# Molecular characterization of the *Schizosaccharomyces pombe* *nbs1*$^{+}$ gene involved in DNA repair and telomere maintenance

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**Abstract**

The human MRN complex is a multi-subunit nuclease that is composed of Mre11, Rad50 and Nbs1 and is involved in homologous recombination and DNA damage checkpoints. Mutations of the MRN genes cause genetic disorders such as Nijmegen breakage syndrome (NBS). Here we identified an *Schizosaccharomyces pombe nbs1*$^{+}$ homologue by screening for mutants that were methyl methane sulfonate (MMS)-sensitive and synthetically lethal with *rad2Δ* mutation. Nbs1 physically interacts with the C-terminal half of Rad32, *S. pombe* Mre11 homologue, in a yeast two-hybrid assay. *nbs1* mutants showed similar sensitivities to γ-rays, UV, MMS and hydroxyurea and displayed telomere shortening as *rad32* and *rad50* mutants. *nbs1*, *rad32*, and *rad50* mutant cells were elongated and exhibited abnormal nuclear morphology. These findings indicate that *S. pombe* Nbs1 forms a complex with Rad32-Rad50 and is required for homologous recombination repair, telomere length regulation, and the maintenance of chromatin structure. Amino acid sequence features and some characteristics of the DNA repair function suggest that the *S. pombe* Rad32-Rad50-Nbs1 complex has functional similarity to the corresponding MRN complexes of higher eukaryotes. Therefore, *S. pombe* Nbs1 will provide an additional model system for studying the molecular function of the MRN complex associated with genetic diseases.

(**Introduction**)

Nijmegen breakage syndrome (NBS) is an autosomal recessive genetic disease characterized by developmental defects, microcephaly, immune deficiency and a high incidence of cancer (8, 38, 64). Cells from NBS patients show genetic instability and hypersensitivity to γ-ray irradiation and are impaired in cellular responses to γ-ray irradiation including the radio-resistant DNA synthesis (29, 52, 57). These cytogenetic features are indistinguishable from those of another genetic disease, ataxia telangiectasia (AT) (50). The cellular defects in NBS and AT cells are suggested to be caused by defective responses to DNA double-strand breaks (DSBs) due to mutation(s) of the responsible genes, *NBS1* and *ATM*, respectively.

DSBs are not only generated by exogenous DNA damaging agents such as γ-ray irradiation, but they also occur during normal DNA replication. There are two main pathways for the repair of such DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR). The Mre11-Rad50-Xrs2 protein complex (MRX complex) from *Saccharomyces cerevisiae* has been suggested to be involved in the initial steps of both repair pathways (24). In NHEJ, the MRX complex is suggested to stimulate the intermolecular DNA end joining by DNA ligase IV (11). In HR, the processing of DSB ends to produce 3' single-stranded tails is a very important reaction that provides substrates for homologous pairing and strand exchange reactions. From early studies, the MRX complex was thought to function as a nuclease that is involved in such a DNA end processing (24). However, recent studies suggest a structural role of the MRX complex in holding DNA ends and/or sister chromatids together (12, 16, 25, 26, 28, 30).

The Mre11 and Rad50 proteins are highly conserved from yeast to humans.

However, Xrs2 is not well conserved, and the functional counterpart of Xrs2 is considered to be Nbs1 in vertebrates. The similarity of the overall sequences between those two proteins is very weak, and is limited to only the N-terminal FHA domain and a small C-terminal region (56). Although Nbs1 contains a BRCA C-terminal (BRCT) domain in the N-terminal region, Xrs2 does not contain this domain. Nbs1 binds to the Mre11 subunit of Rad50-Mre11 complex via the C-terminal conserved region (18, 56).

The MRX (N) complex possesses exo/endonuclease activity *in vitro* (21, 49, 59, 63). Mre11 contains the phosphodiesterase motif responsible for the nuclease activity (27). Rad50 is related to the SMC proteins, which contain Walker A and B motifs responsible for ATPase activity separated by a long coiled-coil region (26, 27). Rad50 stimulates the nuclease activity of yeast and human Mre11 (48, 60). The biochemical functions of Xrs2 and Nbs1 during the recombination process remain unclear. To date, the only biochemical activity assigned to Nbs1 is stimulation of the unwinding and hairpin cleavage activities of the human Mre11-Rad50 complex (49). The yeast Mre11-Rad50 complex cleaves hairpin structures in the absence of Xrs2 (59). Surprisingly, some mutations that eliminate the nuclease activity of yeast Mre11 do not confer telomere defects or strong DNA repair deficiency (33, 41). Therefore, it seems likely that end processing is not the major role of the complex.

Mutations in the genes that encode components of the MRX(N) complex result in DNA-damage sensitivity, genome instability, telomere shortening and aberrant meiosis (14). These phenotypes are considered to be related to deficiencies of DNA repair abilities involving the DNA-processing activity of the MRX (N) complex. In addition, recent studies have shown other functions for the Mre11 complex in checkpoint signaling and DNA replication (6, 31, 37, 39, 68). Hypomorphic mutations

in the human *MRE11* gene cause ataxia telangiectasia (AT)-like disorder (ATLD), whose symptoms are very similar to those of NBS and AT (51). In the cells from these patients, DNA synthesis is not arrested in response to ionizing radiation (IR). Yeast mutants with mutations of *mre11*, *rad50* or *xrs2* are hypersensitive to hydroxyurea (HU) and show no delay in DNA synthesis in the presence of HU or bleomycin, indicating a defect in the intra-S-checkpoint (15, 23). Mre11 and Xrs2/Nbs1 have been shown to be phosphorylated in response to IR, and this phosphorylation is dependent on checkpoint kinase function (22, 34, 70).

In the fission yeast *Schizosaccharomyces pombe*, which is distantly related to *S. cerevisiae*, the *rad32*$^+$ gene was first identified as a gene corresponding to a mutant that showed hyper-sensitivity to DNA damage and was later found to be the structural and functional homologue of *MRE11* (58, 65, 66). The *rad50*$^+$ gene in *S. pombe* was identified through its sequence similarity with the *S. cerevisiae*, *Caenorhabditis elegans*, and human *RAD50* genes. Both *rad32*$^+$ and *rad50*$^+$ are required for DNA repair and telomere maintenance (25). However, no Nbs1/Xrs2 homologue in *S. pombe* has been reported; one of the reasons for this is that no structural homologue can be found in the database even though the whole genome sequence of *S. pombe* has been determined (67).

To identify novel genes involved in homologous recombination and recombination repair in *S. pombe*, we previously isolated seven *slr* mutants, whose growth was dependent on the *rad2*$^+$ function (*slr* stands for synthetic lethalality with *rad2*.) (62). The *rad2*$^+$ gene encodes a structure-specific endonuclease homologous to mammalian Fen-1, that is required for Okazaki fragment maturation (46). Yeast cells defective in Fen-1 nuclease activity are non-viable in combination with mutations that

inactivate HR (17, 54). Thus, *slr* mutants are often considered to be defective in HR and recombination repair. Indeed, *S. pombe rhp51* (*RAD51* homologue), *rhp54* (*RAD54* homologue), *rhp57* (*RAD57* homologue), *rad50* and *rad32* mutations cause lethality when combined with the *rad2* mutation (25, 45, 58, 62). In an earlier study, we characterized two genes, *rhp57*$^+$ and *rad60*$^+$, identified among the seven *slr* mutants. The former is a structural and functional homologue of *S. cerevisiae RAD57* and the later is a novel recombination gene for which no structural homologue has been found in the database (43, 62).

Since the *slr* screening is an effective mechanism for isolating recombination-related genes, we performed a second large-scale screening and obtained further *slr* candidates. In this study, we characterize the eighth *slr* mutant, *slr8*. DNA sequencing of complementing plasmids revealed that *slr8*$^+$ encodes a protein with a forkhead associated (FHA) domain in the N-terminal region and an Mre11-binding consensus sequence in the C-terminal region. Indeed, Slr8 protein was found to physically interact with the Rad32 via the C-terminal region of Rad32 using a yeast two-hybrid assay. *slr8Δ* mutants showed very similar DNA repair defect phenotypes to *rad32Δ* and *rad50Δ* mutants, and showed epistatic interactions with these mutations. Although the overall sequence similarities of the three proteins, *S. cerevisiae* Xrs2, Slr8 and vertebrate Nbs1, are limited, our data allow us to conclude that *slr8*$^+$ is a functional homologue of *XRS2/NBS1* and thus we have designated it *nbs1*$^+$ in *S. pombe*.

## MATERIALS AND METHODS

***S. pombe* strains, media and genetic methods.** The *S. pombe* strains used in this study are listed in Table 1. All strains are derivatives of 972 $h^-$, 975 $h^+$ or JY741 $h^-$. Standard procedures and media were used for propagation and genetic manipulation (42). To measure the sensitivity of cells to γ-rays or UV light, exponentially growing cells were irradiated with γ-rays from a $^{60}$Co source at a dose rate of 100-200 Gy/hour or with UV light from a germicidal lamp (UVP UV-CROSSLINKER, CL-1000) at a dose rate of 50-100J/m$^2$/min. Duplicates of irradiated cells and unirradiated cells were plated on YPAD medium plates and incubated at 30 ˚C for 4 days, and the colonies were counted. For semi-quantitative analysis of DNA repair activity, the spot assay was employed as described (62). Briefly, 3 μl of 10-fold dilutions of log-phase cells (0.5x10$^7$ cells/ml) were spotted onto a YPAD (agar 2%) plate or YPAD plate containing the indicated concentration of MMS or HU. All experiments were repeated at least twice and gave similar results.

***slr* mutant isolation**. *S. pombe* strain SP184 (*h⁻ smt-0 ade6-704 ura4-D18 leu1-32 rad2:: ura4*$^+$) carrying pAUR2 , which is a derivative of pUR18 that carries the *rad2*$^+$ gene and *ura4*$^+$ as a selective marker (62), was mutagenized with *N*-methyl-*N*'nitro-*N*-nitroguanidine as described by Moreno et al. (42). Initially, approximately 100,000 colonies were roughly examined for the plasmid-dependency of their growth by replica plating on EMM plates containing 0.1% 5-fluoroorotic acid (5-FOA), and 521 5-FOA-sensitive colonies were isolated. These isolates were examined for methylmethane sulfonate (MMS) sensitivity by replica plating on EMM plates containing 0.004% MMS, and 92 MMS-sensitive colonies were obtained. Their 5-FOA sensitivity were confirmed by a spot assay on EMM plates containing 5-FOA,

and 42 of the isolates were found to be sensitive to both MMS and 5-FOA. In this study, we analyzed 15 of those isolates that showed much higher sensitivity to MMS than the others. These 15 isolates were then crossed with SP185 (*h*$^{+}$ *ade6-704 ura4-D18 rad2::ura4*$^{+}$ ) carrying pAUR2. Three isolates were found to produce progeny with both phenotypes of MMS-sensitivity and 5-FOA-resistance, indicating that the growth of the three strains was not dependent on pAUR2. The remaining 12 strains were backcrossed three times with the wild-type strain, and the final progeny that were obtained had the phenotypes *slr*$^{-}$ *rad2*$^{+}$ *ade*$^{-}$ *ura*$^{-}$ and *leu*$^{-}$.

**Cloning of the *slr* gene**. Each of the new *slr* mutants was transformed with the *S. pombe* genomic libraries constructed in the laboratories of Dr. H. Masukata (Osaka University) and Dr. A. M. Carr (University of Sussex), and spread on EMM plates containing appropriate supplements and MMS (0.004%). The transformants were examined for plasmid-dependent MMS resistance and the plasmids which complemented the MMS sensitivity were recovered as described previously (43).

***slr8* cDNA cloning**. The cDNA corresponding to the *slr8*$^{+}$ N-terminal region was amplified by PCR with a sense primer, IWA 252, (5'-CTCATATGAACAGACAAGCTGGGTCCAG-3') and an antisense primer, IWA184, (5'-GAGGATCCTTAAAAGTGAAACTTGAGATCATTAAATTCATCG-3'), using the *S. pombe* cDNA library (Clontech) as template. The PCR product was cloned into the *Nde* I- *Bam* HI site of the vector pBSNde, giving the plasmid pNT139. pBSNde was constructed by insertion of an *Nde* I linker (5'-CCCATATGGG-3' ) into the *Hin*c II site of pBluescript ll SK (+). The cDNA corresponding to the C-terminal region was amplified by PCR with a sense primer, IWA 256, (5' –CTCCATATGTGGATAATTGAGGCTGAGGCTGAGGGTGACATTC-3') and an

antisense primer, IWA37, (5'-CAGAGTCATATACTGCGTTG-3') and the product was cloned into the *Nde* I- *Bam* HI site of pBSNde, giving the plasmid pNT136. The full-length open reading frame (ORF) of the cDNA was constructed by insertion of the *Nde* I –*Eco* RV fragment encoding the N-terminal portion of *slr8*$^{+}$ from pNT139 into the *Nde* I –*Eco* RV site of pNT136, giving pNT140. The nucleotide sequence of the full-length ORF was determined using an automated DNA sequencer (ABI PRISM 3100) and shown to match with SPBC6B1.09c in cosmid c6B1. The exons predicted at the Sanger Centre Database (http://srs.ebi.ac.uk/srs6bin/cgi-bin/wgetz?-e+[{EMBL%20EMBLNEW}-acc:AL021838]] and the exons predicted by our cDNA sequencing are shown below. Exons predicted in the Sanger Center database. Join(complement(26318..26354), complement(26205..26258), complement(26078..26166), complement(25825..25988), complement(24495..25785), complement(24185..24448)). Exons predicted by our cDNA sequencing. Join(complement(26318..26354), complement(26205..26258), complement(26078..26166), complement(25825..25973), complement(25703..25785), complement(24495..25660), complement(24185..24448)). The numbers used above correspond to the numbering used in cosmid c6B1 in the Sanger Centre Database. The cDNA sequence data are available from the DDBJ/EMBL/GenBank nucleotide databases (accession no. AB099299)**.**

**3'- and 5'-RACEs**. Total RNA was prepared from wild-type *S. pombe* strain 968 ($h^{90}$) by using an RNeasy Mini kit (QIAGEN) and then the mRNA was purified using an OligotexTM-dT30<Super>mRNA Purification kit (TaKaRa). 3'-RACE and 5'-RACE experiments were carried out by using a 3'-Full RACE Core Set (TaKaRa) and 5'-Full RACE Core Set (TaKaRa), respectively,

according to the manufacturer's instructions. The primer sets used for 3'-RACE and 5'-RACE were as follows:

IWA328 (sense primer for 3'-RACE),

GTCATCCGAGAAATCGAATGCTAACAGTA

IWA318 (reverse transcription primer for 5'-RACE), 318CGTATCGAGGTCCTTTAC

IWA321 (1st sense primer for 5'-RACE), GCCATGCTCGTTTTACG

IWA320 (1st antisense primer for 5'-RACE), CGAATCGTCAGATACATTTC

IWA322 (2nd sense primer for 5'-RACE), GTGAGAAAGACTACTTTACC

IWA319 (2nd antisense primer for 5'-RACE), CCTACAATATAAGTTCCTGG.

**Yeast two-hybrid analysis.** Gal4-based Matchmaker Two-Hybrid system 3 (Clontech) was used for the yeast two-hybrid assay according to the manufacturer's instructions. *S. cerevisiae* strain AH109 was used as the reporter strain, The indicated proteins were fused to the GAL4 activation domain (AD) in pGADT7 vector and the GAL4 DNA-binding domain (DBD) in pGBKT7, and expressed in AH109. The reciprocal combinations of fusions with AD and DBD were examined. The interactions were judged by a spot test on 3 types of dropout (DO) plates: 4DO (SD -adenine, - histidine, - leucine and - tryptophan; high stringency condition), 3DO (4DO + adenine; medium stringency) and the control 2DO (SD - leucine and - tryptophan to select plasmids).

**Disruption of the *nbs1*$^+$ gene**. An Nbs1 knockout plasmid, pNT117, was constructed as bellows. A 0.8-kb fragment containing the sequence upstream of the *nbs1*$^+$ ORF, which was amplified by PCR using genomic DNA and primers IWA 180 (5'-CACAAGCTTGTATACACATACTTCTCCAG-3') and IWA 181 (5'-CACCTCGAGGGTTTAGTAGATTTAGCTTC-3') was subcloned into pBluescript

II SK (+) giving the plasmid pNT115. A 1.0-kb fragment containing the sequence downstream of the *nbs1*$^+$ ORF, which was amplified by PCR using genomic DNA and primers IWA 178 (5'-CACGGATCCGCTCTTCTTCCAAGATTTTG-3') and IWA 179 (5'-CACAAGCTTGACTACTTACCTGAGATATC-3') was subcloned into pBluescript II SK (+), giving a the plasmid pNT114. Then *Hind* III-*Bam* HI fragment containing the sequence downstream of the *nbs1*$^+$ ORF from pNT114 was inserted into the *Hind* III-*Bam* HI site in pNT115, giving pNT116. Next the 1.8-kb *ura4*$^+$ gene was inserted into the *Hind* III site in pNT116, giving the knockout plasmid pNT117. An *Xho* I-*Bam* HI fragment carrying the *nbs1::ura4*$^+$ construct derived from the knockout plasmid pNT117 was transformed into haploid strain SPN124 (*smt-0 ura4-D18 leu1-32 his3-D1 arg3-D1*) by using the lithium acetate method (42). Stable transformants were isolated, and gene disruption was confirmed by Southern blot analysis.

**Measurement of telomere length.** Telomere length was measured by Southern hybridization according to the procedure described previously (13) by using with the AlkPhos DirectTM kit module (Amersham Pharmacia Biotech). Briefly, chromosomal DNA, which was digested with *Apa* I and separated by electrophoresis on a agarose gel, was probed with a 0.3-kb DNA fragment containing the telomeric repeat sequences, which was derived from pNSU70 (a gift from Dr. N. Sugawara).

## RESULTS AND DISCUSSION

**Newly isolated *slr* mutants.** To identify novel genes involved in homologous recombination and recombination repair of *S. pombe,* we isolated *slr* mutants as described in *MATERIALS AND METHODS*. From approximately 100,000 cells mutagenized with *N*-methyl-*N'*nitro-*N*-nitroguanidine, 12 colonies were identified as *slr* mutants (Table 2). Complementation analysis of MMS- and UV-sensitivities using the cloned genes *rhp51*$^+$, *rhp55*$^+$ and *rhp57*$^+$ revealed that mutant number 11 in Table 2 was an allele of *rhp57*$^+$. We subsequently attempted to isolate plasmids that complemented the MMS-sensitivity of the 11 remaining mutants from two genomic libraries. Four genes have been isolated to date. Mutant number 6, 7, and 23 were complemented by plasmids carrying the *rad32*$^+$ gene. Sequencing of the genomic DNA revealed that the *rad32*$^+$ gene was mutated in these strains. This is consistent with a previous report demonstrating that *rad32* mutations are lethal in combination with a *rad2* mutation (58).

One plasmid that complemented the sensitivity of mutant number 27 was isolated (pNT101). pNT101 carried a 3.3-kb genomic DNA fragment of cosmid c6B1. Since there are no known genes involved in DNA repair and recombination in this region, we renamed it *slr8*$^+$ (since we had already isolated 7 *slr* mutants (62)), and further analyzed it in this study.

**Identification of the *nbs1*$^+$ gene in *S. pombe*.** Complementation analysis using several deletion plasmids derivated from pNT101 revealed that *slr8*$^+$ corresponds to the open reading frame, SPBC6B1.09c. The cDNA was prepared and its sequence revealed that *slr8*$^+$ contained six introns, although only five of these were predicted in

the Sanger Centre Database (http://srs.ebi.ac.uk/srs6bin/cgi-bin/wgetz?-e+[{EMBL%20EMBLNEW}-acc:AL021838]) (see *MATERIALS AND METHODS*). RACE experiments revealed that mRNA for *nbs1*$^+$ was transcribed from 82 bp upstream from the putative initiation codon to 110 bp downstream from the predicted stop codon. Since there was a stop codon and no ATG codons in-frame upstream of the putative ATG, this should be the actual initiation codon. These cDNA analyses indicate that the *slr8*$^+$ gene product consists of 613 amino acids. Genomic DNA sequencing of the *slr8* locus revealed that the codon for amino acid gln-88 (CAA) in Slr8 protein is mutated to a nonsense sequence (TAA) in the *slr8* mutant, suggesting that the gene is responsible for the *slr8* mutation. The features of the amino acid sequence of Slr8 (see below) suggest that this protein could be an Nbs1 homologue, and therefore we designated Slr8 as Nbs1 in *S. pombe*.

**Stucture of *S. pombe* Nbs1/Slr8 protein and Nbs1 homologues.**

Vertebrate Nbs1 proteins contain an FHA domain in the N-terminal region, followed by a BRCT domain, and a C-terminal conserved domain (CCD) (Fig. 1A). The *S. pombe* Slr8 also contains FHA domain in the N-terminal region (1-103 aa) and a CCD in the C-terminal region (470-531 aa), which show 26 % and 23 % identities to FHA and CCD in human Nbs1, respectively (Figs. 1AB and D). The domain search program at the NCBI server (http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi?cmd=rps) did not detect a BRCT domain in Slr8. However, a hydrophobic cluster analysis (7) revealed that Slr8 contains all of the five motifs (designated *A*-*E*) characteristic of the BRCT domain (Fig. 1C). In addition to these five motifs, the sequence corresponding to the alpha helix ($\alpha$2) in the BRCT domain is highly conserved in Slr8 (Fig. 1C). This region (106-199 aa) shows 18% identity to the BRCT domain of human Nbs1 (Fig.

1C). These sequence features suggest that Slr8 contains a BRCT domain in this region. Therefore, we conclude that Slr8 is a structural homologue of Nbs1, although the overall similarity is very limited. *S. cerevisiae* Xrs2, which is considered an Nbs1 counterpart, does not seem to possess a BRCT domain (data not shown and (14)). Therefore, Nbs1 is a more appropriate name than Xrs2 for the Slr8 protein.

The FHA domains in human Chk2 and *S. cerevisiae* Rad53 have been shown to bind to phosphorylated proteins (1, 53). The BRCT domain is also predicted to be a protein-protein interaction domain (69). Indeed, the recombinant fragment containing the FHA/BRCT domain of human Nbs1 bind to phosphorylated histone H2AX (γ-H2AX) (32). Therefore, *S. pombe* Nbs1 most probably binds to phosphorylated histone H2A. The physiological significance of this interaction remains unclear, and will be the subject of future studies.

A database search revealed that CG6754-PB protein from *Drosophila melanogaster* contains the three domains (FHA, BRCT and CCD), suggesting that it could be the Nbs1 homologue in the fly (Fig. 1A). Moreover, two protein fragments (We designated them as drNBS1-N or drNBS1-C) predicted from cDNA clones in Zebrafish (*Danio rerio*), BI984731 and BM775439, and two protein fragments (designated as xlNBS1-N or xlNBS1-C) predicted from cDNA clones in the frog (*Xenopus laevis*), CA988284 and BG022948, showed high similarity to the human NBS1 N-terminal fragment and C-terminal fragment, respectively (Fig. 1A). These fragments contain either the FHA/BRCT domains or CCD, which show high similarity to those of human Nbs1 (Figs. 1B-D). These amino acid sequence features strongly suggest that frog and zebrafish contains NBS1 proteins, although the full-length cDNAs those encode frog or zebrafish NBS1 have not been identified. These cDNA

fragments containing FHA/BRCT domains and CCD might be truncated products from the same genes.

We also found that the amino acid sequence (427-484) of ScaA protein in the filamentous fungus *Aspergillus nidulans,* which is suggested to be related to the human NBS1(5), shows high similarity to CCD (637-695) in human NBS1 (Fig. 1D). Strains carrying the *scaA* mutation are hypersensitive to camptothecin, MMS, UV light and γ-rays (5). The sequence of amino acids 230-513 of ScaA shares 20% identity and 38% similarity with that of amino acids 280-572 of *S. pombe* Nbs1. These facts further suggest that ScaA is most probably a functional Nbs1 homologue in *A. nidulans,* although ScaA possesses neither an FHA nor a BRCT domain.

**C-terminal half of Nbs1 binds to Rad32 in a two-hybrid assay.** Since human Nbs1 and *S. cerevisiae* Xrs2 physically interact with Mre11 (10, 21, 63), we tested whether *S. pombe* Nbs1 also interacts with Rad32, an *S. pombe* homologue of Mre11. A yeast two-hybrid assay was performed in which reciprocal combinations of fusions of Nbs1 and Rad32 with the GAL4-activation domain (AD) and the GAL4 DNA binding domain (DBD) were examined (Fig. 2). A DBD-Nbs1 fusion protein interacted with AD-Rad32 fusion at high stringency (4DO). Although we did not detect interaction between the AD-Nbs1 and DBD-Rad32 under "high stringency" conditions, we did observe this at lower stringency (3DO). The inability to detect interactions when using particular combinations of AD and DBD fusion proteins may have been due to a very low level of expression of the fusion protein. Indeed, Western blotting analysis revealed that the protein band corresponding to AD-Nbs1 was barely detectable in the extract from the *S. cerevisiae* tester strain (data not shown). Notably, the two-hybrid interactions were observed between the C-terminal half of Nbs1 and

Rad32 under high stringency conditions, while the N-terminal half of Nbs1 did not interact with Rad32 (Fig. 2B). This is consistent with the proposal that Nbs1 interacts with Mre11 via its small C-terminal conserved region (56). These two-hybrid interactions between Nbs1 and Rad32 suggest that *S. pombe* Nbs1 functions together with Rad32 *in vivo*.

Since the CDDs are highly conserved among Nbs1/Xrs2 proteins, and the conservation is much higher than that of the FHA or BRCA domains (Fig. 1D), it is suggested that the molecular mechanism of the interaction between Nbs1 and Mre11 is conserved in eukaryotes. We also suggest that CDD could be used for searching for Nbs1 homologues in other organisms as described above.

**Construction of *nbs1* disruptants**. To study the *in vivo* function of $nbs1^{+}$ of *S. pombe*, we made a heterozygous strain ($nbs1^{+/-}$) using a one-step gene replacement procedure, in which one of the chromosomal $nbs1^{+}$ genes was replaced with a $ura4^{+}$ cassette. Spores derived from the heterozygote were viable regardless of auxotrophy for uracil, indicating that *nbs1* is not essential (data not shown). However, the growth of the *nbs1* disruptant was poor compared with that of the wild-type strain. The synthetic lethality of the *nbs1* disruptant with *rad2Δ* was confirmed by analyzing the segregants of $nbs1^{+/-}$ $rad2^{-/-}$ heterozygotes (data not shown). We also constructed the *nbs1* null mutant from a haploid strain as described in *MATERIALS AND METODS* and used it for further characterization.

**DNA repair activity of *nbs1Δ* mutants.** We first characterized the DNA repair activity of *nbs1* null mutants. An *nbs1* single mutant showed high sensitivity to γ-ray irradiation, indicating that Nbs1 is involved in DSB repair (Fig. 3A). In addition, the mutant was very sensitive to UV, MMS, and HU, suggesting that Nbs1 is also

involved in the repair of many types of DNA damage in addition to DSBs (Figs. 3B-D). The HU-sensitivity of the *nbs1* mutant might imply the involvement of *S. pombe* Nbs1 in S-phase checkpoint. The sensitivities of the *nbs1* single mutant to these DNA damages were very similar to those of *rad32* and *rad50* single mutants and combinations of these mutations with an *nbs1* mutation did not affect these sensitivities (Figs. 3A-D). These results suggest that Nbs1 functions together with Rad32 and Rad50, perhaps via a complex similar to the Mre11-Rad50-(Xrs2/Nbs1) complex.

Rhp51 protein from *S. pombe* is the functional homologue of *S. cerevisiae* Rad51, which plays central roles in the homology search and DNA strand exchange reactions during homologous recombination and recombination repair (44). The sensitivities to γ-ray and UV of an *nbs1 rhp51* double mutant were very similar to those of the *rhp51* single mutant (Figs. 3E and F). Taken together, our results suggest that that $nbs1^+$ belongs to the same epistatic group in DNA repair as $rad50^+$, $rad32^+$ and $rhp51^+$ and that $nbs1^+$ is involved in recombination repair.

Treatment with MMS, HU or UV-irradiation, unlike γ-ray irradiation, does not directly produce DSBs. However, the fact that *rad22*, *rhp51* and *rhp54* mutants all have significantly increased sensitivities to these treatments indicates that recombination is very important for the recovery from the damage caused by those agents in *S. pombe*. These treatments cause replication fork arrest. When replication forks are stalled, Holliday junctions or chicken-foot structures of DNA are thought to be produced, and those would either be reversed by Rqh1 or resolved by Mus81-Eme1 endonuclease (4, 35). In the latter pathway, it is thought that DNA DSBs are generated upon cleavage by Mus81-Eme1(19). The Rad32-Rad50-Nbs1 complex might be required for the processing of these DSBs at the replication fork to initiate

recombination-dependent replication. It is also possible that the Rad32-Rad50-Nbs1 complex, rather than Mus81-Eme1 endonuclease, is directly involved in the cleavage at collapsed replication forks since it has been suggested that Rad32 cleaves the hairpin structure generated on the lagging DNA strand during S phase (20).

**Nbs1 is involved in telomere length regulation.** As *S. pombe* Rad50 and Rad32 are involved in telomere length regulation (25, 66) and Rad32 has been shown to associate with telomeres (47), we examined the telomere length of *nbs1* mutants. The telomeres of the *nbs1*□mutant□□were shorter than those of the wild-type strains (Figs. 4). The telomere length of *nbs1* mutants was almost the same as those of *rad32*and *rad50* single mutants and *nbs1 rad50* double mutants. These results indicate that Nbs1 is required for telomere length maintenance. Recently we have found that deletion of *taz1*$^+$ significantly increases the single-stranded G-rich overhang at telomere ends and that this overhang disappears by concomitant deletion of *rad32*$^+$, *rad50*$^+$ (in press) or *nbs1*$^+$(data not shown). This result suggests that Nsb1 function together with Rad32 and Rad50 at telomere ends. Human Mre11 and Rad50 bind to telomere ends in a cell-cycle-independent manner, while human Nbs1 binds to telomeres only during S phase, suggesting that the MRN complex plays an important role in telomere maintenance in S phase (71). Consistent with this, it has been suggested that *S. cerevisiae* MRX complex may be required for recruitment of the telomerase components to telomeres during S phase (55, 61).

***nbs1Δ* cells are elongated and exhibits abnormal nuclear morphology.** Since the growth of the *nbs1Δ* cells were fairly poor, we attempted to observe the cell morphology by microscopy. The *nbs1Δ* cells, as well as the *rad32Δ* cells and *rad50Δ* cells, were significantly elongated (Fig. 5A). *rhp51Δ* cells and *rhp54Δ* cells

are also elongated (45). As the Rad32-Rad50-Nbs1 complex, Rhp51 and Rhp54 are required for recombination repair, elongation of the cells may be due to the accumulation of DNA damage during normal mitotic growth. Such DNA damage would activate the DNA damage checkpoint, which would retard the cell cycle and cause elongation of the cells (9). Consistent with this, we have observed a slight activation of the checkpoint kinase Cds1 in *rad50* mutants in the absence of any DNA-damaging agents (data not shown and (3)).

In addition, most of the *nbs1Δ* cells had aberrant nuclear morphology as visualized by DAPI-staining, whereas the nuclear chromosomal domain of wild-type cells is hemispherical or round (Fig. 5B). The nuclei of most *nbs1* mutant cells appeared diffuse, and a compressed and crescent-like structure was observed within them. The *rad32Δ* cells and *rad50Δ* cells had also similar aberrant nuclear morphology (Fig. 5B). These results indicate that Nbs1, probably in a protein complex with Rad32-Rad50, is required for maintaining chromosomal structure.

**Perspectives.** The abnormal nuclear morphology of the *rad32*, *rad50* and *nbs1* mutants might be related to a defect in chromatin cohesion. The depletion of a component of the cohesin complex, Rad21, from cells has been shown to result in disturbance of the nuclear organization (2) and a functional link between Rad21 and Rad50 has also been suggested (25). Such a link between the cohesin complex and the MRN complex has also been suggested in mammals. For example, human SMC1 is phosphorylated in response to IR in an NBS1-dependent manner (31, 68). More recently, a direct interaction between Mre11-Rad50 complex and cohesin at DNA damage sites has been reported (30). However, the exact roles of the cohesin complex in DNA repair and the maintenance of nuclear organization remain unclear. More

extensive investigations of the roles of *S. pombe* Nbs1 in the DNA damage response would reveal the molecular details of the DNA damage checkpoint pathway and of the possible functional link between the Nbs1 complex and cohesin in DNA repair and the maintenance of nuclear organization.

Although both the *S. cerevisiae* MRX complex and vertebrate MRN complexes have been suggested to be involved in both DNA repair and checkpoint signaling (14), there are several differences in the roles of these complexes in DNA repair and checkpoint signaling. For example, the *S. cerevisiae* MRX complex is reported to be partially required for phosphorylation of the Rad53 checkpoint kinase in response to DSBs (23). On the other hand, activation of human CHK2, a functional homologue of *S. cerevisiae* Rad53, in response to IR is intact in NBS1 cells (68). In addition, the *S. cerevisiae* MRX complex is required for NHEJ repair, while chicken Nbs1 is not required for NHEJ repair (40, 57). Similarly to vertebrate MRN complexes, *S. pombe* Rad50 is not required for NHEJ repair (36). Moreover, the sequence features of *S. pombe* Nbs1 are closer to those of homologues in higher eukaryotes than to those of *S. cerevisiae* Xrs2 (Fig. 1), suggesting that the *S. pombe* Rad32-Rad50-Nbs1 complex may have functional similarity to the MRN complexes of higher eukaryotes rather than to the MRX complex of *S. cerevisiae*.

**Conclusion.** We succeeded in identification of the Nbs1 homologue in *S. pombe*. *S. pombe nbs1*$^{+}$ is required for DNA repair, telomere length regulation, and maintenance of chromatin structure. The amino acid sequence features and the DNA repair function suggest that the *S. pombe* Rad32-Rad50-Nbs1 complex have functional similarities to the MRN complexes of higher eukaryotes. Accordingly, *S. pombe* Nbs1 will become one of the convenient model systems for studying the molecular function

of the Mre11 (Rad32)-Rad50-Nbs1 complex, which plays very important roles in DNA repair, DNA damage checkpoint, and the maintenance of telomere and chromatin integrity. In addition, it will also provide clues to understanding the molecular mechanism of cancer development observed in AT and NBS patients.

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## FIGURE LEGENDS

Fig. 1. Amino acid sequence analysis of *S. pombe* Nbs1. Alignment was performed as described previously (64). (A) Overall structural comparison among several Nbs1 and related proteins. (B) FHA (forkhead-associated) domain. (C) BRCT (BRCA1-C terminus) domain. The five conserved regions, designated *A-E* (7), of the BRCT domain based on the hydrophobic cluster analysis (HCA) are shown by a dotted arrow. The secondary structure of the XRCC1 BRCT domain (69) is shown at the bottom of the alignment. (D) CCD (C-terminal conserved domain). Abbreviations are as follows: human (h), mouse (m), chicken (ch), *Aspergillus nidulans* (an), *Drosophila melanogaster* (dm), *Danio rerio* (dr), *Xenopus laevis* (xl), *S. pombe* (sp), and *S. cerevisiae* (sc). Coloring of conserved positions (at most, two deviating amino acids for the FHA domain, four deviating amino acids for the BRCT domain, three deviating amino acids for the CCD): green, hydrophobic (FYWILMVTAC); red, acidic (DEQN); blue, basic (HKR); magenta, glycine (G); brown, serine or threonine (ST); yellow, proline (P). Sequences are denoted by species identification prefixes (*C. elegance* (ce), *A. thaliana* (at)) and by protein acronyms (hNBS1 (GenBank BAA28616); spNBS1 (GenBank AB099299); drNBS1-N (GenBank BI984731); drNBS1-N (GenBank BM775439); xlNBS1-N (GenBank CA988284); xlNBS1-C (GenBank BG022948); scXRS2 (GenBank AAB64805); mNBS1 (GenBank AB016988); chNBS1 (GenBank AAG47947); dmNBS1 is also named CG6754-PB protein (GenBank NM_143716); predicted translation products of the cosmids F37D6.1 (GenBank CAA99847) and T10M13.12 (GenBank T01512); the CAG trinucleotide repeat containing cDNA CAGF28 (GenBank AAB91434); the BRCA-associated RING finger domain protein BBARD (GenBank Q99728); the DNA repair proteins XRCC1 (GenBank A36353) and

REV1 (GenBank S67255); the oncoprotein ECT2 (GenBank S32372); the breast cancer susceptibility type 1 protein BRCA1 (GenBank U14680); the radiation-sensitive checkpoint protein RAD9 (GenBank M26049); campothecin-resistance-conferring protein SCAA (GenBank AAF81094), which has moderate structural similarity to hNBS1). Numbers in the alignment denote amino acids omitted from it.

Fig. 2. Yeast two-hybrid interaction between Nbs1 and Rad32. (A) A simple scheme of the protein structure and constructs of Nbs1 designed for yeast two-hybrid analysis. Nbs1-FL, full-length Nbs1 (1-613 aa); Nbs1-N, N-terminal half of Nbs1 (1-252 aa); Nbs1-C, C-terminal half of Nbs1 (252-613 aa). FHA domain (1-103 aa), BRCT domain (106-199 aa), and C-terminal conserved domain (CCD) (470-531 aa) are indicated. (B) Two-hybrid interaction of Nbs1 and Rad32. Pairwise interaction of pGBKT7 and pGADT7 was judged by a spot test on 3 types of dropout (DO) plates: 4DO (high stringency), 3DO (medium stringency) and 2DO (control) (see *MATERIALS AND METHODS*).

Fig. 3. DNA damage sensitivity of *nbs1Δ* cells and epistasis analysis. (A) The sensitivities to γ-ray of *nbs1Δ* cells (SPN100), *rad50Δ* cells (KT120), and *rad50 nbs1* double mutants (KW015). JY741 was used as a wild-type strain. (B) The sensitivities to UV light of the *nbs1Δ* cells, the *rad50Δ* cells, *rad32Δ* cells (142) and the *rad50 nbs1* double mutants. (C, D). The sensitivities of the *nbs1Δ* cells, the *rad50Δ* cells, the *rad32Δ* cells and the *rad50 nbs1* double mutants to MMS (C), and HU (D) determined in a spot test. (E, F). Epistasis between *nbs1Δ* cells and *rhp51Δ* cells for γ-ray (E) and UV (F) sensitivity. Wild-type cells (SPN124), *nbs1Δ* (SPN100), *rhp51Δ*

(B54), *nbs1 rhp51* (SPN103). For genotypes, see Table 1. Standard deviations are shown by error bars.

Fig. 4. Nbs1 is involved in telomere length maintenance. The telomere length of wild-type cells, *nbs1Δ* , *rad50Δ*, *rad32Δ*, and *rad50 nbs1* double mutant cells was evaluated by Southern hybridization. Lane 1, wild-type cells (JY741); Lane 2, *nbs1Δ* (SPN100); Lane 3, *rad50Δ* (KT120); Lane 4, *rad32Δ* (142); Lane 5, *rad50 nbs1* double mutants (KW015); Lane 6, *rad3Δ* (rad3D); Lane 7, wild-type cells (JY741). Genomic DNA was digested with *Apa* I, and separated by electrophoresis on a 2 % agarose gel. Telomeres are indicated by an arrow. For genotypes, see Table 1.

Fig. 5. The *nbs1Δ*, *rad50Δ*, and *rad32Δ* cells are all elongated and exhibit abnormal nuclear morphology. (A) Cell length of wild-type cells (SPN114), *nbs1Δ* (SPN138), *rad50Δ* (SPN148), *rad32Δ* cells (SPN141) was determined by microscopy. (B) Nuclear morphology of the wild-type cells, the *nbs1Δ*, the *rad50Δ* , and the *rad32Δ* cells. The cells were fixed with 2.5% glutalardehyde and stained with 1 μg /ml DAPI. Fluorescence images obtained with an epifluorescence microscope are shown. Arrows indicate nucleus with abnormal morphology. For genotypes, see Table 1.

TABLE 1. *S. pombe* strains used in this study

| Strain | Genotype | Source |
|---|---|---|
| 142 | *h$^{+}$ leu1-32 ura4-D18 ade6-M210 rad32::ura4$^{+}$* | A. Matsuura |
| 968 | *h$^{90}$* | NCYC |
| 972 | *h$^{-}$* | NCYC |
| 975 | *h$^{+}$* | NCYC |
| B54 | *h$^{-}$ smt-0 leu1-32 ura4-D18 his3-D1 arg3-D1 rhp51::his3$^{+}$* | This study |
| JY741 | *h$^{-}$ leu1-32 ura4-D18 ade6-M216* | M. Yamamoto |
| KT120 | *h$^{+}$ leu1-32 ura4-D18 ade6-M210 rad50::LEU2* | Our lab stock |
| KW015 | *h$^{-}$ smt-0 leu1-32 ura4-D18 his3-D1 ade6-M210 nbs1::ura4$^{+}$ rad50::LEU2* | This study |
| SP154 | *h$^{+}$ ura4-D18* | NCYC |
| SP184 | *h$^{-}$ smt-0 ade6-704 leu1-32 ura4-D18 rad2::ura4$^{+}$ / pAUR2* | This study |
| SP185 | *h$^{+}$ ade6-704 ura4-D18 rad2::ura4$^{+}$ / pAUR2* | This study |
| SPN100 | *h$^{-}$ smt-0 leu1-32 ura4-D18 his3-D1 arg3-D1 nbs1::ura4$^{+}$* | This study |
| SPN103 | *h$^{-}$ smt-0 leu1-32 ura4-D18 his3-D1 arg3-D1 nbs1::ura4$^{+}$ rhp51::his3$^{+}$* | This study |
| SPN114 | *h$^{+}$ mat1PD::LEU2 leu1-32 ura4-D18 his3-D1 arg3-D1* | Our lab stock |
| SPN124 | *h$^{-}$ smt-0 leu1-32 ura4-D18 his3-D1 arg3-D1* | Our lab stock |
| SPN138 | *h$^{+}$ mat1PD::LEU2 leu1-32 ura4-D18 his3-D1 arg3-D1 nbs1::ura4$^{+}$* | This study |
| SPN141 | *h$^{+}$ mat1PD::LEU2 leu1-32 ura4-D18 his3-D1 arg3-D1 rad32::ura4$^{+}$* | This study |

| | | |
|---|---|---|
| SPN148 | *h*$^{+}$ *mat1PD::LEU2 leu1-32 ura4-D18 his3-D1 arg3-D1 rad50::ura4*$^{+}$ | This study |
| YA137 | *h*$^{+}$ *mat1PD::LEU2 ura4-D18 leu1-32 rad2::ura4*$^{+}$ | This study |
| Rad3D | *h*$^{-}$ *ura4-D18 leu1-32 ade6-704 rad3::ura4*$^{+}$ | A. Carr |

NCYC: National Collection of Yeast Culture

TABLE 2. Summary of *slr* mutant isolation

| mutant number | mutant gene | *S. cerevisiae* homologue |
|---|---|---|
| 6 | *rad32* | *MRE11* |
| 7 | *rad32* | *MRE11* |
| 11 | *rhp57* | *RAD57* |
| 14 | ND | |
| 16 | ND | |
| 17 | ND | |
| 23 | *rad32* | *MRE11* |
| 27 | *slr8* (This study) | *XRS2* |
| 28 | ND | |
| 29 | ND | |
| 34 | ND | |
| 45 | ND | |

ND: not determined

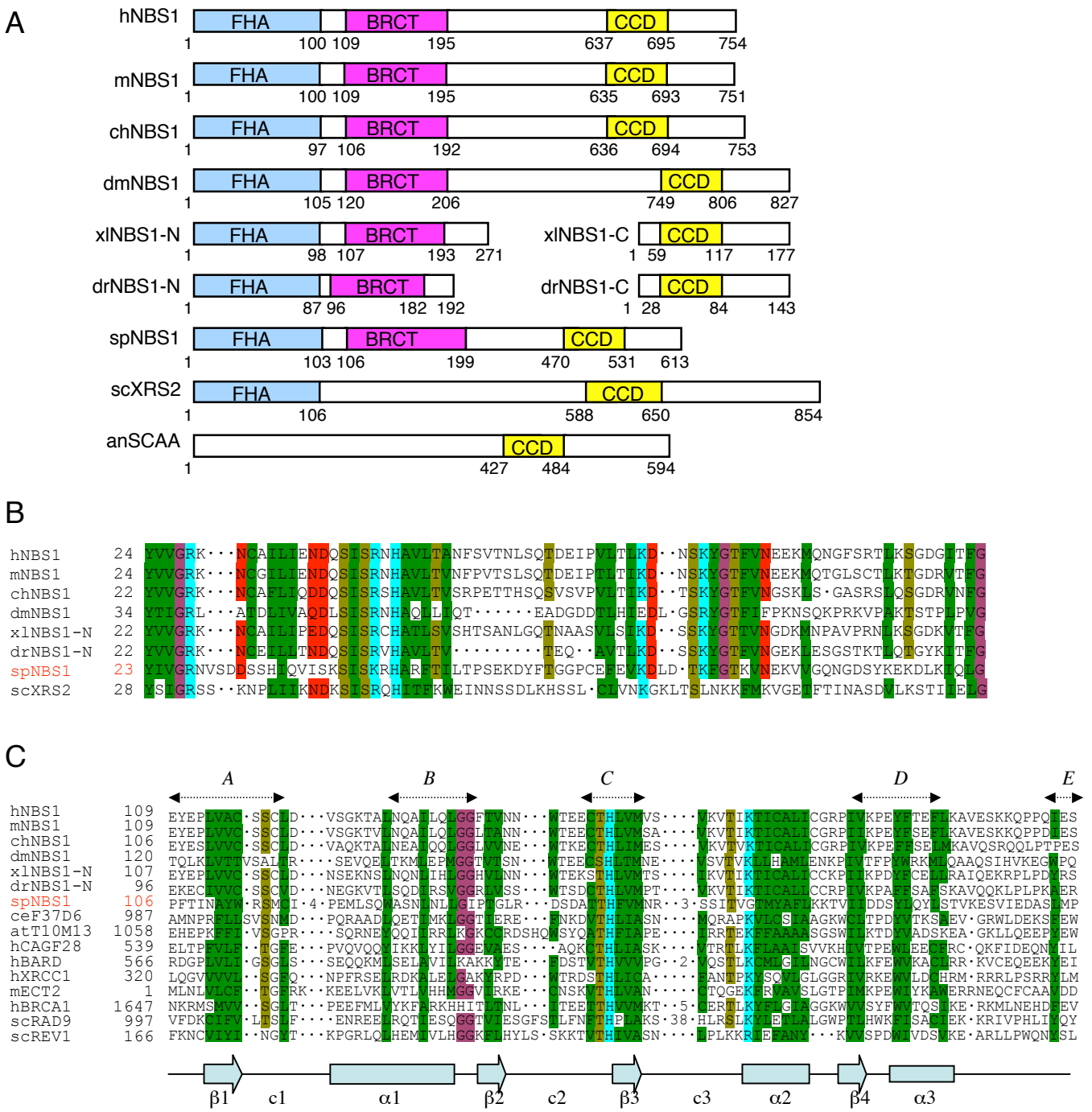



Fig. 1 Ueno et al.

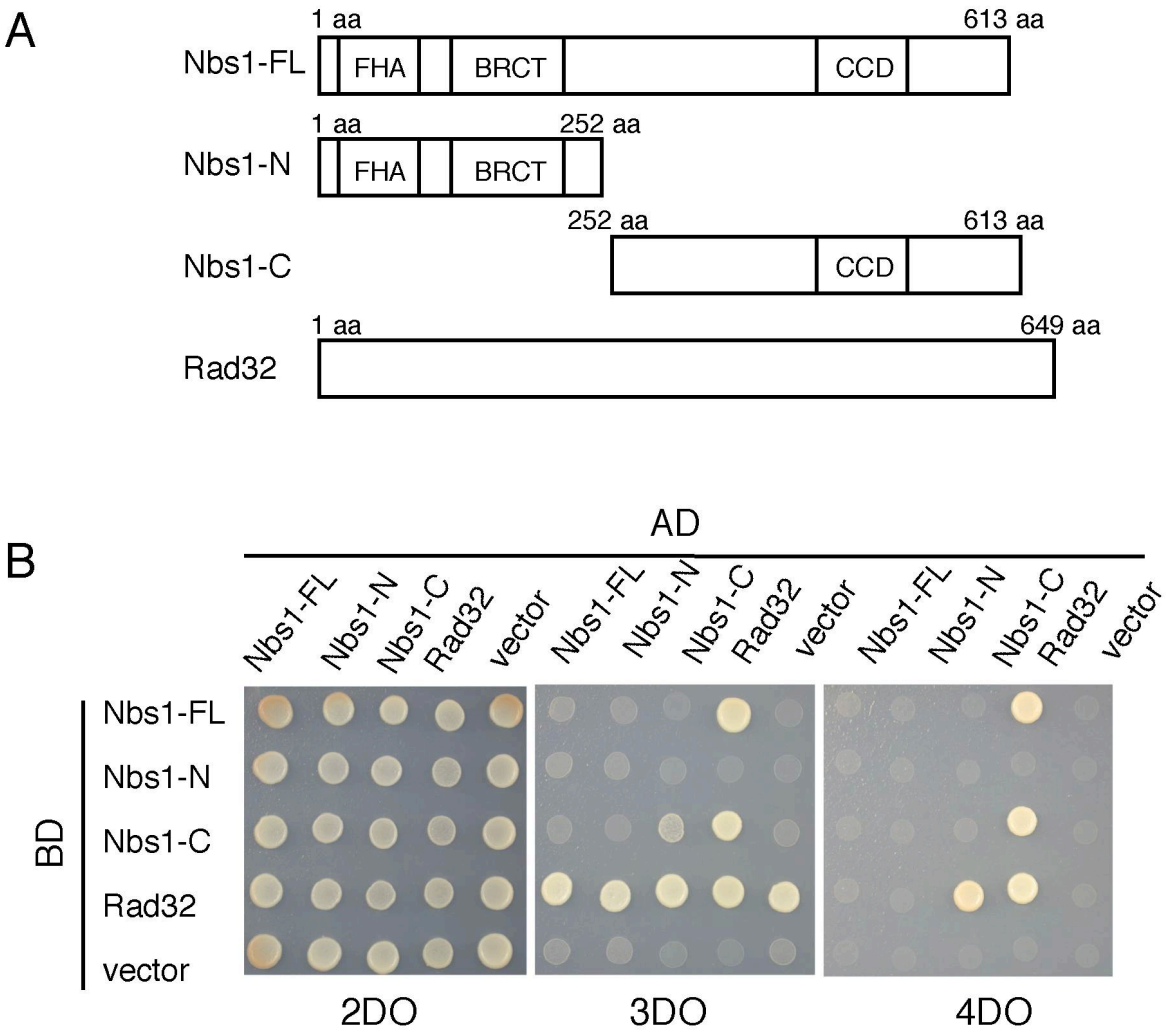



Fig. 2 Ueno et al

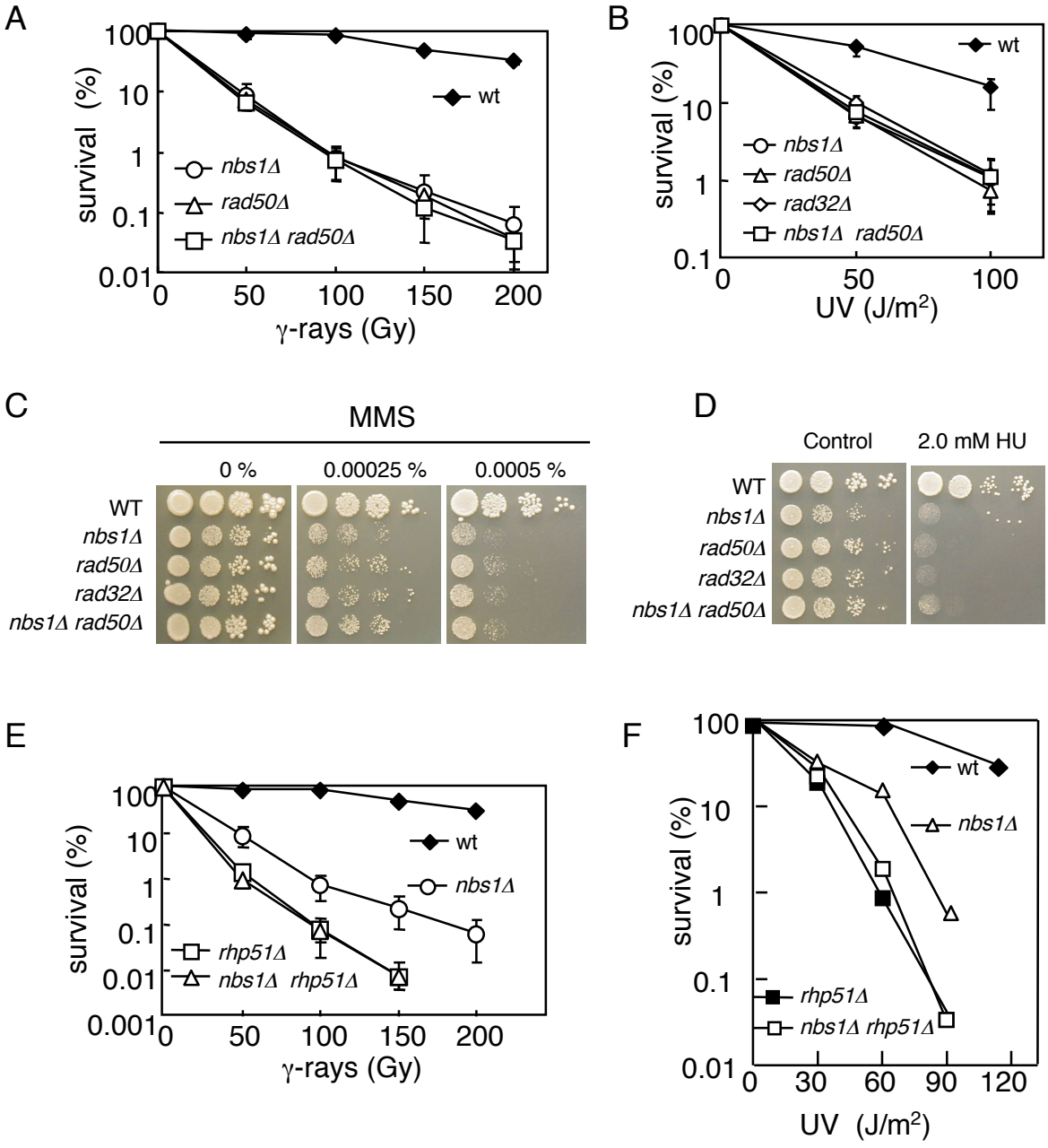



Fig. 3 Ueno et al.

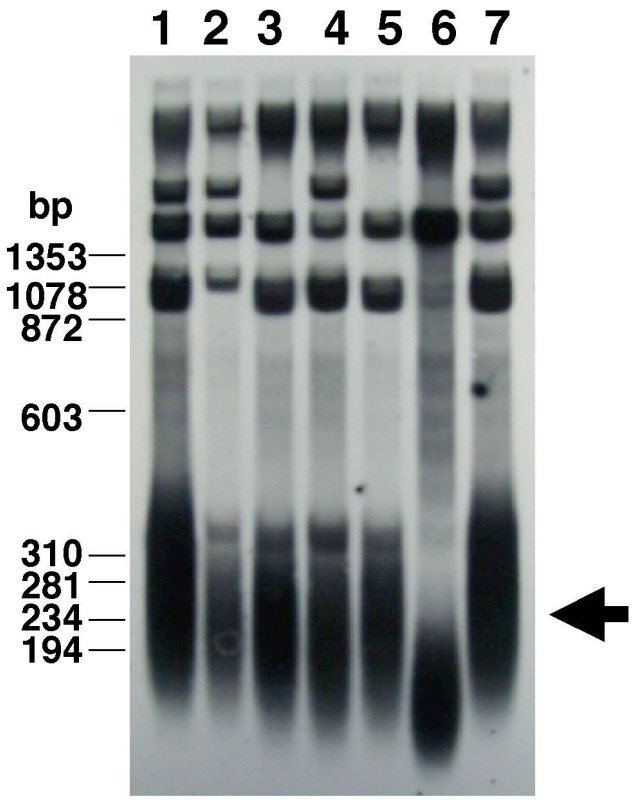
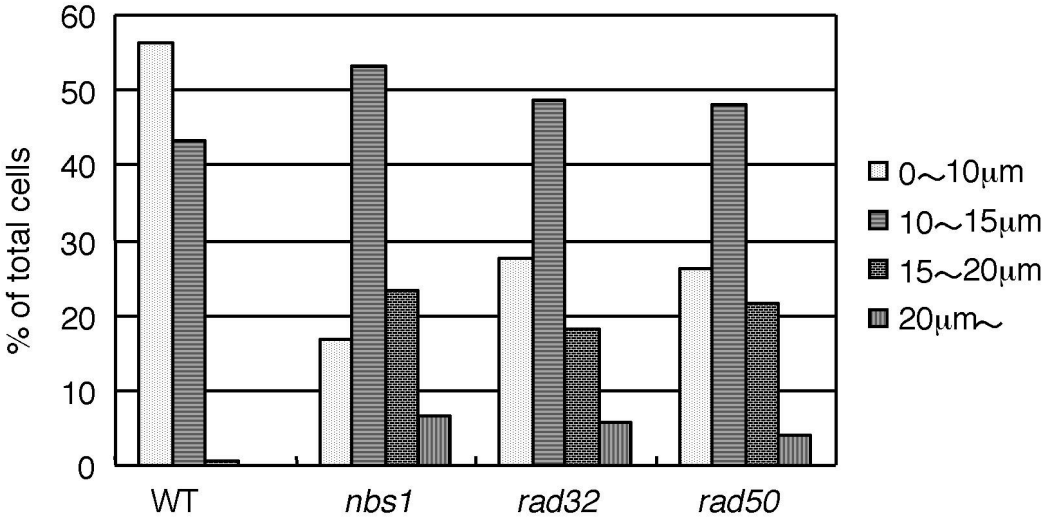



Fig. 4 Ueno et al

A




B

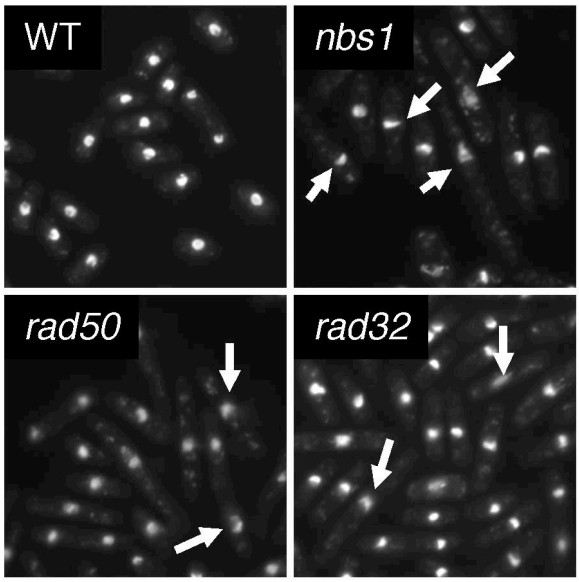



Fig. 5 Ueno et al.