0.05*, ****p<0.001*, mean ± s.d., Two or three independent experiments in triplicate). (A and B) *WT* SV40-MEFs generated with littermate embryo of each genotype were used for comparison. (C) ATM signaling in *Nbs1$^{-/mid8}$* SV40-MEFs was assessed by Western blot for the phosphorylation of ATM substrates, KAP1 (S824) and Chk2, after IR treatment. (D) Analysis of G2/M checkpoint in *Nbs1$^{-/mid5}$* and *Nbs1$^{-/mid8}$* SV40-MEFs. Mitotic cells were detected by measuring mitosis-specific phosphorylation of histone H3 (Ser10). *Nbs1$^{+/mid8}$*, *Nbs1$^{-/-}$*, *ATM$^{-/-}$* SV40-MEFs were used for controls. *P-value* was determined by unpaired t-test (****p<0.001*, mean ± s.d., n=3).

Figure S3

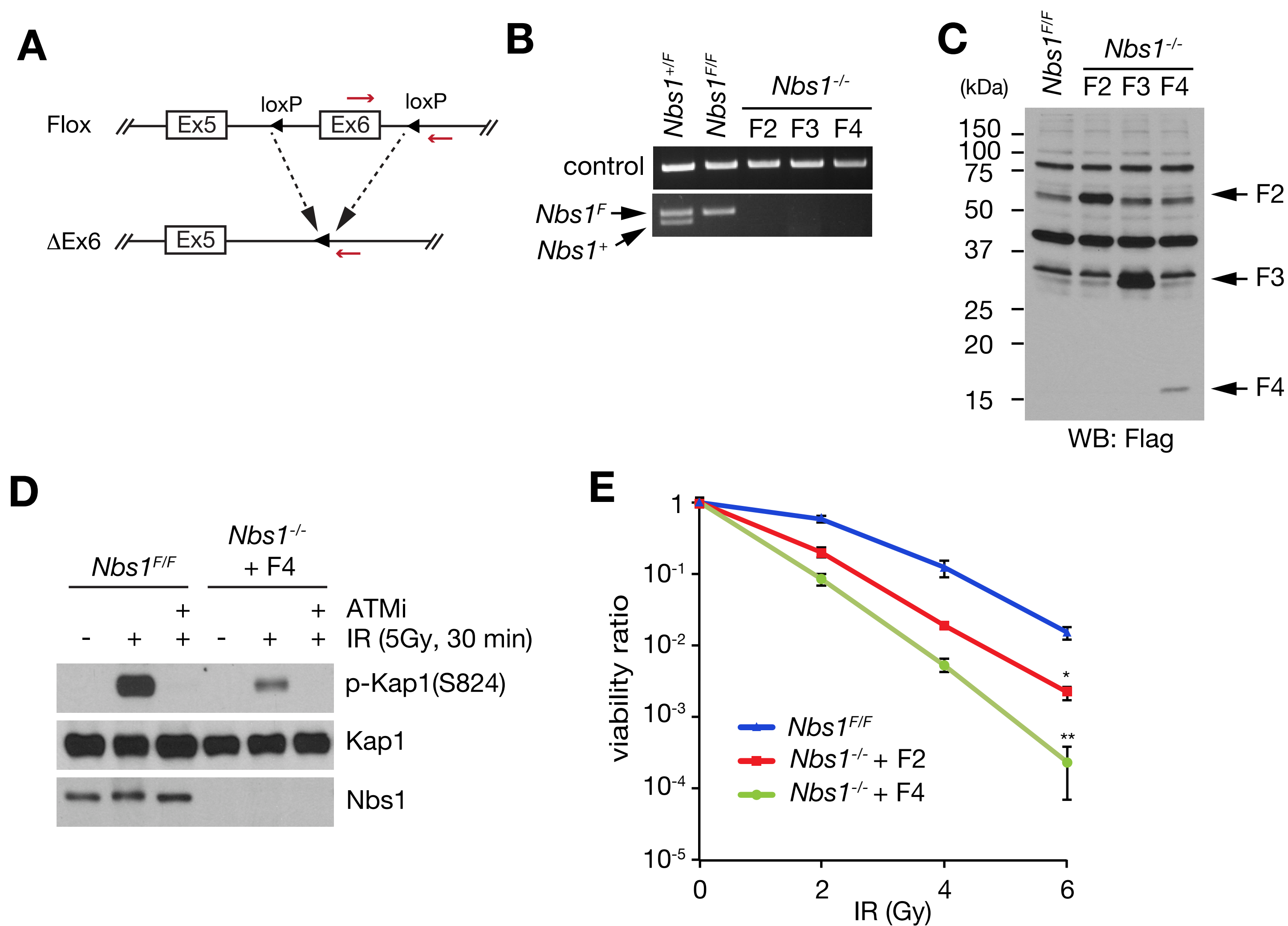

Figure S3. Verification and analysis of rescue cell lines, Related to Figure 3.
(A) *Nbs1*^{F} allele and genotype PCR primers are indicated. Primers are indicated in red arrow and sequences were previously described (Frappart et al., 2005). (B) PCR genotyping of *Nbs1*$^{-/-}$ rescue cells. (C) Expression of rescue fragments was shown by Western blot using anti-Flag antibody. (D) IR-induced p-Kap1(S824) was assessed in the absence or presence of KU-55933 ATM inhibitor (10 μM 1 hr pretreated). Nbs1 level shows the absence of endogenous Nbs1 in *Nbs1*$^{-/-}$ + F4 cells. Colony formation assay to determine the DNA damage sensitivity of *Nbs*$^{-/-}$+ F2 and *Nbs*$^{-/-}$+ F4 SV40-MEFs. Parental *Nbs*$^{F/F}$ SV40-MEFs were used for comparison. Cells treated with different dose of IR were grown for 10 days and survived colonies were counted. *P-value* was determined by unpaired t-test (**p<0.05* for *Nbs*$^{F/F}$ vs. *Nbs*$^{-/-}$+ F2; ***p<0.01* for *Nbs*$^{-/-}$+ F2 vs. *Nbs*$^{-/-}$+ F4, mean ± s.d., Two independent experiments in triplicate).

Figure S4

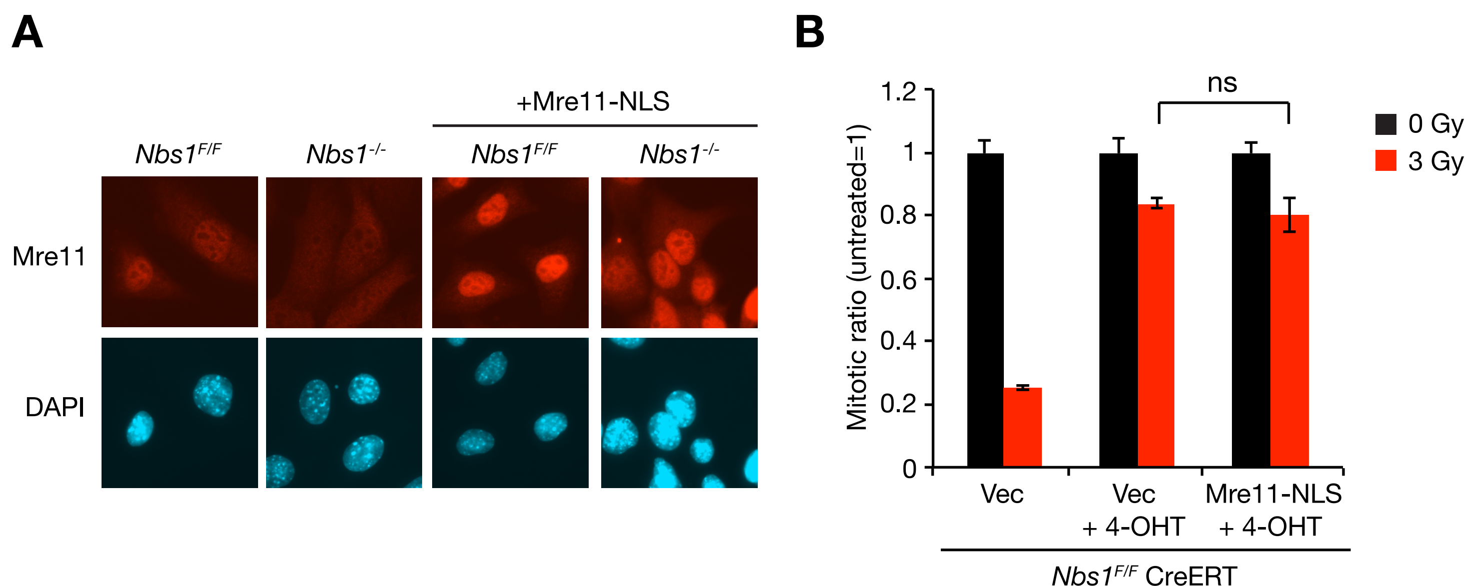

Figure S4. Mre11-NLS expression fails to rescue Nbs1 deficiency, Related to Figure 3.
(A) Immunofluorescence cell staining of Mre11-NLS in *Nbs1*$^{-/-}$ SV40-MEFs. Nbs1 deficiency was achieved by cre induction acutely. Nuclei are shown by DAPI (4',6-diamidino-2-phenylindole) staining. (B) IR-induced G2/M cell cycle checkpoint of *Nbs1*$^{-/-}$-Mre11-NLS SV40-MEFs. Mitotic cells were detected by measuring mitosis-specific phosphorylation of histone H3 (Ser10). Three independent clones of *Nbs1*$^{-/-}$-Mre11-NLS SV40-MEFs were analyzed and combined for graph presentation. *P-value* was determined by unpaired t-test (ns: not significant).

Figure S5

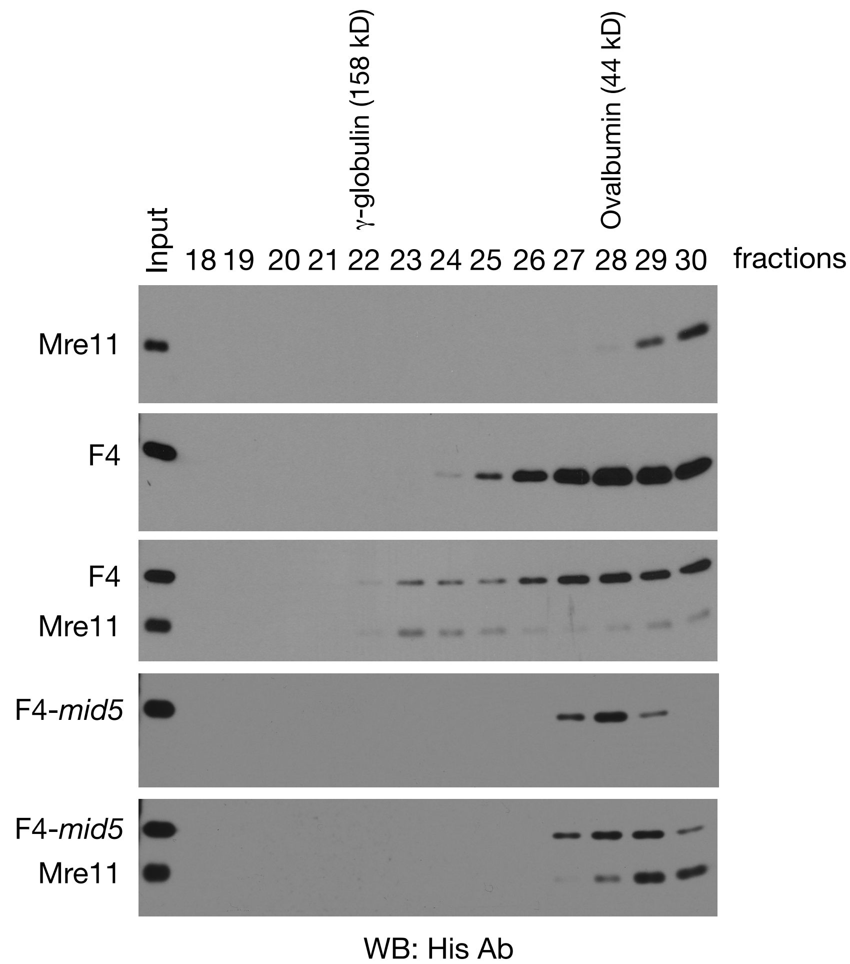

Figure S5. Gel filtration of Mre11 and Nbs1-F4 complex by Superdex 200, Related to Figure 4. Equimolar mixture of human Mre11 (1-411aa) and *WT* or *mid5* Nbs1-F4 protein were used at 0.5 μM. Elution fractions were visualized by Western blot using His antibody. Molecular weight was estimated by gel filtration standard.

Figure S6

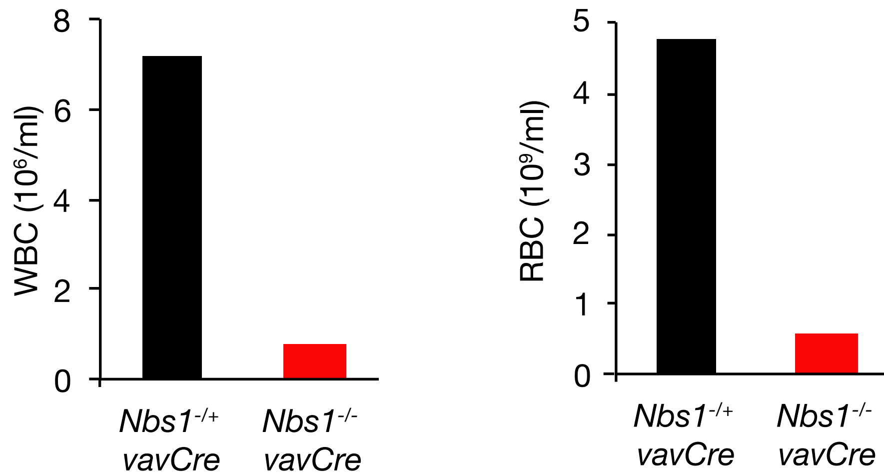

Figure S6. Hemavet quantification of whole blood cell numbers from 12 days-old *Nbs1*$^{-/+}$ *vavCre* and *Nbs1*$^{-/-}$ *vavCre* mice, Related to Figure 6. Peripheral blood from mouse of each genotype was obtained from the tail vein and complete blood cell analysis was performed on Hemavet (Drew Scientific). WBC= white blood cells, RBC=red blood cells.

Table S1. Pathology of *Nbs1*$^{+/+}$ *p53*$^{-/-}$, *Nbs1*$^{mid3/mid3}$ *p53*$^{-/-}$ and *Nbs1*$^{mid4/mid4}$ *p53*$^{-/-}$ mice, Related to Figure 2.

Nbs1$^{+/+}$ *p53*$^{-/-}$

case	Sex	Age (days)	Histological types
1	male	102	lymphoma
2	female	117	ND
3	female	123	lymphoma
4	male	145	lymphoma
5	female	152	lymphoma
6	female	165	lymphoma
7	female	173	lymphoma
8	male	180	lymphoma
9	female	181	ND
10	female	188	lymphoma
11	male	194	lymphoma
12	male	200	lymphoma
13	female	209	lymphoma
14	male	212	lymphoma
15	male	266	lymphoma

Nbs1$^{mid3/mid3}$ *p53*$^{-/-}$

case	Sex	Age (days)	Histological types
1	male	67	leiomyosarcoma
2	male	90	lymphoma
3	female	126	lymphoma, squamous cell carcinoma
4	male	127	lymphoma
5	female	128	lymphoma
6	male	131	lymphoma
7	male	135	lymphoma
8	female	156	lymphoma
9	male	162	lymphoma
10	male	167	lymphoma

Nbs1$^{mid4/mid4}$ *p53*$^{-/-}$

case	Sex	Age (days)	Histological types
1	male	76	lymphoma
2	male	95	lymphoma
3	male	97	lymphoma
4	female	102	lymphoma
5	male	110	hemangiosarcoma
6	male	111	lymphoma
7	male	124	lymphoma
8	male	129	lymphoma
9	male	140	lymphoma
10	male	154	lymphoma
11	female	154	lymphoma
12	male	159	rhabdomyosarcoma
13	male	161	lymphoma
14	male	162	lymphoma
15	female	170	lymphoma
16	male	176	lymphoma
17	male	181	histiocytic sarcoma
18	male	186	lymphoma
19	male	186	lymphoma
20	male	195	ND

ND : not determined.

Supplemental Experimental Procedures

Cell lines

Primary mouse embryonic fibroblasts (MEFs) were derived from E13.5 embryos and maintained in DMEM/10% fetal bovine serum/antibiotics. SV40 transformed MEFs were maintained in DMEM/10% cosmic calf serum.

Inducible MEFs were derived by crossing of *Nbs1*$^{+/mid5\ or\ mid8}$ mice with *Nbs1*^{F} mice. MEFs in which deletion of *Nbs1*^{F} allele is 4-hydroxytamoxifen (4-OHT) inducible were generated by stable expression of MSCV CreERT2 puro (a gift from Tyler Jacks; Addgene plasmid #22776). Deletion was carried out by 1 day exposure with 100 nM 4-OHT treatment followed by removal for additional 3 days to minimize Cre toxicity.

Nbs$^{-/F}$ SV40-MEFs were kindly provided by Titia de Lange (Rockefeller University, USA).

Rescue cells were generated by expression of Flag-SV40 NLS (PKKKRKV) containing Nbs1 fragments followed by deletion of endogenous *Nbs1*^{F} allele. Nbs1 fragments were cloned into pMIG-W-IRES-GFP plasmid (a gift from Luk Parjis; Addgene plasmid #12282). Deletion of endogenous *Nbs1*^{F} allele and expression of exogenous Nbs1 fragments were confirmed by Western blot and PCR genotyping. Mre11-NLS construct was made by insertion of 2 copies SV40 NLS (PKKKRKV) at C terminus of Mre11 cDNA.

Cellular assay

Immunoprecipitaions (IP) were performed with 500 μg of Flag-tagged Nbs1 expressing cells extracts using 1X PBS/0.5% (v/v) TritonX-100/400 mM NaCl/Protease

inhibitor cocktail buffer (Roche). Flag peptide (F3290, Sigma) was added to *WT* sample at 100 μg/ml for a Flag IP control.

Western blots were carried out by standard protocol. Briefly, total cell extracts were prepared in SDS lysis buffer (60 mM Tris-HCl pH 6.8, 2% SDS) and 20-40 μg of extracts were analyzed with specific antibodies. Antibodies used in this study were Mre11 (custom made), Nbs1 (custom made), Rad50 (custom made), p-Kap1 S824 (ab70369, Abcam), total Kap1 (NB500-159, Novus), Chk2 (05-649, Millipore), p-ATM S1981 (#4526L, Cell signaling), total ATM (#2873S, Cell signaling), and Flag (F3165, Sigma).

G2M cell cycle checkpoint assay was performed by flow cytometry measuring Ser10 phosphorylation of histone H3 with anti-phospho-Ser10-Histone H3 antibody (06-570, Millipore) 1 hr after 3 Gy of IR exposure.

For colony formation assay, cells were plated with different IR treatments and cultured for 10 days. Colonies were visualized by crystal violet stain and counted.

For micronuclei staining, cells were fixed with 4% (v/v) formaldehyde in PBS for 15 min at RT and permeabilized in PBS containing 0.5% (v/v) TritonX-100. Cells were mounted with ProLong® Gold Antifade DAPI-Mountant (Life technologies).

For metaphase spread, cells were treated with 100 ng/ml of KaryoMAX colcemid (Life technologies) for 1 hr and harvested. Cells were swelled in 0.075 M KCl for 15 min at 37°C and fixed in ice-cold 3:1(v/v) methanol: acetic acid. Dropped samples on slides were stained with 5% Giemsa (Sigma) and mounted with Permount medium (Fisher Scientific). More than 40 spreads were analyzed per each sample.

Fetal liver cell (FLC) transplantation

For donor FLCs, fetal liver cells were isolated from E13.5 *Nbs1$^{F/F}$vavCre* embryos and red blood cells were lyzed in ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). FLCs were maintained in FLC media (DMEM-IMDM1:1/10% FBS/4 mM L-glutamine/50 mM β-mercaptoethanol, 10 ng/ml IL-3, 10 ng/ml IL-6, 20 ng/ml SCF). For transplantation, donor *Nbs1$^{F/F}$vavCre* FLCs were infected by spin infection (1800 rpm for 45 min with 8 μg/ml of polybrene) with retrovirus expressing Nbs1 fragment-IRES-GFP. As the viability of *Nbs1$^{F/F}$vavCre* FLCs dropped during the culture after isolation, cells were infected and cultured only for 3 days before transplantation. Due to the mouse strain of donor *Nbs1$^{F/F}$vavCre* FLCs, we used F1 hybrid of C57BL/6 x 129 as a recipient. Briefly, 6-week old F1 hybrid mice were irradiated at lethal dose (5 Gy x 2 times) and 3×10^5 FLCs were transplanted via tail vein injection. After 10 weeks post transplantation, thymocytes and splenocytes were isolated and analyzed to assess GFP^+ cells that were derived from donor FLCs expressing Nbs1 fragment-IRES-GFP. Rescued cells by Nbs1 fragment-IRES-GFP were confirmed by flow cytometry and PCR genotyping using primers for *vavCre* or exogenous Nbs1 rescue fragments that are specific for donor cells. Primers for PCR genotyping are *vavCre* allele and Nbs1 rescue fragments are 5'-CAAGTGACAGCAATGCTGTTTCAC-3', 5'-CAGGTATCTCTGACCAGAGTCATC-3' for Cre and 5'-CAGTGAGGAGCTGCCACGGAAACT-3', 5'-TCTAACTCGGTATTCTTTCGAGCATGGT-3' for Nbs1 rescue fragments.

Protein purification and analysis

Bacterial expression vector for N-terminal his-tagged human Mre11 (2-411aa) was gifted from Dr. John Tainer (Lawrence Berkeley National Laboratory, USA). With C-

terminal his-tag, human Nbs1 (F4, 622-729aa) was constructed in pMAL vector (New England BioLabs) for N-terminal MBP-tag for its solubility.

For purification, Mre11 protein was purified by serial FPLC purification using HisTRAP (GE Healthcare) and HiTRAP Q FF (GE Healthcare) column followed by Superdex S200 (GE Heathcare) gel filtration. FPLC running buffers are; 20 mM Tris-HCl pH8, 0.5 M NaCl, 0.5 mM DTT, 5 mM imidazole for HisTRAP; 20 mM Tris-HCl pH 8, 0.1 mM EDTA, 2 mM DTT for HiTRAP Q FF ; 20 mM Tris-HCl pH 8, 0.1 mM EDTA, 0.2 M NaCl, and 2 mM DTT for Superdex S200. Nbs1 proteins were purified by batch affinity purification methods using amylose resin (New England BioLabs) followed by Nickel-affinity purification (Qiagen) by manufacturer's standard protocol. Then, the samples were subject to FPLC Superdex S200 gel filtration to remove imidazole from the elution buffer. Mre11 and Nbs1 proteins were eluted at the single peak as a monomer at the given gel filtration condition.

For electrophoretic mobility shift assay (EMSA), the indicated amounts of purified Mre11 and Nbs1 proteins were incubated with ^{32}P-end labeled dsDNA or hairpin DNA probe in the binding condition of 25 mM Tris-HCl pH8, 100 mM NaCl, 1 mM DTT for 20 min at RT. The sequences of ds DNA is 5'-GTCTTCAGGACAGCAGTGAGGAGAACCCACGGAAACTGCTGCTGACTGA-3', and hairpin is 50 nt (DAR134) previously used (Paull and Gellert, 1998). The reactions were loaded in to 5% native-PAGE and run for 1hr at 200 V in 0.5x TBE buffer. After drying gel, the probes in the gel were visualized by phosphorimager (Fujifilm). For supershift, 1 μg of MBP (sc-32747, Santa Cruz) or control antibodies (sc-2025, Santa Cruz) were preincubated 10 min at RT before adding DNA probe.

Nuclease assay

Nuclease assay were performed as described in previous literatures (Anand et al., 2016; Cannavo and Cejka, 2014). Briefly, 3'-end radiolabelled 1 nM of biotinylated 70 bp-long DNA oligonucleotide substrates were incubated with streptavidin (15 nM, Sigma) to block the ends of substrates. Purified recombinant proteins were then added to the reaction for 30 min at 30°C in nuclease reaction buffer containing 25 mM Tris-acetate pH 7.5, 25 mM Manganese acetate, 1 mM Magnesium acetate, 5 mM Dithiothreitol, 1 mM ATP, 1 mM Bovine serum albumin (New England Biolabs), 0.25 mg/ml Phosphoenolpyruvate, and 1 mM Pyruvate kinase (Sigma). Reaction products were analyzed on 15 % polyacrylamide denaturing urea gels (19:1 acrylamide-bisacrylamide, Bio Rad) and scanned by Typhoon phosphor imager (GE Healthcare).

Immunofluorescence staining

Cells were fixed with 4% (v/v) formaldehyde in PBS for 15 min at RT and permeabilized in PBS containing 0.5% (v/v) TritonX-100. All staining were done with PBS buffer containing 1% BSA, 0.1% (v/v) Tween 20. Primary antibodies were used at 1:10000 (Mre11; custom made) or 1:2000 (Rad50; IHC-00076, Bethyl) and secondary antibodies (Alex Fluor-594; Life technologies) were used at 1:1000 dilutions. Cells were mounted with ProLong® Gold Antifade DAPI-Mountant (Life technologies).

Histopathology

Tissue samples from the sacrificed mice were fixed with 4% (v/v) formaldehyde overnight at 4°C and stored at 4°C in 70% ethanol. Paraffin embedded samples were prepared by eight-micrometer section and were subjected to pathological analysis

after Hematoxylin and eosin (H&E) staining. All sample preparation and pathological analysis were performed by Histoserv, Inc. (Maryland, USA).

Supplemental References

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Cannavo, E., and Cejka, P. (2014). Sae2 promotes dsDNA endonuclease activity within Mre11-Rad50-Xrs2 to resect DNA breaks. Nature *514*, 122-125.

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