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ORIGINAL ARTICLE

Homologous recombination repair is regulated by domains at the N- and C-terminus of NBS1 and is dissociated with ATM functions

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The proteins responsible for radiation sensitive disorders, NBS1, kinase ataxia-telangiectasia-(A-T)-mutated (ATM) and MRE11, interact through the C-terminus of NBS1 in response to the generation of DNA double-strand breaks (DSBs) and are all implicated in checkpoint regulation and DSB repair, such as homologous recombination (HR). We measured the ability of several NBS1 mutant clones and A-T cells to regulate HR repair using the DR-GFP or SCneo systems. ATM deficiency did not reduce the HR repair frequency of an induced DSB, and it was confirmed by findings that HR frequencies are only slightly affected by deletion of ATM-binding site at the extreme C-terminus of NBS1. In contrast, The HR-regulating ability is dramatically reduced by deletion of the MRE11-binding domain at the C-terminus of NBS1 and markedly inhibited by mutations in the FHA/BRCT domains at the N-terminus. This impaired capability in HR is consistent with a failure to observe MRE11 foci formation. Furthermore, normal HR using sister chromatid was completely inhibited by the absence of FHA/BRCT domains. These results suggested that the N- and C-terminal domains of NBS1 are the major regulatory domains for HR pathways, very likely through the recruitment and retention of the MRE11 nuclease to DSB sites in an ATM-independent fashion.
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Introduction

DNA double-strand breaks (DSBs), which can be induced by ionizing radiation (IR) and which also occur during normal DNA processing, pose a considerable threat to cell viability. To maintain genomic integrity, eukaryotic cells respond to DSBs through a variety of pathways such as cell-cycle checkpoints, induction of apoptosis and direct DNA repair reactions. One of the known key factors affecting the cellular response to DSBs is NBS1, the protein mutated in Nijmegen breakage syndrome (NBS), which is characterized by immunodeficiency and a high frequency of malignancies (The International Nijmegen Breakage Syndrome Study Group, 2000). NBS1 is a component of the RAD50/MRE11/NBS1 (R/M/N) complex and directly binds to the MRE11 nuclease through its conservative region at the C-terminus. The cell lines derived from NBS patients show elevated sensitivity to IR, chromosome instability and defects in cell-cycle checkpoints after DNA damage (Kobayashi *et al*., 2004). This cellular phenotype is similar to that observed in MRE11-deficient disease, so-called ataxia-telangiectasia-like disease and also ataxia-telangiectasia (A-T), another cancer-prone genetic disorder caused by mutation of the PI3-like kinase A-T-mutated (ATM) (Shiloh, 2003). It has been demonstrated that NBS1 is phosphorylated by ATM on Ser278 and Ser343 when cells are irradiated and that these phosphorylation events in NBS1 are critical for intra-S-phase checkpoint activation as a downstream event of ATM (Lim *et al*., 2000). However, recent reports showed that NBS1 physically interacts with ATM at the extreme C-terminus of NBS1 and recruits it to damaged sites, suggesting that NBS1 can also function as an upstream damage sensor for ATM signaling (Uziel *et al*., 2003; Lavin, 2004; Falck *et al*., 2005).

In addition to a role in cell-cycle checkpoint and damage recognition, NBS1 also plays an important role in DSB repair. In higher eukaryotes, there are at least two pathways for DSB repair: homologous recombination (HR) and non-homologous end joining. It is well known that DSB repair through HR maintains genomic integrity through precise repair via gene conversion (Jasin, 2002). It has been reported that NBS1 is essential for DSB repair through HR from studies using the

Nbs1-deficient chicken DT40 cell line (Tauchi *et al*., 2002). In yeast HR repair process, Mre11 complex may recruit Exo1 and gives rise to the single-strand regions from the ends of DSB, which initiate recombination by invasion of sister chromatids and, hence, yeast Mre11 mutants are deficient in HR and sensitive to IR (Bressan *et al*., 1999). However, it seemed unlikely that elevated radiation sensitivity is a marker of mammalian HR, as a recent report indicated the involvement of ATM in a pathway of non-homologous end-joining (NHEJ) (Riballo *et al*., 2004). It remains uncertain as to whether all the proteins, NBS1, MRE11 and ATM, are required for mammalian HR repair and if so, which protein is upstream of which in the hierarchy of the repair process. The present results demonstrate that NBS1 is a major regulator of HR repair by mediating the localization of MRE11 to sites of DNA damage and, contrary to its role in cell-cycle checkpoint regulation, ATM was dispensable for the regulation of HR repair by an induced DSB.

Results

Human NBS fibroblast cells show a decreased frequency of HR repair

We have previously shown in hyper-recombinogenic chicken DT40 cells that chicken Nbs1 is essential for normal HR repair of an induced DSB in the SCneo sequence (Tauchi *et al*., 2002). On the basis of this finding, it was of interest to determine whether NBS patient cells are characterized by the impaired HR repair of DSBs. In the present study, HR repair frequency in a human NBS fibroblast cell line was examined with the DR–GFP system (Pierce *et al*., 1999) (Figure 1a). In the absence of I-*Sce*I expression, GFP-positive cells are rare in each cell line. When I-*Sce*I expression was induced, the NBS cells (ND#17 and ND#26) showed a three- to fourfold lower frequency in HR repair compared with the clones complemented with wild-type human NBS1 cDNA (ND#17-G, ND#26-A). NBS1 was expressed at a level similar to that in normal cells (Supplementary Figure 1A) and the radiation hypersensitivity of the DR-GFP containing NBS patient cell lines was corrected by the introduction of wild-type NBS1, whereas the sensitivities varied from clone to clone (Figure 1b). Similar to that observed with the DR-GFP assay, a decreased HR repair frequency was also revealed in NBS cells by SCneo analysis. NBS cells showed a repair frequency of about 1/40 of that seen in normal MRC5 cells (Supplementary Figure 4). These observations suggest that NBS1 is required for normal HR repair pathway function by an induced DSB.

Human A-T fibroblast cells show normal frequency of HR repair of an induced DSB

Subsequently, the HR repair frequency in human A-T fibroblast AT5BIVA cells was examined. Figure 2a shows HR repair frequency of DR-GFP containing A-T cells and the ATM-complemented lines. In parallel to the results from the NBS1-mutant assay, the expression of ATM only slightly enhanced the HR repair frequency of an induced DSB in DR-GFP containing A-T clones (Figure 2a), whereas the radiation sensitivity was fully corrected (Figure 2b). Similar results were obtained in A-T cells containing SCneo cassette and ATM-complemented cells (Figure 2c). It is notable that the HR activity in A-T patient cells is almost normal with an HR frequency similar to that seen in normal MRC5SV cells. In these ATM-complemented cells, ATM was expressed at a level similar to that in normal cells (Supplementary Figure 1B) and the radiation sensitivity was restored to normal levels (Figure 2d). This unchanged level of HR was confirmed by ATM inactivation using the inhibitor, KU-55933 (Supplementary Figure 2). These results suggest that ATM is dispensable for HR repair of an induced DSB.

To examine the DSB repair in AT cells, we amplified the genomic region surrounding the I-*Sce*I site of repair products, of which site is lost after completion of several types of repair: HR, single strand annealing (SSA), NHEJ (Figure 3a). The amplified products were subsequently treated with I-*Sce*I and additionally with

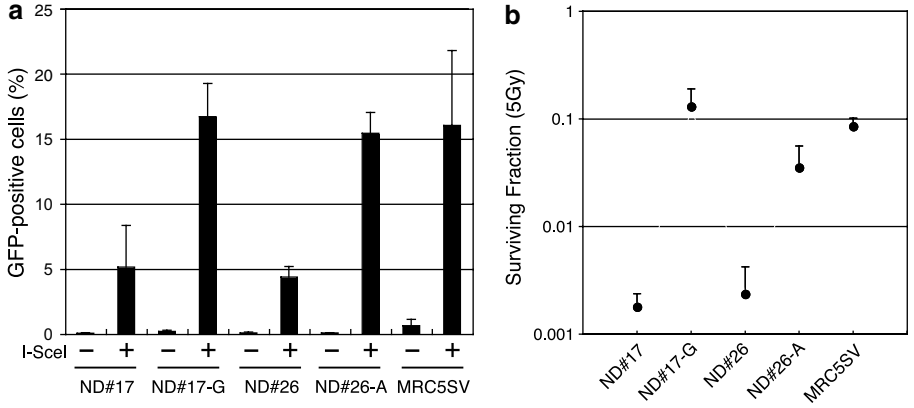

Figure 1 HR repair activity of NBS cells analysed with the DR-GFP assay. (**a**) HR repair frequency in NBS cells (ND#17 and ND#26) and complemented lines (ND#17-G and ND#26-A) were measured with the DR-GFP assay. Bars indicate standard deviation of the means obtained from at least three independent experiments. (**b**) Radiation sensitivity of NBS cells and complemented lines were assayed after 5 Gy of γ-radiation.

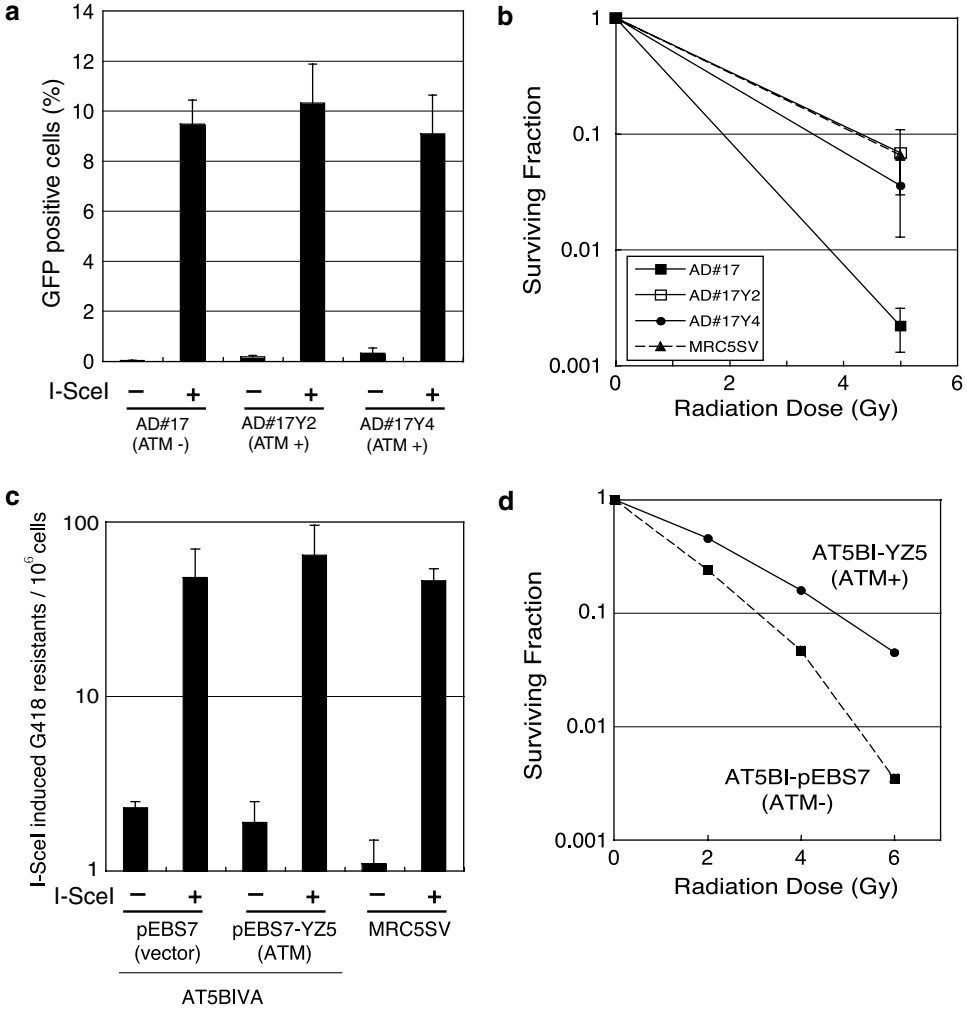

Figure 2 An ATM defect does not reduce the HR frequency in A-T patient fibroblast cells. (**a**) HR repair frequency in A-T cells (AD#17) and complemented lines (AD#17Y2 and AD#17Y4) measured with the DR-GFP assay. Bars indicate standard deviation of the means obtained from at least three independent experiments. (**b**) Radiation sensitivity of A-T cells and complemented lines. Clonogenic survival was assayed as described in Figure 1b. (**c**) HR repair frequencies of A-T cells transfected with the empty vector (pEBS7) or a complemented cell line expressing ATM cDNA (pEBS7-YZ5) were measured with the SCneo assay. Bars are the same as in (**a**). (**d**) Radiation sensitivity of A-T cells and complemented lines. Clonogenic survival was assayed as described in Figure 1b.

BcgI. The amount of uncleaved DNA after digestion with both *Bcg*I and I-*Sce*I indicates the level of NHEJ and the subtraction from the portion of uncleaved DNA after digestion with I-*Sce*I alone provide the level of homologous repair, such as HR and SSA. As a result, AT cells showed higher frequency in NHEJ than in homologous repair (Figure 3b and c). The expression of ATM exaggerated NHEJ repair, whereas the HR remains almost unchanged. They are confirmed by the experiments using ATM inhibitor, KU-55933, which significantly reduced NHEJ level but did not alter HR level (Supplementary Figure 2). Furthermore, this ATM inactivation did not affect HR activity of NBS cells (Supplementary Figure 2), indicating a pivotal role of NBS1 in HR repair in the absence of ATM.

Functional domains of NBS1 in HR repair

To gain insights into the mechanistic role of NBS1 in HR and the possible relevance of ATM and MRE11 to this process, we generated a series of human NBS1-mutant cDNAs and measured their ability to regulate HR repair. The NBS1 mutants used in this study are shown in Figure 4a. Point mutations were introduced within the FHA (FHA-2D; G27D/R28D), BRCT (BRCT-2D; L150D/V151D) at the N-terminal region and for the ATM phosphorylation sites (S343A or S278A/S343A) in NBS1. We also generated a short deletion in the MRE11-binding domain (682–693aa) at the C-terminal region and in the ATM-binding domain at the extreme C-terminal region (703–754aa). The expression levels in the various mutants were assessed with immunoblotting using anti-myc antibodies (Supplementary Figure 1C). Consistent with A-T cells, mutations in the ATM-phosphorylation sites (S278A/S343A) and deletion of the ATM-binding domain (Del703) did not change the capacity of HR compared with that of full-length NBS1-expressing cells (Figure 4b), suggesting that ATM is not essential for

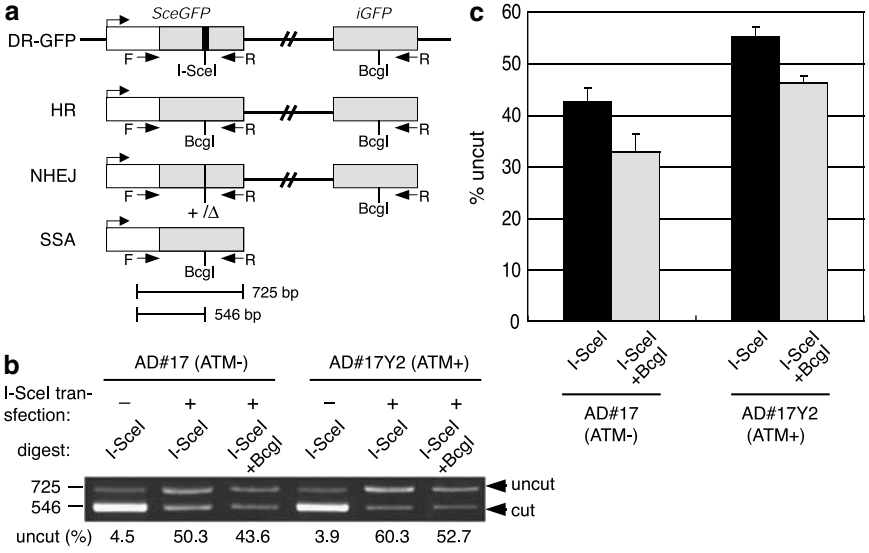

Figure 3 PCR analysis of DR-GFP products in A-T cells. (**a**) Diagrammatic scheme of amplified region surrounding the I-*Sce*I site after rejoined by HR, NHEJ and SSA. (**b**) PCR products (primers F and R) from A-T cells and complemented cell line transfected with an I-*Sce*I expression vector. Amplified products from the same reaction were digested with I-*Sce*I or with I-*Sce*I and *Bcg*I. (**c**) The ratio of NHEJ with homologous repair (HR and SSA) in A-T cells and the complemented cells were calculated from the amount of uncleaved DNA after digestion with I-*Sce*I or with I-*Sce*I and *Bcg*I. Bars represented standard deviations of four experiments.

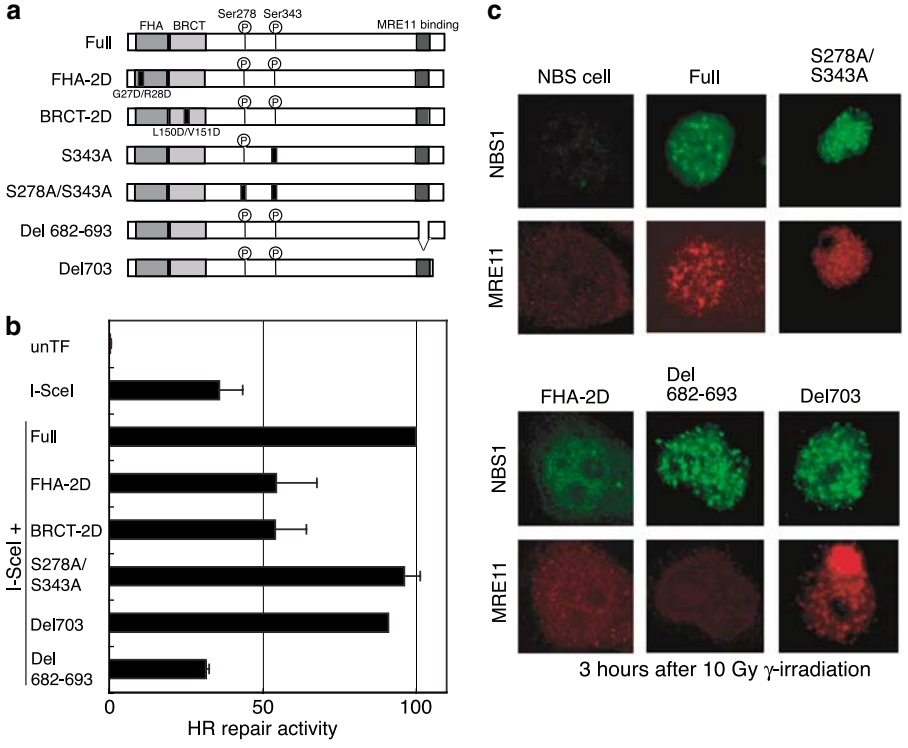

Figure 4 MRE11 foci formation is correlated with HR activity in NBS1 mutants. (**a**) A series of NBS1 mutant cDNAs. Point mutations, which were introduced into the FHA domain and BRCT domain and at ATM phosphorylation sites are indicated by a bold line. A mutant lacking residues 682–693 of the MRE11-binding domain (Del682–693) and the extreme C-terminal mutant (Del703) were also established. (**b**) HR repair activity of various NBS1 mutants identified with the DR-GFP assay. Bars indicate standard deviation of the means obtained from at least three independent experiments. (**c**) Ionizing radiation-induced foci formation of NBS1 and MRE11 in NBS cells (GM7166VA7) or in cells transfected with various mutants of NBS1. Cells irradiated with 10 Gy of γ-rays were stained at 3 h post-treatment.

HR, whereas the ATM-binding domain is crucial for intra-S checkpoint (Supplementary Figure 3). Although coexpression of wild-type NBS1 with I-*Sce*I resulted in a threefold increase in HR frequency (Figure 4b), no such increase was observed with the coexpression of the Del682–693 mutant, suggesting that MRE11 binding is essential for HR activity. In the case of N-terminal mutants (FHA-2D and BRCT-2D), coexpression with I-*Sce*I resulted in only a small increase of the HR repair frequency. The reduced HR activity of the BRCT-2D mutant was not caused by decreased protein level, as NBS1 protein at a similar level did not affect the capability of HR (Supplementary Figure 1D). Thus, two N-terminal-conserved domains, FHA and BRCT, are both important for the HR-enhancing activity of NBS1, in addition to the MRE11-binding domain. Similar results were obtained using SCneo analysis (Supplementary Figure 4).

To compare a level of HR repair with the localization of NBS1 and MRE11 at the damaged sites, we determined the radiation-induced foci formation in NBS1 mutant clones with different HR activities. Both NBS1 and MRE11 foci did not form when NBS cells were irradiated with γ-rays and they were clearly observed in NBS cells transfected with full-length NBS1 and phosphorylation site mutants (Figure 4c). Significant formation of radiation-induced MRE11 foci was observed in a mutant of NBS1 containing the MRE11-binding domain but devoid of ATM-binding region (Del703, Figure 4c), in which a level of HR repair is normal. The ability of MRE11 foci formation in this clone is consistent with the normal foci in the absence of ATM (Mirzoeva and Petrini, 2001). On the other hand, the lack of the MRE11-binding domain resulted in failure of the MRE11 foci formation (Del682–693, Figure 4c) and a level of HR repair was reduced. As NBS1 forms normal foci in this mutant, a key event for HR repair could be MRE11, instead of NBS1, localization as visible-size foci at damaged sites. As we have shown previously, the FHA-2D N-terminal mutant could not form both foci of MRE11 and NBS1 in response to DNA damage (Figure 4c) and a level of HR was reduced (Figure 4b). Thus, MRE11 foci formation at damaged sites correlated well with restoration of HR capability (Supplementary Table 1), suggesting that MRE11 localization at damaged sites is a crucial factor for HR repair and it is regulated by NBS1 through FHA/BRCT domains in ATM-independent modes.

Long-tract gene conversion/sister chromatid exchange events were inhibited by the mutations of FHA/BRCT domain

To extend these investigations of NBS1 functions in HR repair of DSBs, we analysed HR repair products from SCneo in NBS or in mutant NBS1-expressing cells by digestion of S2neo fragment with *Nco*I or I-*Sce*I. Table 1 shows the summarized results of HR-product analysis. Products, which were aberrant, are often seen in NBS patient cell lines and expression of full-length NBS1 dramatically reduced the percentage of these aberrant products. Although it could not be determined whether these aberrant recombination products resulted from nonspecific background events owing to the very low HR frequency, the results indicate the importance of the NBS1 complex in normal HR events. The frequency of aberrant recombination products was also dramatically decreased when partially complementing NBS1 mutants, such as S278A/S343A and S703, were expressed. They were consistent with the findings that it is rare in A-T cells. Interestingly, FHA or BRCT mutants generated no long-tract gene conversion/sister chromatid exchange (LTGC/SCE) products, suggesting that the reduction of HR frequency in those mutants was caused by an abnormality in the formation or processing of recombination intermediates between sister chromatids. This is a reasonable hypothesis because these domains are required for chromatin binding or appropriate recruitment of R/M/N complexes in response to DSB formation.

Discussion

We have previously shown that NBS1 is required for MRE11 foci formation at sites of DNA damage through

Table 1 Analysis of HR-products from SCneo in NBS or in mutant NBS1-expressing cells

Cell line	*No. of HR clones analysed*	*STGC (%)*	*LTGC(SCE) (%)*	*Aberrant HR*[a]	*LTGC ratio of clones versus MRC5SV cells*
MRC5 SV	25	16 (64)	9 (36)	0	1.0
NBS cells	14	1 (7)	2 (14)	11 (79)	0.39
Full	79	60 (76)	17 (22)	2 (3)	0.61
FHA-2D	20	17 (85)	0 (0)	3 (15)	0[b]
BRCT-2D	48	48 (100)	0 (0)	0	0[b]
S278A/S343A	48	23 (48)	20 (42)	5 (10)	1.16
del 682–693	11	2 (18)	8 (73)	1 (9)	2.02
del 703	42	33 (79)	9 (21)	0	0.58
A-T cells	23	8 (35)	15 (65)	0	1.81
+ATM	26	18 (69)	8 (31)	0	0.86

Abbreviations: A-T, ataxia-telangiectasia; HR, homologous recombination; NBS, Nijmegen breakage syndrome; LTGC(SCE), long-tract gene conversion (sister chromatid exchange); STGC, short-tract gene conversion. [a]Clones that do not fit either STGC or LTGC. [b]Significant at $P<0.01$.

R/M/N complex formation (Tauchi *et al*., 2001). The present results, moreover, demonstrated that foci formation of MRE11 appears to be crucial for HR repair, because either mutation at FHA/BRCT domains or deletion of the MRE11-binding domain abolished MRE11 foci formation and HR activity of NBS1 (Figure 4b and c). It is noted that the initial recruitment of repair proteins to DSB does not correlate with their foci formation at damage sites, as the cells lacking histone H2AX can recruit them but cannot form their foci (Celeste *et al*., 2003). This disappearance of the foci might be because of failure in retaining the repair protein at the vicinity of DSB and it is substantiated by observation at laser-irradiated NBS cells (Kim *et al*., 2002). These evidences suggest that initial recruitment of MRE11 is not enough for proper HR events and it is required to accumulate and retain at the vicinity of DSB, in which R/M/N complex facilitate HR repair possibly by physical interaction with several HR-related proteins, such as BRCA1 (Greenberg *et al*., 2006).

An obvious question is how does the N-terminus of NBS1 contribute to HR repair? Zhao *et al*. (2002) showed that these N-terminal domains are important for optimal chromatin binding. However, it should be noted that chromatin binding of the R/M/N complex is not dependent on exposure to IR. Thus, the functional relationship between the HR repair activity of NBS1 and biochemically defined chromatin binding is unclear. On the other hand, we previously demonstrated that the FHA/BRCT domain binds directly to phosphorylated histone H2AX and recruits and retains the R/M/N complex to the vicinity of sites containing DSBs (Kobayashi *et al*., 2002). Therefore, NBS1 can form the foci even in the absence of MRE11-binding region, whereas the mutant NBS1 foci are dispensable for HR repair (Supplementary Table 1). Interestingly, a recent report indicated that H2AX serine 139 is involved in the efficient HR repair of DSBs using the sister chromatid as a template (Xie *et al*., 2004). Taking these findings together, it can be hypothesized that NBS1 functions in HR repair using the sister chromatid through recruitment or retention of MRE11/RAD50 to the vicinity of a DSB site by binding to γ H2AX. This hypothesis is consistent with the results of repair product analysis (Table 1), which indicated that N-terminal mutants generated no LTGC/SCE products.

Because NBS and A-T cells have similar phenotypes, it seems reasonable to expect that there must be an interaction or collaboration between NBS1 and ATM in cellular responses to DSBs. Definite conclusions about the role of ATM in the HR repair of an I-*Sce*I-induced DSB still remain to be established, but there are two reports with different conclusion. Golding *et al*. (2004) showed that inhibition of ATM by dominant-negative ATM mutations or caffeine treatment decreased the HR repair frequency in human glioma cells using the DR-GFP assay. In contrast, a report by Bolderson *et al*. (2004), using the SCneo assay, found that human A-T fibroblast cell lines are proficient in HR repair. Furthermore, ATM$^{-/-}$ DT40 cells showed twofold decrease in HR based on the targeted integration at specific loci (Takao *et al*., 1999), and this is in contrast to NBS1$^{-/-}$ DT40 cells, in which HR was reduced to 200-folds (Tauchi *et al*., 2002). It should be considered that the changes in HR frequency of ATM$^{-/-}$ DT40 cells were unable to detect in human A-T cells, because the repressed level of HR was reduced from 200-fold in NBS1$^{-/-}$ DT40 cells to three- to fourfold in human NBS cells (present results). This is also substantiated by comparison of HR levels between human FA-D2 cells (Nakanishi *et al*., 2005) and FANCD2$^{-/-}$DT40 cells (Yamamoto *et al*., 2005). In this study, a normal HR repair frequency in human A-T fibroblast cells was found (Figure 2a and c) and it is confirmed by the findings that only a slight decrease in HR repair ability was seen in S278A/S343A and Del703 mutants (Figure 4b). Therefore, it suggests that ATM is dispensable for the HR repair of an induced DSB and that NBS1 can function in DSB repair using HR in an ATM independent manner. This is consistent with that H2AX phosphorylation and thereby MRE11 and NBS1 foci formation at damage sites occurred in the absence of ATM (Mirzoeva and Petrini, 2001; Kobayashi *et al*., 2002).

A-T cells showed normal HR repair frequency with severe radiation hypersensitivity (Figure 2). One explanation for this difference is that the HR repair pathway is dispensable for cellular radiation sensitivity and that other DSBs repair pathways, such as NHEJ are the major pathways that determine radiation sensitivity. For instance, a recent report showed that an ATM-dependent DSB end-joining pathway is critical for cellular radiation sensitivity (Riballo *et al*., 2004), and that NBS1 is also required for this pathway. This is supported by our polymerase chain reaction (PCR) analysis of repair products that showed the enhanced NHEJ in ATM-complemented cells (Figure 3). Another explanation is that ATM is important for radiation-induced damage, but not for I-*Sce*I-induced DSB, because it is well known that radiation-induced damage is more complex than endonuclease-induced DSB. The majority of DSB generated by radiation has frequently 3′-phosphate or 3′-phosphoglycolate end, which requires processing before ligation, whereas I-*Sce*I-induced DSB has 3′-hydroxyl moieties.

Recent intensive studies have revealed a functional link between ATM and NBS1 at the signal transduction. Serine residues at positions 278 and 343 in NBS1 are phosphorylated by ATM both *in vitro* and *in vivo*, and these phosphorylation events are strongly associated with an intra-S cell-cycle checkpoint (Lim *et al*., 2000). Furthermore, Falck *et al*. (2005) reported that NBS1 binds to ATM through its conserved C-terminal motif and this motif is essential for efficient recruitment of ATM to sites of DNA damage. Consistent with this, present results indicated that the intra-S checkpoint requires ATM-binding domain on NBS1 in addition to ATM itself (Supplementary Figure 3). However, both mutants at the ATM-binding domain and at the serine 278- and 343-residues allow for normal HR repair (Figure 2a and c). These observations indicate that the

regulatory mechanism of NBS1 in HR repair differs from that involved in the intra-S checkpoint, although the checkpoint must be associated with DSB repair. Among the proteins responsible for NBS/A-T phenotype diseases, NBS1 is a unique protein, which contains the domains for interaction with other two proteins, MRE11 and ATM, and regulates them in a response to DSB (Cerosaletti and Concannon, 2003; Horejsi *et al*., 2004). Therefore, NBS1 has a dual function in HR repair (Figure 1) and in the intra-S checkpoint (Supplementary Figure 3), providing a crossroad between checkpoint control and DNA repair.

In conclusion, our data demonstrate that HR requires FHA/BRCT domains and MRE11-binding domain of NBS1, which regulate the MRE11 recruitment and retention, suggesting that MRE11 foci formed at damaged sites is a crucial step for HR repair. Furthermore, the function of the R/M/N complex in HR repair is independent of ATM, possibly owing to ability of normal MRE11 foci formation in the absence of ATM. Further studies are still required to evaluate the significance of the NBS1-dependent HR pathway on cellular radiation sensitivity and chromosomal instability, which are major phenotypes of NBS/A-T diseases.

Materials and methods

Plasmid and DNA manipulations

ATM cDNA was kindly supplied by Dr Y Shiloh. Wild-type, Del703, and Del683–692 NBS1 cDNAs were cloned into the pIRES-hyg vector (Clontech, Mountain View, CA, USA) as described previously (Tauchi *et al*., 2001). The missense mutations, FHA-2D (G27D, R28D), BRCT-2D (L150D, V151D) and S278A/S343A, were generated by PCR using primers containing point mutations with Pyrobest DNA polymerase (TaKaRa, Kyoto, Japan) and cloned into the pIRES-hyg vector (Clontech). For tagging with Myc-His, cDNA was subcloned into the *Bam*HI site and the *Xho*I site of the pcDNA3.1/Myc-His (A) vector (Invitrogen, Carlsbad, CA, USA). The entire cDNA insert was confirmed by DNA sequencing.

Cell culture and transfection

GM7166VA7 cells from an NBS patient were used as the recipient for all the mutant NBS1 cDNAs. AT5BIVA and MRC5SV cells were used as an A-T cell line and a control cell line, respectively. Cell cultures were maintained in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA). The vectors were transfected into GM7166VA7 and AT5BIVA cells with electroporation using a GenePulser (Bio-Rad, Hercules, CA, USA) and stable transformants were selected by incubation in medium containing 400 μg/ml hygromycin B (Wako, Osaka, Japan).

Immunostaining

Immunostaining was performed as described previously (Tauchi *et al*., 2001). The primary antibodies used were as follows: anti-NBS1 (Novus Biologicals, Littleton, CO, USA) and Alexa-488-conjugated anti-rabbit IgG (Molecular Probes, Carlsbad, CA, USA), or anti-MRE11 (Novus Biologicals) and Alexa-594-conjugated anti-rabbit IgG (Molecular Probes) was used for visualization of NBS1 or MRE11. The excited fluorescence from the Alexa-488 or 594 was visualized with a laser-scanning microscope (Olympus, Tokyo, Japan).

DR-GFP assay

Stable integrants were obtained by electroporating cells with linearized phprtDR-GFP (Pierce *et al*., 1999). Puromycin-resistant colonies were screened with Southern blots for an intact DR-GFP reporter. To measure the repair of an I-*Sce*I-generated DSBs, 50 μg of the I-*Sce*I expression vector pCBASce was introduced to 5×10^6 cells by electroporation. To determine the amount of HR repair, the percentage of cells that were GFP-positive was quantitated with flow cytometric analysis 4–7 days after electroporation on a Becton Dickinson FACScan. The HR ability was compared with NHEJ in NBS and A-T cells, as described by others (Nakanishi *et al*., 2005). Briefly, genomic DNA was used as template and the PCR products were digested overnight with 10 units of I-*Sce*I (Roche Molecular Biochemicals, Indianapolis, IN, USA), then half volume of products was digested with *Bcg*I (NEB, Beverly, MA, USA). The digested products were separated and the fluorescence signals for the enzyme-resistant band were quantified for determination of ratio of HR and NHEJ ability.

SCneo assay

SCneo analysis was performed as described elsewhere (Tauchi *et al*., 2002). For analysis of the HR products, the S2neo sequence in G418-resistant clones was amplified by PCR using a specific primer set (5′-CGTCGAGCAGTGTGGTTTTCA-3′ and 5′-AAAGCACGAGGAAGCGGTCAG-3′) and Ex Taq DNA polymerase (TaKaRa). The amplified DNA was digested with *Nco*I or I-*Sce*I to identify two types of the HR products: short-tract gene conversion or LTGC/SCE.

Cell survival assay

Exponentially growing cells were trypsinized and then an appropriate number of cells were plated. The cells were then irradiated with ^{137}Cs γ-rays. After 18 days of incubation, the surviving fractions were calculated by the number of colonies.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).