Conditional deletion of Nbs1 in murine cells reveals its role in branching repair pathways of DNA double-strand breaks

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NBS1 forms a complex with MRE11 and RAD50 (MRN) that is proposed to act on the upstream of two repair pathways of DNA double-strand break (DSB), homologous repair (HR) and non-homologous end joining (NHEJ). However, the function of Nbs1 in these processes has not fully been elucidated in mammals due to the lethal phenotype of cells and mice lacking Nbs1. Here, we have constructed mouse Nbs1-null embryonic fibroblasts and embryonic stem cells, through the Cre-loxP and sequential gene targeting techniques. We show that cells lacking Nbs1 display reduced HR of the single DSB in chromosomally integrated substrate, affecting both homology-directed repair (HDR) and single-stranded annealing pathways, and, surprisingly, increased NHEJ-mediated sequence deletion. Moreover, focus formation at DSBs and chromatin recruitment of the Nbs1 partners Rad50 and Mre11 as well as Rad51 and Brca1 are attenuated in these cells, whereas the NHEJ molecule Ku70 binding to chromatin is not affected. These data provide a novel insight into the function of MRN in the branching of DSB repair pathways.

The EMBO Journal (2006) **25**, 5527–5538. doi:10.1038/sj.emboj.7601411; Published online 2 November 2006

Subject Categories: genome stability & dynamics

Keywords: DNA double-strand break; homology-directed repair; NBS1; nonhomologous end-joining; single-stranded annealing

Introduction

The NBS1 protein (hypomorphically mutated in Nijmegen Breakage Syndrome; NBS) forms a complex (MRN) with Mre11 (mutated in A-T-like disorder, A-TLD) and Rad50 in

mammalian cells (D'Amours and Jackson, 2002). The functional or physical interactions between the MRN complex with ATM, ATR, MDC1, H2AX, BRCA1 and FANCs suggest how MRN may coordinate checkpoint molecules and the double-strand break (DSB) repair machinery (Shiloh, 2003; Lavin, 2004). Thus, NBS cells also show checkpoint defects in cell cycle progression (Shiloh, 2003). NBS1 has been shown to directly activate ATM and to stimulate the kinase activity of ATM towards its substrates *in vitro* (Lee and Paull, 2004, 2005; Falck *et al*, 2005). Consistent with such a role, activation of ATM targets is impaired in human NBS cells or mouse Nbs1-deficient B cells (Carson *et al*, 2003; Uziel *et al*, 2003; Difilippantonio *et al*, 2005).

DNA DSBs repair is carried out by two major pathways: homologous repair (HR) and non-homologous end joining (NHEJ) (West, 2003). HR is conducted by Rad52 epistasis group molecules, including Rad51, Rad54, Rad59, XRCC2/3 and BRCA1/2 (Symington, 2002), while NHEJ repair is carried out by the Ku complex consisting of the Ku70/Ku80 heterodimer and the catalytic subunit of DNA-PK (DNA-PKcs), which recruit XRCC4, DNA Ligase IV and artemis (Weterings and van Gent, 2004). However, how a cell chooses to undergo repair by either pathway remains largely unexplained. It is proposed that cell cycle phase is one factor that modulates the choice of repair pathway (Lieber *et al*, 2003). The repair of DSBs in G0/G1 cells is carried out mainly by NHEJ because of the limited supply of homology template, whereas HR would become the major form of repair in S/G2 cells when sister chromatids are available. Other factors may also influence the use of pathways. For example, the CDK1 inhibitor represses HR in budding yeast arrested in G2 phase but results in a compensatory increase in NHEJ (Ira *et al*, 2004). Different from other DSB molecules, MRN is proposed to act at the branching point upstream of both HR and NHEJ. However, there is conflicting evidence on the role of MRN in HR and NHEJ. In budding yeast *Saccharomyces cerevisiae*, the Mre11–Rad50–Xrs2 complex is required for both HR and NHEJ, whereas in fission yeast *Schizosaccharomyces pombe*, the Rad32(Mre11)–Rad50 complex is dispensable for NHEJ (Manolis *et al*, 2001; Tomita *et al*, 2003; see review by Khanna and Jackson, 2001). Finally, although Nbs1-deficient chicken DT-40 cells showed defects in homology-directed repair (HDR) (Tauchi *et al*, 2002), the precise role of Nbs1 in mammalian cells and in particular repair steps have not been studied.

Although the function of mammalian NBS1 has been extensively studied using biochemical approaches and using cell lines derived from NBS patients who carry NBS1 hypomorphic mutations (Maser *et al*, 2001), the biological role of the entire Nbs1 protein in the repair of DSBs remains elusive. One of the reasons is that null mutation of Nbs1 and members of the MRN complex in mammals cause a lethal phenotype at the cellular or mouse level (Xiao and Weaver,

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Received: 27 August 2006; accepted: 6 October 2006; published online: 2 November 2006

1997; Luo *et al*, 1999; Zhu *et al*, 2001; Dumon-Jones *et al*, 2003). We and others have applied Cre-loxP technology to overcome the lethality and to delete Nbs1 in specific tissues of mice (Demuth *et al*, 2004; Frappart *et al*, 2005; Kracker *et al*, 2005; Reina-San-Martin *et al*, 2005) and studied the biological function of Nbs1 in lymphoid development and in neurogenesis. In the present study, we engineered an *Nbs1*-null cellular system by the constitutive (gene targeting) and inducible (via Cre-loxP technology) deletion of the gene in embryonic stem (ES) cells and fibroblasts (MEFs). Using these cells, we have investigated the role of Nbs1 in the regulation of DSB repair pathways.

Results

Generation of inducible *Nbs1*-null mouse embryonic fibroblast cells

In order to study the function of the entire Nbs1 protein in DNA repair in mammals, we constructed MEFs with an inducible *Nbs1* deletion by disrupting exon 6 of the *Nbs1* gene (the murine homolog of *NBS1*) using the Cre-loxP technology (Supplementary Figure S1A). We isolated MEFs from E13.5 fetuses of *Nbs1*^{f6/f6} and *Nbs1*^{neo/f6} genotypes (Dumon-Jones *et al*, 2003; Frappart *et al*, 2005) and immortalized them according to the 3T3 protocol. These MEFs were then stably transfected with the Cre-ER vector expressing Cre recombinase (CER clones) or an empty vector (PSG clones) (Figure 1A). The inducibility of the disruption of the *Nbs1* locus by 4-hydroxytamoxifen (OHT) in CER clones was analyzed by Southern blotting and RT-PCR (Figure 1B). *Nbs1* deletion was further confirmed by Western blotting (Figure 1B, lower panel). Genetically, the *Nbs1*^{neo} and *Nbs1*^{Δ6} alleles are null because homozygous mutant embryos (*Nbs1*^{neo/neo}; *Nbs1*^{Δ6/Δ6}; *Nbs1*^{neo/Δ6}) died at the peri-implantation stage (Dumon-Jones *et al*, 2003; Frappart *et al*, 2005). These molecular and genetic data confirm that these *Nbs1*^{Δ6/Δ6} and *Nbs1*^{neo/Δ6} CER cells are *Nbs1*-null mutants and thus designated as 'inducible' *Nbs1*-null MEF cells.

Characterization of DNA damage response of inducible *Nbs1*-null MEF cells

We first examined the effect of Nbs1 null on DNA damage response and chromosome stability. To this end, we applied inducible *Nbs1*-null MEF cells to study the effect of Nbs1 depletion on cell proliferation, survival and chromosome stability. After depletion of Nbs1 by OHT treatment, CER clones (*Δ6/Δ6*) completely lost clonogenic formation ability (Figure 1C). To test whether this phenotype was due to proliferation and/or apoptosis, we analyzed the proliferation profile as well as apoptosis. As expected, Nbs1 deletion in these cells resulted in defective proliferation (Figure 1D) and increased TUNEL-positive populations, which were further enhanced by DNA damaging agent adriamycin (ADR) (*Δ6/Δ6* + ADR) (Figure 1E). Moreover, cytogenetic analysis revealed that inducible Nbs1 deletion in MEF CER cells (*Δ6/Δ6*, *neo/Δ6*; after OHT treatment) caused a high degree of spontaneous chromosomal aberrations and micronuclei (Figure 1F and G). The findings support the proposed role of Nbs1 in cell viability and DNA damage response.

Impaired HDR of DSBs in MEFs after induced *Nbs1* deletion

To investigate repair pathways of DSBs, we constructed PSG (without Cre) and inducible *Nbs1*-null CER (Cre) cells in which a single copy of the HDR reporter substrate DRGFP was integrated into the genome (Figure 2A). To characterize the HR pathway, we used seven CER clones that carried a single intact copy of DRGFP and were treated with or without OHT. After expression of the I-SceI endonuclease that induces a single DSB in the substrate, HDR activity can be monitored by flow cytometry for GFP-positive cells (Pierce *et al*, 1999). We found that GFP-positive populations were significantly lower in all seven inducible *Nbs1*-null CER cells (*Δ6/Δ6*) compared to *Nbs1*-proficient MEF cells (*f6/f6*; Figure 2B). In contrast, almost none of GFP-positive MEFs were detected among mock-transfected cells (Figure 2B, upper panel; data not shown).

To monitor the transfection efficiency, we transfected a GFP-expressing plasmid and found similar populations of these inducible *Nbs1*-null and -proficient MEFs that were GFP-positive (data not shown), suggesting no difference in the uptake of the I-SceI-expressing plasmid in both genotypes. Thus, the HDR deficiency observed in inducible *Nbs1*-null MEFs was solely due to the depletion of the Nbs1 protein. Taken together, these findings indicate that Nbs1 is required for efficient HDR of DSBs in mammalian cells.

Decreased single-stranded annealing of DSBs in inducible *Nbs1*-null MEFs

To determine the specific step of HR pathways in which Nbs1 is involved, we analyzed single-stranded annealing (SSA) in MEFs after induced Nbs1 deletion. To this end, using the primers DF and SAR (Figure 2C), we amplified SSA products from inducible *Nbs1*-null CER cells after I-SceI transfection, and compared them to total PCR products (including HDR, SSA, NHEJ and uncut substrate DRGFP) generated using primers DF and DR (see Figure 2C). In the HDR reporter system used here, either HR (HDR and SSA) or NHEJ can repair I-SceI-induced DSBs (Figure 2C; see Yang *et al*, 2005). Using these assays, we found that SSA-generated products were significantly decreased (about 2.5-fold) in inducible *Nbs1*-null CER MEFs (*Δ6/Δ6*) compared to *Nbs1*-proficient control clones (*f6/f6*) (Figure 2D).

To test the potential contribution of LTGC (long-tract gene conversion) that can be amplified by primers DF and SAR to the authentic SSA events, we grew cells in the presence of puromycin after DSBs repair, which would eliminate cells undergoing SSA that caused the loss of the puromycin resistance gene. Primers (DF and SAR) then would solely amplify the regions around the *BcgI* site generated via LTGC (Figure 2C; Nakanishi *et al*, 2005; Yang *et al*, 2005). This assay did not detect any PCR products (data not shown), ruling out a significant contribution of the LTGC event to the SSA products shown in Figure 2D.

To exclude the possibility of experimental bias, we next constructed new *Nbs1* CER cells containing a single intact copy of a specific SSA reporter substrate SAGFP (Figure 2E; Stark *et al*, 2004). After induction of Nbs1 deletion, in the presence of I-SceI, numbers of SSA-generated GFP-positive cells were reduced significantly among *Nbs1*-null MEFs

Figure 1 Generation and characterization of inducible Nbs1 deletion in mouse 3T3 MEFs. **(A)** Protocol used for generation of 'inducible' Nbs1-null MEFs. **(B)** Southern blot, RT-PCR and Western blot analysis of Nbs1 deletion after OHT treatment in 'inducible' Nbs1-null CER MEFs. The 'f6' and 'Δ6' alleles are indicated in the Southern blot. The genotype of each sample after OHT treatment is indicated at the bottom of the gels. *Hprt* served as an internal control for RT-PCR analysis. Actin is the loading control for Western blotting. **(C)** Colony formation assay of Nbs1 deletion in MEFs. Representative plates (upper panel) and quantification (lower panel) of colony formation assay are shown. Bars represent the mean of six clones of each sample from two independent experiments. **(D)** The proliferation curve of Nbs1-proficient (PSG ± OHT) and null (CER + OHT, Δ6/Δ6) MEFs. Curves represent the mean of duplicate samples from two independent experiments. **(E)** TUNEL analysis of inducible Nbs1-null MEFs (Δ6/Δ6) after treatment with adriamycin (+ADR) or not (-ADR) for 3 h (Time 0). TUNEL staining was performed at the indicated time points after drug removal. **(F)** Cytogenetic analysis of Nbs1^{f6/f6} or Nbs1^{neo/f6} CER cells after OHT-mediated Nbs1 deletion. Forty metaphases of each cell line were analyzed. **(G)** Spontaneous and ADR-induced micronucleus formation in the absence of Nbs1. Bars represent the mean of three clones of each genotype from two experiments.

(Δ6/Δ6), compared to wild-type cells (f6/f6) (Figure 2F). In control experiments, without I-SceI expression, almost no GFP-positive cells were detected for both genotypes (data not shown). Thus, the absence of Nbs1 affects the SSA step, consistent with the proposed role of the MRN complex in the resection of DNA DSBs.

Imprecise NHEJ is increased in inducible Nbs1-null MEF cells

We next used two independent approaches to analyze the involvement of the mammalian MRN complex in the NHEJ pathway after I-SceI induced DSBs. First, we amplified the genomic region surrounding the I-SceI site in DRGFP using

Figure 2 Nbs1 depletion compromised both HDR and SSA in inducible *Nbs1*-null MEFs. **(A)** The HDR assay substrate DRGFP. **(B)** *Nbs1*-proficient or -null cells carrying a single intact copy of substrate DRGFP were analyzed by flow cytometry. Upper panel: representative flow cytometric analysis for HDR in MEFs after I-SceI expression. The GFP-positive population, reflecting HDR repair events, is separated from GFP-negative population. FL1, green fluorescence; FL2, orange fluorescence. Lower panel: The HDR events were scored as the percentage of GFP-positive populations after I-SceI expression. The percentage of GFP-positive cells in inducible ($\Delta 6/\Delta 6$) *Nbs1*-null mutants was set as 1. Bars represent the mean of seven MEF clones of each genotype from two independent experiments. **(C)** Strategy for analysis of SSA products in the DRGFP substrate. Four putative products surrounding the original genomic region of the I-SceI site: HDR, SSA, NHEJ and uncut DRGFP are shown. SSA was quantified by the products from primers DF and SAR, compared to that of the total PCR products from primers DF and DR (including HDR, SSA, DRGFP and NHEJ products). **(D)** Representative PCR-Southern blotting image (left panel) and quantification of SSA products (right panel) after I-SceI expression. The density of SSA products (DF-SAR) was normalized to the total PCR products (DF-DR) within the same sample. The SSA value of inducible ($\Delta 6/\Delta 6$) *Nbs1*-null mutants was set as 1. Bars represent the mean of three MEF clones of each genotype from two independent experiments. **(E)** SSA assay substrate SAGFP was stably integrated into MEFs and SSA was analyzed by flow cytometry after I-SceI transfection. **(F)** Summary of SSA in *Nbs1*-null ($\Delta 6/\Delta 6$) and -proficient (f6/f6) MEF cells. The SSA events were scored by the percentage of GFP-positive populations. The percentage of GFP-positive cells in *Nbs1*-null mutants was set as 1. Bars represent the mean of seven MEF clones of each genotype from two independent experiments. *P*-values by *t*-test are indicated in (B, D, F).

PCR primers (DF and DR; see Figure 2C). The NHEJ products were significantly increased in inducible *Nbs1*-null MEF cells ($\Delta 6/\Delta 6$), compared to *Nbs1*-proficient controls (f6/f6)

(Figure 3A). After subcloning into the pGEM-T vector, direct sequencing of these NHEJ products from 31 wild-type and 35 *Nbs1*-null plasmid clones revealed deletions ranging

Figure 3 Increased NHEJ in MEF cells after induction of Nbs1 deletion. **(A)** PCR-Southern blot analysis of NHEJ. Representative gel (left panel) and quantification of NHEJ deletion products (right panel) after I-SceI expression. The PCR primers are shown in Figure 2C. The NHEJ frequency was examined by normalizing the density of NHEJ deletion products (Δ10–25 bp) to that of the 723-bp PCR product within the same sample. The NHEJ value of *Nbs1*-proficient cells (f6/f6) was set as 1. **(B)** Southern blot strategy for NHEJ analysis. NHEJ products are resistant to double digestion with I-SceI and *Bcl*I enzymes. Note: the first *Hind*III site (star) is located outside the DRGFP substrate. Black filled box, I-SceI site arisen from uncut DRGFP; striped box, *Bcl*I site retrieved from HDR and SSA products; empty box, NHEJ products resistant to both I-SceI and *Bcl*I digestion. **(C)** Southern blot analysis of NHEJ using the strategy shown in (B). Representative Southern blot image (left panel) and quantification of total NHEJ products (right panel) in the DRGFP substrate after I-SceI expression. The NHEJ efficiency was examined by normalizing the density of NHEJ products (resistant to both I-SceI and *Bcl*I) to that of cleaved product (0.9 kb, sensitive to both I-SceI and *Bcl*I) within the same sample. The NHEJ value of *Nbs1*-proficient cells (f6/f6) was set as 1. Bars in (A, C) represent the mean of three MEF clones of each genotype from two independent experiments. *P*-values by *t*-test in (A, C) are indicated.

from 10 to 25 bp around the original I-SceI site (data not shown). However, no such deletion was detectable in mock controls without I-SceI expression (Figure 3A; data not shown).

To confirm these findings, we adopted another approach to examine directly the total NHEJ events by digesting the genomic DNA of mock or I-SceI-transfected cells with I-SceI, followed by *Bcl*I and *Hind*III enzymes (Figure 3B). The repair of a DSB in the DRGFP substrate via HDR, SSA, or imprecise NHEJ pathways generates I-SceI-site loss leading to a resistance to I-SceI digestion. HDR and SSA generate a *Bcl*I site that replaces the I-SceI site in the substrate, leading to sensitivity to *Bcl*I digestion (Figure 3B; Yang *et al*, 2005). Therefore, the uncleaved products resistant to both I-SceI and *Bcl*I enzymes arose solely through NHEJ. The relative amount of I-SceI/*Bcl*I-resistant DNA, indicative of this process, was significantly increased in inducible *Nbs1*-null CER MEFs (Δ6/Δ6), as we found about three-fold increase of NHEJ products in these cells compared to the *Nbs1*-proficient

counterpart (f6/f6) (Figure 3C). In mock-transfected cells, genomic DNA was completely cleaved by I-SceI (Figure 3C; data not shown). Together, these results suggest that Nbs1 represses the NHEJ pathway.

Generation and characterization of constitutive *Nbs1*-null mouse ES and MEF cells

To verify DNA repair defects of inducible *Nbs1*-null MEFs, we also generated ES and MEF cells containing 'constitutive' *Nbs1* deletion following the strategy outlined in Figure 4A and Supplementary Figure S1B. By sequential gene targeting in ES cells (Supplementary Figure S1B), one homozygous *Nbs1*^{neo/hyg} mutant clone was obtained. To obtain an independent *Nbs1*-null ES cell line, we isolated blastocysts (E3.5 embryos) from intercrosses of heterozygous *Nbs1*^{+/neo} (Dumon-Jones *et al*, 2003) and *Nbs1*^{+/f6} (Frappart *et al*, 2005) mice, from which *Nbs1*^{neo/f6} ES clones were established. Following transfection of a Cre-expressing plasmid, the *Nbs1*^{neo/Δ6} mutant ES cells were identified by Southern blotting (data not shown). Western blot analysis did not detect the full-length nor truncated form of the Nbs1 protein in these ES cells (Figure 4B). We next isolated MEFs from E13.5 chimeric fetuses that were generated by injection of *Nbs1*^{neo/hyg} ES cells into wild-type blastocysts. The *Nbs1*^{neo/hyg} MEFs were selected against G418 to eliminate wild-type cells before immortalization (Figure 4A). Consistent with their ES cell origin, these immortalized *Nbs1*-null MEF cells (*Nbs1*^{neo/hyg}) were devoid of the Nbs1 protein (Figure 4C) and thus these ES and MEF cells (*Nbs1*^{neo/hyg}, *Nbs1*^{neo/Δ6}) were designated as 'constitutive' *Nbs1*-null mutants.

We next examined proliferation of these *Nbs1*-null cells and found no difference in proliferation and normal cell cycle distribution compared to wild-type controls (Figure 4D and E). The proliferation potential of *Nbs1*-null ES cells was further tested using chimeric embryo assays by injecting *Nbs1*-null ES cells into wild-type blastocysts. The presence of mutant alleles (*neo* and *hygro*) in a given tissue was quantified and compared to the wild-type allele in the same tissue by image analysis of the Southern blot. It was noted that *Nbs1*-null ES cells could differentiate into various cell types and participate in the formation of various tissues although the capacity varied (Supplementary Figure S2).

To test the DNA damage response of these mutant cells, we performed the colony formation assay using constitutive *Nbs1*^{neo/hyg} MEFs. After treatment with ADR and hydroxyurea (HU), constitutive *Nbs1*-null MEFs completely lost their clonogenic capacity (Figure 4F). In addition, flow cytometric analysis revealed that *Nbs1*-null ES cells showed similar apoptosis (sub-G1) in spontaneous cell population compared to wild-type controls. However, after 10 Gy of ionizing radiation (IR), more *Nbs1*-null ES cells accumulated in the sub-G1 phase, indicating an increased apoptosis (Figure 4G). Cytogenetic analysis revealed that *Nbs1*-null ES cells contained high levels of chromosome breaks and fusions after 10 Gy of IR although without IR treatment their basal levels of chromosome aberrations were similar to those of wild-type counterparts (Figure 4H and I). We also found a higher rate of sister chromatid exchange (SCE) in the presence and absence of mitomycin C (MMC) in constitutive *Nbs1*-deficient MEFs compared to *Nbs1*-proficient controls (Figure 4J).

Figure 4 Generation and characterization of mouse ES cells and MEFs carrying constitutive Nbs1 deletion. (A) Protocol used to establish constitutive Nbs1-deleted MEFs from chimeric fetuses. (B) Western blot analysis of *Nbs1*-null ES cells. PARP-1 is a loading control. (C) Western blot analysis of *Nbs1*-null ES cells and 3T3 MEFs. Actin serves as a loading control. (D) Proliferation curve of constitutive *Nbs1*-null MEF cells. Curves represent the mean of triplicate samples from two independent experiments. (E) FACS analysis of cell cycle distribution of *Nbs1*-null ES cells. (F) Colony formation assay was performed to examine viability of constitutive Nbs1 deleted MEFs after ADR (0.2 μg/ml) or HU (2 mM) treatment. (G) Histogram shows the percentage of ES cells in sub-G1 phase extracted from FACS analysis at the indicated time points after 10 Gy of IR. Representative images of FISH analysis (H) and quantification (I) of wild type (+/+) and *Nbs1*-null (neo/hyg) ES cells subjected to or not to 10 Gy of IR. Br: breaks; Fu: fusions. Between 16 and 19 metaphases from each treatment were analyzed and the results are shown as the mean ± standard deviation. (J) SCE analysis of constitutive *Nbs1*-null MEFs with or without MMC treatment. At least 31 metaphases of each sample were scored and the *t*-test was applied for statistical analysis.

Repair defects of *Nbs1*-null cells are independent of the cell proliferation status

To avoid the argument that the DSB repair defects seen in 'inducible' Nbs1 deleted MEFs is secondary to the impact of Nbs1 deletion-induced cell proliferation arrest (e.g., Figure 1C and D), we took advantage of proliferating constitutive *Nbs1*-null cells for investigation of the role of Nbs1 in the repair pathways. To characterize the DSB pathway, we established six *Nbs1*-null and six wild-type ES clones containing a single intact substrate DRGFP. After expression of the I-SceI, we found that GFP-positive populations were significantly lower in constitutive *Nbs1*-null ES cells (neo/hyg) than in controls (+/+) (Figure 5A). To test the SSA pathway, we used two different assays (see Figure 2C and E) and found that SSA-generated products were significantly less in constitutive Nbs1-deleted ES cells (neo/hyg), compared to their *Nbs1*-proficient counterparts (+/+) (Figure 5B and C). These defects were corrected by the reintroduction of

mouse *Nbs1* cDNA into these mutant cells (Figure 5D–F). In these experiments, we used Fanca null cells as controls because we demonstrated recently that these cells are defective in both HDR and SSA (Nakanishi *et al*, 2005; Yang *et al*, 2005).

We also analyzed the NHEJ activity in constitutive *Nbs1*-null cells by two approaches. We first analyzed DSB repair products in DRGFP in ES cells by PCR-Southern blotting. After transfection of I-SceI, we amplified the genomic region surrounding the I-SceI site in the substrate using PCR primers (DF and DR; see Figure 2C). The PCR products generated by NHEJ deletion were significantly increased in proliferating *Nbs1*-null ES cells (neo/hyg), compared to wild-type controls (+/+) (Figure 5G). In the second approach, we used the same strategy as shown in Figure 3B and C. Similarly, imprecise NHEJ pathways generate I-SceI-site loss leading to a resistance to I-SceI digestion, which can be visualized by uncleaved products

Figure 5 DSB repair defects in constitutive *Nbs1*-null ES cells. (A) The HDR events after *I-SceI* expression were analyzed by flow cytometry (see Figure 2B) and scored as the percentage of GFP-positive populations. The percentage of GFP-positive cells in *Nbs1*-null (*neo/hyg*) mutants was set as 1. Bars represent the mean of six ES clones of each genotype from two independent experiments. (B) Representative PCR-Southern blot image (left panel) and quantification of SSA products (right panel) in the HDR substrate DRGFP. The density of SSA products (DF-SAR) was normalized to the total PCR products (DF-DR) within the same sample. The SSA value of constitutive *Nbs1*-deleted mutants (*neo/hyg*) was set as 1. Bars represent the mean of three ES clones of each genotype from two independent experiments. (C) SSA analysis of *Nbs1*-null (*neo/hyg*) and -proficient (+/+) ES cells (see Figure 2E). The percentage of GFP-positive cells (SSA events) in *Nbs1*-null mutants was set as 1. Bars represent the mean of six ES clones of each genotype from two independent experiments. (D) Western blot analysis of transient ectopic expression of mouse *Nbs1* cDNA in constitutive *Nbs1*-null ES cells harboring DRGFP. Defects in HDR (E) and SSA (F) are rescued by transient ectopic expression of mouse *Nbs1* cDNA. One of two independent experiments are shown for (E) and (F). Fanconi anemia-A mutant fibroblasts (*Fanca*-/-; Yang *et al*, 2005) were used as a negative control. (G) PCR-Southern blot analysis of NHEJ activity in proliferating *Nbs1*-null ES cells as described in Figure 2C. Representative Southern blot image (left panel) and quantification of NHEJ deletion products (right panel). The NHEJ value of *Nbs1*-proficient cells (+/+) was set as 1. (H) Southern blot strategy for NHEJ analysis. Note: NHEJ products resistant to both *I-SceI* and *BclI* digestions are ≥ 4.8 kb. (I) Representative Southern blotting image (left panel) and quantification of total NHEJ products (right panel). The NHEJ efficiency was examined as described in Figure 3C. The NHEJ value of *Nbs1*-proficient cells (+/+) was set as 1. Bars in (G, I) represent the mean of three ES clones of each genotype from two independent experiments. The *t*-test was applied for statistical analysis.

(≥ 4.8 kb) (Figure 5H). The relative amount of *I-SceI*/*BclI*-resistant DNA, indicative of this process, was significantly increased in proliferating constitutive *Nbs1*-null ES cells (*neo/hyg*), compared to that of their wild-type counterparts (+/+) (Figure 5I). In mock-transfected cells, genomic DNA was completely cleaved by *I-SceI* *in vitro* (Figure 5I). Taken together, these data are consistent with the results obtained using inducible *Nbs1*-deleted MEF cells, confirming that the observed DSB repair phenotype is not a consequence of the cell cycle arrest, but rather intrinsic to the *Nbs1* function.

Deletion of *Nbs1* compromises recruitment of MRN and HR repair proteins to DSBs

We further examined the effect of *Nbs1* null on the recruitment of its partners Mre11 and Rad50 to DNA breaks. As expected, ADR and HU treatment resulted in Rad50 foci in *Nbs1*-proficient MEFs (*t6/t6*; Supplementary Figure S3, left panel). However, Rad50 focus formation was completely abolished in both inducible ($\Delta 6/\Delta 6$) and constitutive (*neo/hyg*) *Nbs1*-null MEFs (Supplementary Figure S3, left panel). Similarly, Mre11 foci were also abolished in *Nbs1*-null cells, and instead, Mre11 was dispersed throughout the cytoplasm

Figure 6 Defective focus formation of DSB repair molecules in MEF cells lacking Nbs1. Immunofluorescence analysis of Rad51 (A) and Brca1 (B) focus formation in inducible ($\Delta 6/\Delta 6$) and constitutive (neo/hyg) *Nbs1*-null MEFs after treatment with 0.2 μ g/ml ADR for 3 h. Lower panels of (A) and (B) are quantifications of DNA damage-induced foci. Bars represent means $\pm$ s.d. are shown for duplicate samples from two independent experiments. For each sample at least 500 nuclei were scored. (C) Western blot analysis of the chromatin-enriched fraction of MRN and Ku70 proteins. 0 h, 0 h after removal of ADR; 6 h, 6 h after removal of ADR. PARP-1 is served as a control.

and also in the nucleus (Supplementary Figure S3, right panels). To investigate the molecular basis of the reduced overall HR and increased NHEJ in *Nbs1*-null cells, we next studied the impact of *Nbs1*-null mutation on focus formation of two key players in HR, Rad51 and Brca1. After DNA damage by ADR, Rad51 focus-positive cells was significantly less in *Nbs1*-null genotype ($\Delta 6/\Delta 6$) compared to *Nbs1*-proficient cells (f6/f6) (Figure 6A). We also found fewer Brca1 focus-containing cells among *Nbs1*-null MEFs ($\Delta 6/\Delta 6$) compared to *Nbs1*-proficient (f6/f6) cells (Figure 6B). Focus formation of these two DNA repair molecules in response to DNA damage was not altered in PSG control MEFs in the presence or absence of OHT (data not shown), ruling out any interference of OHT in focus formation. To rule out potential influence of *Nbs1* deletion-induced cell cycle arrest (see Figure 1E) on the low number of Rad51 and Brca1 foci, we performed these experiments with proliferating *Nbs1*-null MEFs (neo/hyg) (see Figure 4D) and found similar reduction of these foci in the absence of *Nbs1* (Figure 6A and B).

***Nbs1* is required for the chromatin loading of MRN, but dispensable for Ku70**

To further characterize the recruitment of MRN to DSBs, we performed chromatin fractionation analysis. Although the

basal level of total Rad50 was unaltered in *Nbs1*-null MEF cells (data not shown), Rad50 was almost absent in chromatin-enriched fractions of *Nbs1*-null cells (neo/hyg) with or without DNA damage (Figure 6C). The trace amount of Rad50 seen in the chromatin-bound fractions correlated with immunofluorescence images (see Supplementary Figure S3), which may be due to the slow turnover of this protein in the nucleus. In contrast to the MRN molecules, the chromatin-bound form of the NHEJ molecule Ku70 (Figure 6C) was apparently unaffected in the absence of *Nbs1*, which may correlate with NHEJ activities seen in these cells (see Figures 3 and 5). These results, together with focus formation experiments, suggest that *Nbs1* is essential for MRN stability and recruitment of HR molecules to DNA breaks; however, its absence does not affect the recruitment of NHEJ molecules to DSBs.

Discussion

Using the Cre-LoxP technology, we have generated mouse MEFs null for *Nbs1*. These cells have allowed us to demonstrate that *Nbs1* (possibly through MRN) modulates DNA DSB repair pathways in mammalian cells by promoting HR while repressing NHEJ. These results are apparently not secondary to the effects of *Nbs1* deletion-induced cell cycle arrest, since these phenotypes were also seen in proliferating constitutive *Nbs1*-null cells. More importantly, ectopic expression of mouse *Nbs1* cDNA in constitutive *Nbs1*-null cells can correct these DSB defects.

It might be surprising that we could obtain cells null for *Nbs1* because complete deletion of any member of the MRN complex in mammals is lethal both at embryonic and cellular levels (Xiao and Weaver, 1997; Luo *et al*, 1999; Zhu *et al*, 2001; Dumon-Jones *et al*, 2003). Although p53 deficiency can rescue a lethal phenotype of mice lacking DSB repair molecules (e.g., see Lim and Hasty, 1996; Hakem *et al*, 1997; Ludwig *et al*, 1997; Gao *et al*, 2000), p53-null mutation did not rescue the embryonic lethality of *Nbs1* null mice (our unpublished data) and the p53 pathway is functional in our *Nbs1*-null cells (data not shown). These observations suggest that the viability of our *Nbs1*-null cells is p53-independent and other genetic factors must be involved. Nevertheless, using inducible *Nbs1* deletion in MEFs, we have demonstrated an indispensable role of *Nbs1* in cell viability (Figure 1C and D), consistent with the general notion that *Nbs1* is essential for cell life. Despite unknown factors overcoming the lethality of constitutive *Nbs1*-null cells, these cells are hypersensitive to DNA-damaging treatments and reproduce many phenotypes similar to those observed in human NBS cells. Thus, the *Nbs1*-null cellular system is a valuable reagent, given the current absence of MRN-null mutants in mammalian cells, and should greatly facilitate the dissection of the early DNA damage response.

The most intriguing result is that *Nbs1* appears to modulate the branching of DNA DSB repair pathways because, in the cells lacking *Nbs1*, the HR pathway (i.e., HDR and SSA) is severely impaired, whereas imprecise NHEJ events are increased. The current study suggests a different mechanism operated by MRN in both HDR and NHEJ pathways in mammalian cells. Several possibilities could explain how *Nbs1*/MRN modulates the repair pathways. The MRN complex harbors exonuclease and DNA end-processing activity

that are required for the DSB resection into ssDNA, a common step to both HDR and SSA (D'Amours and Jackson, 2002; Shiloh, 2003). Moreover, the lack of Nbs1 abrogates the loading of HR molecules (e.g., Rad51 and Brca1) to DSBs and thereby compromises HR, consistent with the fact that BRCA1 and FancD2 physically interact with Nbs1. In this regard, it is interesting to note that although Rad51 is not involved in SSA, cells mutated in BRCA1 and FANCs (-A/-C/-G/-D2) exhibit defects of both HDR and SSA (Stark *et al*, 2004; Nakanishi *et al*, 2005; Yang *et al*, 2005). Interestingly, *Nbs1*-null cells exhibit a similar defect in HDR and SSA as *Fancd2*^{-/-} cells (see Figure 5E and F), suggesting that the FA complex and MRN are epistatic and may participate in a common repair pathway in dealing with certain type of DNA damage. However, it might be surprising that *Nbs1*-null cells contain elevated MMC-induced SCEs while showing low HDR and less Rad51 foci, because IR-induced SCEs in *S. cerevisiae* is an HR-dependent event abolished when RAD51 is inactivated (Fasullo *et al*, 2001). One possible explanation for this apparent paradox is the nature of DNA breaks generated by different approaches. In the current study, HDR and Rad51 foci were analyzed from 'frank' DSBs induced by I-SceI in the DRGFP substrate and by ADR treatment, respectively. However, SCEs were analyzed by using a classical DNA interstrand crosslink (ICL) agent MMC, which does not introduce 'frank' DSBs. Probably, MMC collapses replication forks and initiates recombination with different requirements as compared to 'frank' DSBs. In this regard, many mutant cells that are hypersensitive to ICLs are not clearly IR-sensitive (e.g. FA cells). On the other hand, it is conceivable that loss of MRN may compromise DNA strand resection and thereby favor the Ku-mediated NHEJ activity. Finally, Nbs1 could destabilize or antagonize NHEJ molecules (e.g., Ku70) at DSBs thereby inhibiting NHEJ activities. In support of this hypothesis is the observation that Nbs1-mediated recruitment of HR molecules (Rad51 and Brca1) to DSBs correlates with the disappearance of Ku, which favors disengagement of NHEJ activities at DSBs (Kim *et al*, 2005).

However, our results seem to be contradictory to the recent study using a transient reporter assay to show that NBS skin fibroblasts contain a low capacity for HDR/SSA and microhomology directed NHEJ (Howlett *et al*, 2006). Discrepancy of these findings may be due to different systems used: while we used a chromosomally integrated substrate where a defined DSB is applied, Howlett *et al*. used episomal linearized plasmid assays where cytoplasmic components may influence the activity. In addition, the NHEJ defect was implied in human NBS cells as well as ATLD cells as judged by impaired γ -H2AX focus formation (Riballo *et al*, 2004). However, since MRN is required for ATM activation and γ -H2AX is a substrate of ATM, the reduced γ -H2AX foci in these human NBS and in ATLD cells may not be scored reliably as DSB rejoining defects. Moreover, our results are surprising given the fact that cells carrying either hypomorphic NBS1 mutation (Lahdesmaki *et al*, 2004) or complete deletion (Kracker *et al*, 2005; Reina-San-Martin *et al*, 2005) have defects in class-switch recombination (CSR), a process that requires NHEJ activities. Despite this observation, V(D)J recombination, another NHEJ-dependent process, is apparently not affected in human NBS cells (Harfst *et al*, 2000). CSR and V(D)J are region-

and cell type-specific recombination events in lymphoid cells. Our NHEJ data were obtained from fibroblasts and ES cells in which lymphoid specific factors are not present. Thus, we cannot rule out the specificity of MRN in CSR (and V(D)J) recombination.

It is well established that HR repair is predominant in S/G2 whereas NHEJ is more frequently used in G0/G1 due to the lack of homology sequences in these phases. It might not have been surprising to see increased NHEJ in *Nbs1*-null cells while HR is suppressed. For example, it has been shown that suppression of NHEJ in mammalian cells by disruption of Ku70, Xrcc4 and DNA-PKcs results in increased levels of HDR, as Ku antagonizes DNA strand resection and thereby inhibits the HDR pathway (Pierce *et al*, 2001; Stark *et al*, 2004). However, the increased NHEJ in our study is not secondary to an *Nbs1*-null-induced proliferation arrest in G0/G1 because we observed these phenotypes also in proliferating *Nbs1*-null cells (see Figure 5). Moreover, since our experimental system provides the homology sequence right next to the DSB site (see Figure 2A), it avoids the consideration that the limited availability of homology sequence itself could promote NHEJ. Nonetheless, we cannot formally rule out the possibility that when HR is reduced, the substrates may be processed through the NHEJ pathway. Since the proliferation and the cell cycle distribution of constitutive *Nbs1*-null cells are similar to wild-type controls (Figure 4D and E) and the reintroduction of Nbs1 cDNA corrected DNA repair defects, it seems that the increased NHEJ activity is cell cycle-independent and is not limited by the substrate. These data thus suggest that MRN acts at the branching point upstream of both DSB repair pathways, that is, HR and NHEJ, which thus makes the MRN complex a good candidate that plays an instrumental role in the cellular decision about the use of these pathways, that is, Rad52 epistasis proteins or the Ku complex.

The current study demonstrates, for the first time in mammalian cells completely lacking the Nbs1 protein, that while this protein (possibly via the MRN complex) represses the NHEJ activity, Nbs1 promotes the processing of DNA DSBs, thereby facilitating recruitments of HR molecules (e.g., Brca1 and Rad51) to DNA breaks to conduct HR. Therefore, mammalian cells have evolved a delicate repair mechanism modulated through the MRN complex: by promoting HR, cells can maximize their capacity to repair lethal DSBs with a high fidelity and, by repressing NHEJ, they minimize the generation of 'dangerous liaisons' between broken chromosomes.

Materials and methods

Generation of inducible *Nbs1*-null MEFs ($\Delta 6/\Delta 6$)

The construction of inducible *Nbs1*-null MEFs by the Cre-loxP technique was carried out as described previously (Herceg *et al*, 2001). We isolated MEFs from E13.5 embryos derived from crosses of *Nbs1*^{+/f6} mice (Frappart *et al*, 2005) with *Nbs1*^{+/f6} or *Nbs1*^{+/neo} mice (Dumon-Jones *et al*, 2003) and immortalized them by the 3T3 protocol. *Nbs1*^{f6/f6} or *Nbs1*^{f6/neo} MEF cell lines were co-transfected with a pCre-ER plasmid (referred to as CER) or an empty pSG9 plasmid (no Cre gene) (referred to as PSG), with a PGK-Hyg plasmid containing the hygromycin-resistance gene. To induce Nbs1 deletion, *Nbs1*^{f6/f6} or *Nbs1*^{f6/neo} CER cells were incubated 2 days with 1 μ M 4-hydroxytamoxifen (OHT) (Sigma, St Louis, USA) that triggers the Cre-mediated deletion of the *Nbs1* exon 6 flanked by two loxP sites to generate *Nbs1* ^{$\Delta 6/\Delta 6$} or *Nbs1*^{neo/ $\Delta 6$} cells, respectively.

Establishment of constitutive *Nbs1*-null cells (*neo/hyg*)

To generate ES cells mutant for *Nbs1*, we designed strategies depicted in Supplementary Figure S1B. The first strategy used a 'conventional' gene targeting approach to disrupt both alleles of exon 6 by sequential electroporation of the targeting vectors pTV-neo and pTV-hyg into D3 ES cells. The second strategy was to isolate *Nbs1*^{neo/hyg} ES cells from E3.5 blastocysts derived from intercrosses of *Nbs1*^{+/16} and *Nbs1*^{+/neo} mice. Following transfection of Cre recombinase, the loxP-flanked exon 6 was deleted. To establish constitutive *Nbs1*-null MEFs, primary embryonic fibroblasts were isolated from E13.5 chimeric embryos and the G418-resistant fibroblasts (*Nbs1*^{neo/hyg}) were immortalized according to the 3T3 protocol (see Figure 4A).

RT-PCR assay

Total RNA was extracted using Tri-Reagent (Sigma), and reversely transcribed into cDNA following the instruction in the kit. To analyze the expression of *Nbs1* transcript, *Nbs1* cDNA was amplified by PCR using primers Nbn658F (5'-ctaagaacagctccagata-3'; corresponding to exon 5) and Nbn905R (5'-attctacatcaacaacgcag-3'; corresponding to exon 7), yielding 248-bp PCR products. For the internal control, the primer pair P3 (5'-ccatcacattggccctctgtgtctca-3') and P4 (5'-gttaagttagagatcatctccacca-3'), corresponding to *hprt* exon 3 and 4, respectively, amplifies a 200-bp PCR fragment.

Cytogenetic analysis

To facilitate visualization and identification of chromosome aberrations, we applied fluorescence *in situ* hybridization using a telomere-specific probe Cy3-PNA (Applied Biosystems, Courtaboeuf, France). Cells were collected for metaphase preparation as described previously (Tong *et al*, 2001). For examination of SCE, MEFs were cultured in the presence or absence of 30 nM MMC (Sigma) and were collected for preparation of chromosome spreads (Wang *et al*, 1997). After staining with 4% Giemsa, SCEs were scored.

Cell survival and proliferation assays

For the colony formation assay, MEFs were plated in duplicate at 1000 cells/10-cm dish. PSG or CER cells were cultured in the presence or absence of 1 μ M OHT for 2 days and then for a further 10 days before fixing in methanol/acetic acid and staining with Giemsa. Colonies per dish were counted and expressed as the percentage of untreated controls. To test DNA damage response of constitutive *Nbs1*^{neo/hyg} cells, cells were treated for 12 h with either 0.2 μ g/ml ADR (Sigma) or 2 mM hydroxyurea (HU, Sigma) before the colony formation assay. To analyze apoptosis in cultured cells, TUNEL-positive cells were determined by *in situ* immunostaining with FITC-conjugated BrdU antibody (Roche, Meylan, France). For the proliferation assay, 1.5×10^5 cells were plated into one well of a six-well plate in triplicate and the cell number was determined at the indicated time points.

Cell cycle analysis

Cells were treated with or without 10 Gy of γ -radiation (¹³⁷Cesium) and collected at the indicated time points. For FACS analysis, the percentage of cells in each phase of the cell cycle was measured by a FACScalibur apparatus equipped with Cellquest software (Becton-Dickinson) and ModFit software (Verity Software House Inc., Topsham, USA).

Construction of stable cell lines for DSB repair assay

Cell lines containing a single intact copy of the HDR reporter substrate DRGFP in a random locus (MEFs) or in the *hprt* locus (ES) were generated as described previously (Yang *et al*, 2005). Cells containing the SSA reporter substrate SAGFP were constructed as described (Stark *et al*, 2004). To analyze DSB repair, I-SceI-expressing plasmid pCBASce, a mock plasmid pCAGGS and the GFP-expressing plasmid pCAGGS-EGFP were electroporated into cells. To perform genetic rescue experiments, the mouse *Nbs1* cDNA from pSK-mNbs1 (kindly provided by Dr Martin Digweed) was subcloned into expression vector pCAGGS, which then was transfected into *Nbs1*-null ES cells harboring DRGFP.

HDR and SSA analysis

Flow cytometric analysis was performed on cells carrying a single intact copy of the DRGFP (for HDR) or SAGFP (for SSA) reporter 2

days after electroporation with I-SceI-expressing plasmid (Stark *et al*, 2004; Yang *et al*, 2005). To analyze SSA in the HDR substrate DRGFP by PCR/Southern blotting, primers DF (gggtcggcttctggcggtg) and SAR (cacaggaaacagctatgacc) were used to generate SSA products and the primers DF and DR (gtagccttcgggcatggcgg) for total PCR products. After Southern blotting, the density of SSA products was quantified by PhosphorImager and normalized to the total PCR products within the same sample.

NHEJ analysis

The first assay was based on PCR and Southern blotting as described previously (Yang *et al*, 2005). The efficiency of sequence deletion via NHEJ was quantified by the density of smaller DNA bands compared to that of the 723-bp PCR products within each sample, using PhosphorImager. For the second approach, the genomic DNA of mock or I-SceI-transfected cells was extracted and digested with I-SceI, followed by BclI and HindIII enzymes. After Southern blotting, blots were probed with a DNA fragment corresponding to the C-terminus of SceGFP. The total NHEJ efficiency was quantified by the density of DNA bands resistant to I-SceI and BclI cleavage, normalized to that of uncut DNA within each sample, using PhosphorImage.

Chromatin fractionation

Chromatin fractionation was carried out as described previously (Mendez and Stillman, 2000) with modifications. For DNA damage treatment, cells were incubated with 0.2 μ g/ml ADR for 3 h. Briefly, 3×10^6 cells were washed with PBS and resuspended in 200 μ l of buffer A (10 mM HEPES at pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 10 mM NaF, 1 mM Na₃VO₄, 1 mM β -glycerolphosphate, protease inhibitors). Triton X-100 was added to a final concentration of 0.1%. Nuclei were lysed in 200 μ l of buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 10 mM NaF, 1 mM Na₃VO₄, 1 mM β -glycerolphosphate, protease inhibitors) and chromatin fractions were then separated from soluble nuclear proteins and sheared by sonication.

Immunoblotting

Fractionized proteins or proteins extracted from cells in RIPA buffer (20 mM HEPES pH 7.6, 20% glycerol, 0.5 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA pH 8.0, 0.5% NP-40, 1 mM DTT, 1 mM PMSF, 5 μ g/ml leupeptin, 2 μ g/ml aprotinin, 1 mM β -glycerolphosphate, 1 mM Na₃VO₄ and 10 mM NaF) were resolved by SDS-PAGE, blotted and stained with antibodies in TBST containing 5% non-fat dried milk (NFDm), followed by incubation with horseradish peroxidase-conjugated secondary antibodies and detected by the ECL reagents (Amersham Biosciences, Buckinghamshire, UK). The following antibodies were used: rabbit anti-Nbs1 serum (1:3000, Oncogene, San Diego, USA); rabbit anti-Nbs1 serum (1:2000; Dumon-Jones *et al*, 2003); mouse anti-Rad50 (1:1000, Upstate, Dundee, UK); mouse anti-PARP-1 (1:2000, R&D systems, Minneapolis, USA); rabbit anti-Ku70 (1:5000, Serotec, Oxford, UK); mouse anti-actin serum (1:10 000, Santa Cruz, Heidelberg, Germany).

Focus formation and cell imaging

Immunofluorescence staining of focus formation was performed as described previously (Yang *et al*, 2004) with modification. Briefly, MEFs were treated with or without 0.2 μ g/ml ADR and washed once in PBS with 1 mM β -glycerolphosphate, 1 mM Na₃VO₄ and 10 mM NaF, 1 mM PMSF, and further treated with a hypotonic lysis solution (10 mM Tris-HCl pH 7.4, 2.5 mM MgCl₂, 1 mM PMSF, 1 mM β -glycerolphosphate, 1 mM Na₃VO₄, 10 mM NaF, and 0.5% NP-40) for 8 min on ice. Subsequently, cells were fixed in ice-cold acetone-methanol (1:1) for 30 min on ice. The slides were then incubated with appropriate primary antibody in TBST containing 5% NFDm and mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). A cell with at least five distinct foci in the nucleus was scored as focus-positive. The following antibodies were used: rabbit anti-Rad51 antibody (1:200, kindly provided by Dr Steve West); rabbit anti-Mre11 (1:200, Novus); mouse anti-Rad50 (1:200, Upstate); rabbit anti-Brcal (1:200, kindly provided by Dr Chu-Xia Deng).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Maria Jasin for pDRGFP, pCBASce and pSAGFP plasmids, Steve West for rabbit anti-Rad51 antibody and Chu-Xia Deng for rabbit anti-Brcal antibody. We also thank Christine Caux for flow cytometric analysis, Dominique Galendo for her excellent assistance in the maintenance of the animal colonies, Cyrille Cuenin and Kathrin Eberhardt for their excellent

technical support. Further thanks are due to Janet Hall for her critical reading of the manuscript and John Cheney for the editing of the manuscript. We thank Tomas Lindahl for his support of the study. We are also grateful to many members of ZQW's laboratory for the helpful discussions. This work was supported in part by the Association pour la Recherche sur le Cancer (ARC), France and the Association for International Cancer Research (AICR), UK.

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