The Mre11-Nbs1 Interface Is Essential for Viability and Tumor Suppression

Graphical Abstract

Interaction compromised (Nbs1\textsuperscript{mid} mutants)

Complex: • Assembled • Functional • Nuclear

Highlights

- TALEN editing used to generate Nbs1\textsuperscript{mid} mutant mice altered the Mre11 interaction

- The Mre11-Nbs1 interaction is essential for embryonic viability and DDR

- The Nbs1 minimal fragment (108 amino acid) is sufficient to sustain viability

- Nbs1 is required for proper assembly and localization of Mre11 and Rad50

Authors
Jun Hyun Kim, Malgorzata Grosbart, Roopesh Anand, Claire Wyman, Petr Cejka, John H.J. Petrini

Correspondence
petrinij@mskcc.org

In Brief
Kim et al. find that Nbs1 promotes the proper assembly and localization of a complex containing Mre11 and Rad50. Nbs1-mediated assembly is required for the function of the complex, and a 108-amino-acid Nbs1 fragment containing the Mre11 interaction domain is sufficient for this essential role.
The Mre11-Nbs1 Interface Is Essential for Viability and Tumor Suppression

Jun Hyun Kim,1 Malgorzata Grosbart,2,3 Roopesh Anand,4 Claire Wyman,2,3 Petr Cejka,4 and John H.J. Petrini1,5,*
1Molecular Biology Program, Memorial Sloan Kettering Cancer Center, New York, NY 10021, USA
2Department of Molecular Genetics
3Department of Radiation Oncology
Erasmus University Medical Center, 3000 Rotterdam, the Netherlands
4Institute for Research in Biomedicine, Università della Svizzera Italiana, Via Vincenzo Vela 6, 6500 Bellinzona, Switzerland
5Lead Contact
*Correspondence: petrinij@mskcc.org
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SUMMARY

The Mre11 complex (Mre11, Rad50, and Nbs1) is integral to both DNA repair and ataxia telangiectasia mutated (ATM)-dependent DNA damage signaling. All three Mre11 complex components are essential for viability at the cellular and organismal levels. To delineate essential and non-essential Mre11 complex functions that are mediated by Nbs1, we used TALEN-based genome editing to derive Nbs1 mutant mice (Nbs1mid mice), which harbor mutations in the Mre11 interaction domain of Nbs1. Nbs1mid alleles that abolished interaction were incompatible with viability. Conversely, a 108-amino-acid Nbs1 fragment comprising the Mre11 interface was sufficient to rescue viability and ATM activation in cultured cells and support differentiation of hematopoietic cells in vivo. These data indicate that the essential role of Nbs1 is via its interaction with Mre11 and that most of the Nbs1 protein is dispensable for Mre11 complex functions and suggest that Mre11 and Rad50 directly activate ATM.

INTRODUCTION

The DNA damage response (DDR) is important for maintaining genomic integrity. It comprises pathways that mediate DNA repair, DNA damage signaling, cell-cycle regulation and apoptosis. Impairment of the DDR is associated with diverse human pathologies such as cancer, neurodegenerative disorders, immune deficiency, and premature aging (Ciccia and Elledge, 2010).

The Mre11 complex—Mre11, Rad50, and Nbs1 (Xrs2 in S. cerevisiae)—influences all aspects of the DDR via its role as DNA double-strand break (DSB) sensor as well as its enzymatic and structural roles in DSB repair (Stracker and Petrini, 2011). Each member of the complex has been identified as the underlying basis of chromosome instability syndromes associated with immunodeficiency, radiosensitivity, cell-cycle checkpoint defects, and cancer predisposition (Stracker and Petrini, 2011). These disorders each exhibit decrements in ataxia telangiectasia mutated (ATM) activation or activity, consistent with the idea that the Mre11 complex is required for the activation of ATM. This conclusion is supported by biochemical and genetic analyses in mice, yeast, and human cells (Cerosaletti and Concannon, 2004; Difilippantonio et al., 2005; Schiller et al., 2012; Shull et al., 2009; Stracker et al., 2008; Theunissen et al., 2003; Waltes et al., 2009; Williams et al., 2002).

Whereas Mre11 and Rad50 orthologs are present in Bacteria, Archaea, and Eukarya, Nbs1 appears to be restricted to Eukarya. Accordingly, the protein appears to influence functions that are unique to eukaryotic cells. Unlike Mre11 and Rad50, Nbs1 does not appear to bind DNA, nor does it specify enzymatic activities relevant to DNA repair. Nbs1 primarily influences Mre11 complex function by mediating protein interactions via its N- and C-terminal domains that influence DNA repair, subcellular localization, and ATM-dependent checkpoint and apoptotic functions (Cerosaletti and Concannon, 2003; Desai-Mehta et al., 2001; Larsen et al., 2014; Lloyd et al., 2009; Saito and Kobayashi, 2013; Stracker and Petrini, 2011; Williams et al., 2008). The mechanistic basis for Mre11-complex-dependent ATM activation remains unclear. It is notable that the appearance of Nbs1 in eukaryotes coincides with the Mre11 complex’s role in promoting DNA damage signaling, as does the Mre11 domain with which Nbs1 interacts. The Mre11 interaction interface of Nbs1 is a bipartite structure comprising Mid1 and Mid2 (Mre11 interaction domain) that is conserved among Nbs1 orthologs (Schiller et al., 2012).

Having previously established that the N and C termini alone and in combination are dispensable for ATM activation (Stracker and Petrini, 2011), we undertook mutagenesis of the Mre11-Nbs1 interface with the goal of impairing the Nbs1-Mre11 interaction while leaving the Nbs1 protein otherwise intact in an effort to define the role of Nbs1 in ATM activation. Using transcription activator-like effector nuclease (TALEN)-based genome editing, we created an allelic series in mice consisting of six mutations (Nbs1mid) within Mid2 that impair the interaction between Nbs1 and Mre11 to varying degrees. The most severe mutants abolished the Mre11-Nbs1 interaction, which resulted in the loss of cellular and organismal viability. These data indicate that the Mre11-Nbs1 interaction is essential, and therefore required, for ATM activation.

Complementation of Nbs1-deficient cells with Nbs1 fragments spanning Mid1 and Mid2 rescued the viability of cultured cells
and hematopoietic cells in vivo. Cells rescued in this manner also exhibited some indices of ATM function. In vitro, the Nbs1 fragments that rescued viability promoted Mre11 dimerization and DNA binding. In addition, they restored the ability of CtIP to activate Mre11 endonuclease activity, a function shown to be dependent on Nbs1. Collectively, these data suggest that the Nbs1-Mre11 interaction is required for proper assembly of the Mre11 complex. Accordingly, that interaction is required for the concerted activities of Mre11 and Rad50 that govern DNA repair and DNA damage signaling and promote viability.

**RESULTS**

**The Evolutionarily Conserved NFKxFxK Motif in Nbs1 Is Essential for Mouse Embryogenesis**

The mammalian Nbs1 protein interacts with Mre11 via a bipartite domain near the C terminus, comprising Mid1 and Mid2 (Figure 1A). Mid2 includes a highly conserved NFKxFxK motif, whereas Mid1 is characterized by a single conserved leucine at position 648 of the mouse protein (Figure 1A). We carried out mutagenesis of the NBS1 cDNA to identify alleles that weakened the Nbs1-Mre11 interaction. Mutation of L648 had a minimal effect on the Mre11-Nbs1 interaction, whereas the interaction was severely impaired by mutation of F686 in Mid2 (Figure 1C). On that basis, we carried out TALEN-based gene editing in mice to induce small deletions within Mid2 and thereby compromise the interaction between Mre11 and Nbs1 (Figure S1A).

We generated seven new Nbs1 mutant mice (Nbs1mid1, Nbs1mid2, Nbs1mid3, Nbs1mid4, Nbs1mid5, and Nbs1mid8) in which the NFKxFxK motif of Mid2 is altered (Figure 1B). The genomic sequences of Nbs1 Exon 13 from founder mutant lines are listed (Figure S1B). These mutations were modeled in cDNA expression constructs. The ability of the corresponding protein products to interact with Mre11 was assessed by co-immunoprecipitation. Nbs1mid1 was indistinguishable from WT, whereas Mre11 interaction was moderately impaired by mutation of F686 in Mid2 (Figure 1C). On that basis, we carried out TALEN-based gene editing in mice to induce small deletions within Mid2 and thereby compromise the interaction between Mre11 and Nbs1 (Figure S1A).

We assessed a range of developmental parameters in Nbs1 mutant mice. Nbs1mid1/mid1, Nbs1mid2/mid2, Nbs1mid3/mid3, Nbs1mid4/mid4, and Nbs1mid5/mid5 mice were born at expected Mendelian frequencies, indicating that the Mre11-Nbs1 interaction is essential for embryonic viability (Figure 1D).
the effects of Nbs1<sup>mid</sup> mutations on ATM activation by examining phosphorylation of the ATM substrates Kap1 (S824) and Chk2. These mutants also exhibited defects in the G2/M checkpoint, indicative of reduced ATM activation. At 1 hr following treatment with 3 Gy IR, the mitotic index of WT cells decreased by 85%–87% (Figure 2A), whereas the decrease was only 64% for Nbs1<sup>mid3</sup> and 72% for Nbs1<sup>mid4</sup> cells with the more severe impairment of Kap1 and Chk2 phosphorylation in Nbs1<sup>mid3</sup> than Nbs1<sup>mid4</sup>. Similarly, colony-formation assays indicated that IR resistance was reduced in Nbs1<sup>mid3</sup> and Nbs1<sup>mid4</sup> cells (Figure S2B).

The defects imparted by Nbs1<sup>mid3</sup> and Nbs1<sup>mid4</sup> increased cancer risk. In our colony, p53-deficient mice present with thymic lymphoma at ~180 days of age. Over a 10-month time course, Nbs1<sup>mid3</sup> mice did not present with malignancy (data not shown); however, when combined with p53 deficiency, the mean tumor-free survival decreased by 27–50 days relative to p53<sup>−/−</sup> mice (**p < 0.01; Nbs1<sup>mid3</sup> p53<sup>−/−</sup> vs. p53<sup>−/−</sup>) (Figure 2B).

In addition to reduced latency, the spectrum of tumors arising in double-mutant mice expanded to include leiomyosarcoma, squamous cell carcinoma, hemangiosarcoma, rhabdomyosarcoma, and histiocytic sarcoma (Table S1). We propose that destabilization of the Mre11-Nbs1 interface impairs ATM activation and modifies the p53<sup>−/−</sup> phenotype.
We were unable to establish embryonic fibroblasts homologous for the Nbs1<sup>mid5</sup> or Nbs1<sup>mid8</sup> alleles. To define the cellular phenotypes of Nbs1<sup>mid5</sup> and Nbs1<sup>mid8</sup>, these mice were crossed with Nbs1<sup>F0</sup> mice in which cre expression inactivates NBS1 (Demuth et al., 2004). Following transduction of a tamoxifen (4-OHT)-regulated cre recombinase into immortalized mouse embryonic fibroblasts (MEFs) from Nbs1<sup>F0mid5</sup> and Nbs1<sup>F0mid8</sup> mice, 4-OHT was added to the media for 24 hr. cre-mediated deletion of Nbs1<sup>F0</sup> was evident within 24–48 hr (data not shown), and the remaining Nbs1<sup>mid5</sup> and Nbs1<sup>mid8</sup> proteins were present at markedly reduced levels, whereas Mre11 and Rad50 levels were unchanged (Figures 2E–2H). These outcomes resemble those observed upon genetic ablation of RAD50, MRE11, or NBS1 (Adelman et al., 2009; Buis et al., 2008; Reina-San-Martin et al., 2005).

ATM activation was assessed in Nbs1<sup>1–mid5</sup> and Nbs1<sup>1–mid8</sup> cells at 4 days following cre. IR-induced Kap1 S284 and Chk2 phosphorylation were nearly undetectable in Nbs1<sup>1–mid5</sup> (Figure 2C) and severely attenuated in Nbs1<sup>1–mid8</sup> cells (Figure S2C), suggesting that impairing the Mre11-Nbs1 interaction compromised ATM activation. Accordingly, IR-induced ATM S1987 autophosphorylation, a direct index of ATM activation (Bakkenist and Kastan, 2005; Paul, 2015), was also sharply decreased in Nbs1<sup>1–mid5</sup> cells relative to Nbs1<sup>1–mid8</sup> controls (Figure 2C). As expected, both Nbs1<sup>mid5</sup> and Nbs1<sup>mid8</sup> alleles exhibit defects in the G2/M checkpoint that were considerably more severe than those observed in Nbs1<sup>mid5mid5</sup> or Nbs1<sup>mid5mid8</sup> alleles (Figure S2D). These assessments may underestimate the severity of the Nbs1<sup>1–mid5</sup> and Nbs1<sup>1–mid8</sup> phenotypes due to the possible presence of residual Nbs1 protein.

**The Nbs1 Minimal Fragment Rescues Nbs1 Deficiency**

Previously, a C-terminal truncation of 100 amino acids of human Nbs1 that included Mid1 and Mid2 was unable to rescue viability of Nbs1-deficient mouse cells (Difilippantonio et al., 2005). Data presented here indicate that the presence of an essentially complete Nbs1 protein that is unable to interact with Mre11 was not sufficient for viability or ATM activation. Given that the N and C termini are dispensable, singularly or in combination, for cell viability and ATM activation (Stracker and Petrini, 2011) (data not shown), we used deletional mutagenesis to define the “minimal Nbs1” required to support viability.

Three Nbs1 gene segments encoding N-terminal truncation fragments, all of which also lacked the C-terminal 24 amino acids of Nbs1, were constructed in a retroviral expression vector. The constructs encode fragments of 388, 188, and 108 amino acids (F2, F3, and F4, respectively) fused to a FLAG epitope and SV40 nuclear localization signal (NLS) at their N termini for nuclear localization (Figure 3A). The Nbs1 gene segments were transduced into Nbs1<sup>F0F0</sup> MEFs, and the ability of the encoded fragments to interact with Mre11 was assessed via FLAG immunoprecipitation. All fragments co-immunoprecipitated with Mre11 and Rad50 (Figure 3B). Moreover, F4 which spans just 108 amino acids inclusive of Mid1 and Mid2 displaced full-length Nbs1 from Mre11 and Rad50, arguing that Nbs1 is unlikely to interact with other domains of the Mre11 complex (Figure 3B).

Subsequently, cre activity was induced with 4-OHT, and the ability of fragment-containing cells to form colonies was assessed. Whereas no colonies formed from control (vector-transduced) cells, all of the cells expressing F2, F3, and F4 were able to form colonies after 10 days in culture. PCR genotyping and western blot confirmed that the introduced fragments were the sole source of Nbs1 protein remaining in the Nbs1<sup>1–</sup> cells—the fragments are hereafter designated “rescue fragments” (Figures S3A–S3C). By cloning the cells in this manner and by propagation in culture over the course of several weeks, any contribution from residual Nbs1-proficient (Nbs1<sup>F0</sup>) cells to colony formation or subsequent phenotypic assessments was excluded. These data indicated that as few as 108 amino acids of Nbs1 spanning the Mre11-Nbs1 interaction interface are sufficient to sustain the viability of cultured cells.

In addition to sustaining viability, the rescue fragments were able to promote ATM activation in Nbs1<sup>1–</sup> cells. The phenotypes of Nbs1-fragment-expressing cells were compared to a culture of Nbs1<sup>F0F0</sup> cells at 4 days after cre induction. Whereas Kap1 S284 phosphorylation was sharply reduced at 0.5 hr after 5 Gy IR in Nbs1<sup>1–</sup>, it was readily evident in F2- and F3-containing cells and, to a lesser extent, in F4 cells (Figure 3C). IR-induced Kap1 S284 phosphorylation of F4 cells was confirmed as ATM-dependent activity by pretreatment with an ATM inhibitor (Figure S3D). Those complemented cells exhibited restoration of the G2/M checkpoint. After 1 hr following treatment with 3 Gy IR, the mitotic index of F2-, F3-, and F4-containing cells decreased by 68%, 66%, and 44%, respectively, while the decrease was only 14% for ATM<sup>–</sup> cells (Figure 3D). These data indicate that a substantial degree of ATM-dependent checkpoint function was retained in rescue-fragment-expressing cells. A fragment of human Nbs1 spanning residues 401–754 was previously shown to suppress ATM activation and nuclear localization defects in Nijmegen breakage syndrome (NBS) patient cells (Cerosaletti and Concannon, 2004).

To obtain a quantitative assessment of DDR function in rescue-fragment-containing cells, the frequency of spontaneous chromosome aberrations was assessed. 100% of Nbs1<sup>1–</sup> cells exhibit widespread chromosome fragility, with more than three aberrations per metaphase spread (Figures 3E and 3F). In contrast, fewer than 21% of F2-, F3-, and F4-containing cells exhibited three or more aberrations (Figure 3F), indicating a substantial degree of residual function.

Nevertheless, rescue-fragment-expressing cells did not necropoy cells expressing wild-type Nbs1. We observed sharply reduced nuclear localization of Mre11 in F4-containing cells relative to F2 or F3-containing cells (Figure 3G), likely accounting for reduced Kap1 phosphorylation in F4-containing cells. Data from budding yeast and human cells indicate that Xrs2 and Nbs1 are required for nuclear localization of Mre11 and Rad50 (Cerosaletti and Concannon, 2004; Cerosaletti et al., 2006), and enforced nuclear localization of Mre11 in S. cerevisiae partially restored function to xrs2Δ mutants (Oh et al., 2016; Tsukamoto et al., 2005). F4-containing cells were more sensitive to IR than F2 cells.
Figure 3. The Nbs1 Minimal Fragment Rescues Nbs1 Deficiency

(A) Structure of Nbs1 fragments used in rescue experiments. Domains are indicated in Figure 1A.
(B) Mre11 interaction of Nbs1 minimal fragments. FLAG-tagged Nbs1 fragments (F2, F3, and F4) were expressed in Nbs1F/F MEFs, and immunoprecipitation with FLAG antibodies was performed followed by western blot for Mre11, Rad50, and Nbs1.
(C) IR-induced ATM signaling in Nbs1+/− MEFs expressing Nbs1 minimal fragments. As an ATM substrate, phosphorylation of KAP1 (S824) was assessed.
(D) IR-induced G2/M cell-cycle checkpoint of Nbs1+/− MEFs expressing Nbs1 minimal fragments. Mitotic cells were detected by measuring mitosis-specific phosphorylation of histone H3 (Ser10). ATM+/− MEFs was used for controls. p value was determined by unpaired t test (**p < 0.01, mean ± SD, three independent experiments).

(E and F) Metaphase spread of Nbs1+/− MEFs expressing Nbs1 minimal fragments. (E) Representative chromosome metaphase images of Nbs1+/− and Nbs1+/− MEFs. (F) The graph indicates the percent ratio of metaphases with aberrations (**p < 0.01 and ***p < 0.001, Fisher’s exact test, more than 65 metaphases from two independent experiments).

(G) Immunofluorescence cell staining of Mre11 in Nbs1+/− MEFs expressing Nbs1 minimal fragments. Nuclei are shown by DAPI (4',6-diamidino-2-phenylindole) staining.
(H) Immunofluorescence cell staining of Rad50 and Mre11 in Nbs1+/− MEFs expressing Mre11-NLS. Nbs1+/−-Mre11-NLS cells were achieved by treating Nbs1+/−creERT2-Mre11-NLS cells with 4-OHT treatment. Cells were stained at day 3 after 4-OHT treatment. Nuclei are shown by DAPI (4',6-diamidino-2-phenylindole) staining.
(Figure S3E), and we reasoned that a contributing factor to the reduced efficiency of F4-dependent restoration of ATM activation might be aberrant localization of Mre11 and Rad50. To determine whether enforced nuclear localization of Mre11 would mitigate the effects of Nbs1 deficiency, we expressed C0 to determine whether enforced nuclear localization of Mre11 in Nbs1−/− cells did not restore nuclear localization of Rad50 (Figure 3H).

Biochemical Effects of the Nbs1 Minimal Fragment

These data suggested that Nbs1 influences the stability and assembly of the Mre11 complex in addition to its subcellular localization. To test this interpretation, we examined the effect of human Nbs1 F4 on the biochemical properties of Mre11 in vitro. The human Mre11 core (1–411 amino acids) (Park et al., 2011) was purified and subjected to size exclusion chromatography. Individually, Mre11 and Nbs1 F4 fused to a maltose-binding protein (F4-MBP) primarily appeared as single, monomeric peaks (apparent molecular weight [MW]: 42 kDa for Mre11 and 59 kDa for F4-MBP). When mixed at a 1:1 ratio, we observed a new peak with an apparent molecular weight of 184 kDa, consistent with the co-elution of Mre11 and Nbs1 dimers (i.e., two F4-MBP and two Mre11 cores) (Figures 4A and 4B). The new peak was not observed in F4-mid5-MBP in which the NFKKFKK motif is deleted (Figure S5).

F4-MBP also stimulated DNA binding by the Mre11 core. Mre11 binds DNA as a dimer (Williams et al., 2008). We found that the binding of Mre11 to double-stranded DNA (dsDNA) (Figure 4C) or a hairpin (Figure 4D) substrate was stimulated by Nbs1 F4 in electrophoretic mobility shift assays (EMSAs). Superh Ishion induced by MBP antisera confirmed that the Mre11-DNA complex contains Nbs1 (Figure 4D).

We next examined the effect of F4-MBP on Mre11 nuclease activity. Phosphorylated CtIP has recently been shown to promote Mre11 endonuclease in a manner that depends on Nbs1 (Anand et al., 2016). The Mre11-Rad50 complex was incubated with a 70-bp radiolabeled dsDNA substrate, the ends of which were blocked by streptavidin to prevent exonuclease degradation (Figure 4E). In the absence of F4-MBP, or in the presence of the non-interacting F4-mid5-MBP fragment (Figure 4F), CtIP did not promote exonuclease cleavage by Mre11 (Figures 4G and 4H). In contrast, wild-type F4-MBP with phosphorylated CtIP promoted the endonuclease of Mre11-Rad50 (Figure 4H).

To further examine the hypothesis that Nbs1 mediates proper assembly of Mre11 and Rad50, we carried out scanning force microscopy (SFM). Previous SFM analysis revealed a stoichiometry of two for four Nbs1 proteins per M2R2 complex (van der Linden et al., 2009). However, the addition of two or four Nbs1 proteins to the globular domain of M2R2 obscured possible structural rearrangement. The minimal Nbs1 fragment identified here allowed analysis of Mre11 changes in complex architecture by SFM imaging. The M2R2 complex is characterized by a single globular domain (Mre11 + Rad50 ATPase domains) with two protruding coiled coils (Figure 5A). The coiled coils are usually apart but 32% of the time appear to be linked by the zinc-hook domains at their apices (de Jager et al., 2004; Moreno-Herrero et al., 2005).

To assess the effect of F4 on Mre11 complex assembly, the full-length human M2R2 complex was incubated at a 1:1 molar ratio with F4-MBP or F4-mid5-MBP (non-binding mutant) before imaging. A striking rearrangement of the Mre11 globular domain was induced in the presence of the F4 peptide, with the globular domain appearing as two distinct but linked globular objects (Figure 5B). The proportion of Mre11 complexes with this conformation increases from 12% to 58% in the presence of the F4 peptide but does not significantly change in the presence of the control F4-mid5 peptide (Figure 5C). This separation into two distinct globular objects is accompanied by an increase in width of the globular domain (Figure 5D), consistent with nanoscale arrangement of Rad50 and Mre11 globular domains with consequent influence on conformational flexibility of the Rad50 coiled coils favoring dimerization of the zinc hooks. Collectively, these data strongly support the view that Nbs1’s influence on the physical disposition of the Mre11 complex constitutes its essential function and that this influence underlies its requirement for nuclear localization, cell viability, and ATM activation.

Nbs1−/− Fetal Liver Cells Reconstitute the Hematopoietic System upon Nbs1 Minimal Fragment Expression

Immortalized cells are likely more tolerant of genotoxic stress than constituents of tissues in vivo. To determine whether the rescue fragments would sustain viability in vivo, we assessed their ability to support the differentiation of lymphocytes. Previous analyses indicated that the Mre11 complex is required for lymphocyte development (Balestrini et al., 2016; Calle´ n et al., 2004; Deriano et al., 2009; Reina-San-Martin et al., 2004). Nbs1−/− mice were crossed to vavCre mice, which express cre recombinase in hematopoietic stem cells (HSCs) (Stadtfeld and Graf, 2005). Hematopoietic Nbs1 deficiency resulted in perinatal lethality due to lack of bone marrow development (Figure S6).

Fetal liver cells (FLCs) from embryonic day 13.5 (E13.5) embryos were isolated and transduced with Nbs1 rescue fragment encoding in an IRES-GFP murine stem cell virus (MSCV) retrovirus prior to transplantation into lethally irradiated mice as depicted (Figure 6A). At 10 weeks after transplantation, spleen was isolated and assessed for GFP-positive cells. Although we did not observe complete reconstitution, GFP-positive, B220-positive cells comprised 6% of splenocytes (Figure 6B). The percentages of GFP-positive, B220-positive cells may underestimate the degree of reconstitution by rescue-fragment-containing HSCs due to silencing of the MSCV retrovirus during hematopoietic differentiation as observed previously (Cherry et al., 2000). The Mre11 complex is dispensable for viability of quiescent cells
Adelman et al., 2009; hence, silencing of F4 expression may be tolerated because splenocytes are largely quiescent.

PCR genotyping confirmed that recipient mice contain differentiated cells derived from Nbs1 fragment containing Nbs1/F4/FLCs and that the fragment-encoding construct is present; the Nbs1F allele was not detected (Figure 6C). This confirms that this rescue is not by remaining undeleted Nbs1F allele in donor FLCs. No reconstitution was observed in control mice transduced with FLCs lacking the rescue fragments (data not shown). These data indicate that as with transformed cells, the 108 amino acids spanning the Mre11 interaction interface of Nbs1 (F4; Figure 3A) are sufficient to promote viability in vivo and, moreover, that minimal Nbs1 fragment was sufficiently functional to support differentiation of HSCs into splenic B cells.

**DISCUSSION**

To examine the role of Nbs1 in Mre11 complex functions, we undertook mutagenesis of Nbs1 in an attempt to weaken the interaction with Mre11 and thereby examine the functionality of the
core Mre11-Rad50 complex disassociated from Nbs1. Mutations that disrupted Mre11 interaction caused inviability. Hence, the presence of a non-interacting but otherwise intact Nbs1 protomer was not sufficient for viability, establishing that Nbs1 interaction per se is essential. Conversely, we found that 108 amino acids of Nbs1 spanning the Mre11 interaction domain were sufficient to promote cell viability. Although ATM activation was reduced in that setting, it was not abolished. Collectively, the data strongly argue that ATM activation is not directly dependent on Nbs1. Instead, we propose that essential functions of Nbs1 are to ensure proper assembly and subcellular localization of the Mre11 complex, which in turn promotes viability and influences ATM activation by Mre11 and Rad50.

The Role of Nbs1 in the Mre11 Complex: Essential Functions

Nbs1 clearly mediates essential as well as non-essential functions. With respect to the former, the data presented here indicate that the Mre11-Nbs1 interaction is specifically required for cellular and organismal viability. The levels of Mre11 and Rad50 protein were not changed in Nbs1mid mutant cells. Hence, it is the loss of interaction, rather than global destabilization of Mre11 complex components that accounts for the loss of viability in non-interacting Nbs1mid mutants. Further, this emphasizes the fact that Mid2 (the conserved NFKxFxK motif, which was the target of the TALEN-based mutagenesis) is the major determinant of Nbs1’s association with the Mre11 complex. These data are consistent with the finding that the human Nbs1tr645 allele in which the C-terminal 100 amino acids of Nbs1 were deleted was unable to support viability of mouse embryos (Difilippantonio et al., 2005).

What are the essential functions of Nbs1 in the Mre11 complex? First, it is clear that the nuclear localization of Mre11 is influenced by Nbs1, but this does not solely depend on the Mre11 interaction domain. Mre11 complex mislocalization is observed in human NBS and A-TLD cells, as well as in Nbs1Δ108/Δ108 and Mre1ATLD1/ATLD1 mouse models of those human mutations, neither of which harbor alterations in their respective interaction domains (Carney et al., 1998; Difilippantonio et al., 2005; Reina-San-Martin et al., 2005; Stewart et al., 1999; Williams et al., 2002). Nbs1 and Mre11 levels are reduced in those settings, raising the possibility that the stoichiometry of complex components may also influence nuclear localization. Alternatively, those mutations may disrupt as-yet-undescribed interactions required for nuclear localization.

Nevertheless, promoting nuclear localization of Mre11 and Rad50 is likely not the only function of Nbs1 required for viability. Enforced nuclear localization by Mre11-NLS could not restore nuclear localization of Rad50 in human NBS cells (Lakdawala et al., 2008), nor did it compensate for Nbs1 deficiency in MEFs (Figure 3H). The data presented here are most consistent with the interpretation that Nbs1 influences the assembly and disposition of the complex. Supporting that view, we showed that dimerization and DNA binding by the N-terminal 411-amino-acid core of Mre11 was enhanced by the F4 fragment (Figures 4A–4D), although it is likely that dimeric assemblies of full-length Mre11 may exhibit greater stability. Further support comes from the fact that only in the presence of Nbs1 or the minimal fragment is...
...which are disrupted in the canonical forkhead-associated (FHA) and BRCA1 C-terminal (BRCT) domains as well as for determining its subcellular localization. The non-essential functions of Nbs1 have been illuminated by genetic and biochemical analyses have shown that Rad50 influences the activation of ATM or its budding yeast ortholog, Tel1 (Alt et al., 2005; Chapman and Jackson, 2008; Kobayashi et al., 2005; Williams et al., 2002). Deletion of the Nbs1 C terminus (the Nbs1<sup>△C</sup> allele), which has been reported to bind ATM (Falck et al., 2005; You et al., 2005), had no effect on ATM activation or cell viability but is required for ATM-dependent apoptosis (Stracker et al., 2007).

The Nbs1<sup>△BC</sup> allele is a composite of the Nbs1<sup>△B</sup> and Nbs1<sup>△C</sup> alleles. The outcomes of Nbs1<sup>△B</sup> and Nbs1<sup>△C</sup> are simply additive in Nbs1<sup>△BC/△BC</sup> mice rather than synergistic; the phenotypic outcomes attributable to Nbs1<sup>△B</sup> and Nbs1<sup>△C</sup> are unchanged in the composite Nbs1<sup>△BC</sup> mice (Shull et al., 2009; Stracker et al., 2007). Therefore, we propose that Nbs1 serves as a platform for Mre11 complex assembly and the recruitment of ATM substrates to enhance access of the activated kinase to substrates that govern ATM- and Nbs1-dependent functions. In this context, we draw a distinction between ATM activation and ATM activities: in the former circumstance, a properly assembled and localized complex is required for ATM activation, whereas in the latter, Nbs1 potentiates ATM activity by promoting access of the active kinase to its downstream effectors.

Although viability and ATM activation are lost upon genetic ablation of Nbs1, the protein does not appear to influence those outcomes directly. Rather, its association with Mre11 and Rad50 via its conserved interaction interface indicates that mutations in Rad50 exert effects on regions distal to the altered residues (Al-Ahmadie et al., 2014; Deshpande et al., 2014; Hohl et al., 2011, 2015; Hopfner et al., 2002). These data underlie the speculation that Rad50 is likely to be the proximal effector of ATM activation.

**The Role of Nbs1 in the Mre11 Complex: Non-essential Functions**

The non-essential functions of Nbs1 have been illuminated by genetic analyses in human cells and mice. Nbs1 contains N-terminal forkhead-associated (FHA) and BRCA1 C-terminal (BRCT) domains, which are disrupted in the canonical Nbs1<sup>△N57△S5</sup> allele inherited by NBS patients and the corresponding Nbs1<sup>△B</sup> allele in mice. Those mutants exhibit defects in DSB end resection, DNA repair, and cell-cycle checkpoint activation, presumably due to the loss of protein interactions mediated by those domains (Al-Ahmadie et al., 2014; Chapman and Jackson, 2008; Kobayashi et al., 2002; Larsen et al., 2014; Lloyd et al., 2009; Maser et al., 2001; Melander et al., 2008; Morishima et al., 2007; Spycher et al., 2008; Williams et al., 2009; Wu et al., 2008, 2012). However, human and mouse cells lacking those domains are viable and retain the ability to activate ATM (Difilippantonio et al., 2005; Williams et al., 2002). Deletion of the Nbs1 C terminus (the Nbs1<sup>△C</sup> allele), which has been reported to bind ATM (Falck et al., 2005; You et al., 2005), had no effect on ATM activation or cell viability but is required for ATM-dependent apoptosis (Stracker et al., 2007).
were previously described (Williams et al., 2002), and Nbs1Mid1 mice were generated by help of the Memorial Sloan Kettering Mouse Genetics core. Detailed protocol will be provided upon request.

EXPERIMENTAL PROCEDURES

For SFM analysis of MR/NBS1 F4-WT or -mid5 complexes, Mre11-Rad50 (MR) protein and Nbs1 F4 fragment were mixed in reaction buffer (20 mM Tris-HCl [pH 8], 0.1 M EDTA, 0.2 M NaCl, and 2 mM DTT) at a molar ratio 1:1 (8 nM of MR to 8 nM F4-WT or -mid5) in total volume of 20 μL and incubated for 5 min on ice. The samples were then diluted five times in final volume of 20 μL in reaction buffer and deposited on freshly cleaved mica. After a 1-min incubation at room temperature, the mica was washed with milliQ water and dried with filtered air. Samples were imaged at room temperature and humidity with a Nanoscope VIII (Digital Instruments) operating in tapping mode. Type NH-C-W silicon tips with resonance frequency 310–372 kHz were obtained from Nanosensors (Veeco Instruments). Images were collected at 2.5 × 2.5 μm, standard resolution 512 lines × 512 rows, and processed only by flattening to remove background slope. Images were quantified first by identifying MR complexes by visual inspection where molecules consisting a large globular domain with two protruding coiled coils were identified as M2R2. These were further classified based on the arrangement of globular domains as dimers with one globular domain or dimers with two distinct linked globular domains. The latter were further categorized according to the arrangement of coiled coils as open, parallel, or hook-linked. The frequency of the different forms was expressed as percentage of total molecules counted. The width of individual globular domains was determined using SFMetrics V4e software (Sanchez and Wyman, 2015) by manually measuring the longest axis across the globular domain.

Figure 7. The Mre11 Interaction Domain of Nbs1 Is Necessary and Sufficient for Mre11 Complex Functions

Disruption of the Mre11-Nbs1 interaction results in cellular and organismal lethality and increased tumorigenesis due to a defect in Mre11 complex function. An Nbs1 minimal fragment spanning just the Mre11-Nbs1 interaction interface is sufficient to sustain the viability of cells and stabilize Mre11 dimer and Mre11 DNA binding and nuclease activity. These data indicate an essential role for Nbs1 is via its interaction with Mre11 and that most of the Nbs1 protein is dispensable for Mre11 complex functions. Mid1, Mid2, and AIM denote Mre11-interacting domain 1, Mre11-interacting domain 2, and ATM interacting motif, respectively.

Manipulation of the DDR for therapeutic benefit offers significant potential (O’Connor, 2015). Accordingly, understanding of ATM activation is an important issue. This study thus provides important mechanistic insight toward that goal by defining the role of Nbs1 in promoting Mre11 complex functions in the DDR.

AUTHOR CONTRIBUTIONS

J.H.K., M.G., and R.A. performed the experiments and analyzed the data. J.H.K., C.W., P.C., and J.H.J.P. designed the experiments. J.H.K. and J.H.J.P. wrote the paper.

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Supplemental Information

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Supplemental Information

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

Jun Hyun Kim¹, Malgorzata Grosbart²,³, Roopesh Anand⁴ Claire Wyman²,³, Petr Cejka⁴, and John H.J. Petrini¹*

¹Molecular Biology Program,
Memorial Sloan-Kettering Cancer Center, New York, NY10021, USA

Department of ²Molecular Genetics and ³Radiation Oncology,
Erasmus University Medical Center, 3000 CA Rotterdam, The Netherlands

⁴Institute for Research in Biomedicine, Università della Svizzera Italiana, Via Vincenzo Vela 6, 6500 Bellinzona, Switzerland

*Correspondence: petrinij@mskcc.org
Figure S1. TALEN targeting for Nbs1\textsuperscript{mid} mutant mouse lines, 
Related to Figure 1.

(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\textsuperscript{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<sup>mid</sup> mutants cells, Related to Figure 2.

(A) Analysis of G2/M checkpoint in Nbs1<sup>mid3</sup> and Nbs1<sup>mid4</sup> 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<sup>mid3</sup> and Nbs1<sup>mid4</sup> 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<sup>-/mid8</sup> 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<sup>-/mid5</sup> and Nbs1<sup>-/mid8</sup> SV40-MEFs. Mitotic cells were detected by measuring mitosis-specific phosphorylation of histone H3 (Ser10). Nbs1<sup>+/mid8</sup>, Nbs1<sup>-/-</sup>, ATM<sup>-/-</sup> SV40-MEFs were used for controls. P-value was determined by unpaired t-test (**p<0.001, mean ± s.d., n=3).
Figure S3. Verification and analysis of rescue cell lines, Related to Figure 3.

(A) *Nbs1*<sup>F</sup> 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*<sup>−/−</sup> 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*<sup>−/−</sup> + F4 cells. Colony formation assay to determine the DNA damage sensitivity of *Nbs1*<sup>−/−</sup>+ F2 and *Nbs1*<sup>−/−</sup>+ F4 SV40-MEFs. Parental *Nbs1*<sup>F/F</sup> 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 *Nbs1*<sup>F/F</sup> vs. *Nbs1*<sup>−/−</sup>+ F2; **p<0.01 for *Nbs1*<sup>−/−</sup>+ F2 vs. *Nbs1*<sup>−/−</sup>+ F4, mean ± s.d., Two independent experiments in triplicate).
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. 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. 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 $Nbs1^{+/+}$, $Nbs1^{mid3/mid3}$, $p53^{-/-}$ and $Nbs1^{mid4/mid4}$ $p53^{-/-}$ mice, Related to Figure 2.

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

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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<sup>+/mid5 or mid8</sup> mice with Nbs1<sup>F</sup> mice. MEFs in which deletion of Nbs1<sup>F</sup> 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<sup>F</sup> 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<sup>F</sup> allele. Nbs1 fragments were cloned into pMIG-W-IRES-GFP plasmid (a gift from Luk Parjis; Addgene plasmid #12282). Deletion of endogenous Nbs1<sup>F</sup> 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

Immunoprecipitations (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<sup>F/F</sup>vavCre embryos and red blood cells were lysed in ACK lysis buffer (150 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 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<sup>F/F</sup>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<sup>F/F</sup>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<sup>F/F</sup>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<sup>5</sup> FLCs were transplanted via tail vein injection. After 10 weeks post transplantation, thymocytes and splenocytes were isolated and analyzed to assess GFP<sup>+</sup> 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’-CAGTGAGGAGCTGCCACGGAACT-3’, 5’-TCTAACTCGGTATTCTTTGCAGCATGGT-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


