An inducible null mutant murine model of Nijmegen breakage syndrome proves the essential function of NBS1 in chromosomal stability and cell viability

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The human genetic disorder, Nijmegen breakage syndrome, is characterized by radiosensitivity, immunodeficiency, chromosomal instability and an increased risk for cancer of the lymphatic system. The NBS1 gene codes for a protein, nibrin, involved in the processing/repair of DNA double strand breaks and in cell cycle checkpoints. Most patients are homozygous for a founder mutation, a 5 bp deletion, which might not be a null mutation, as functionally relevant truncated nibrin proteins are observed, at least in vitro. In agreement with this hypothesis, null mutation of the homologous gene, Nbn, is lethal in mice. Here, we have used Cre recombinase/loxP technology to generate an inducible Nbn null mutation allowing the examination of DNA-repair and cell cycle-checkpoints in the complete absence of nibrin. Induction of Nbn null mutation leads to the loss of the G2/M checkpoint, increased chromosome damage, radiomimetic-sensitivity and cell death. In vivo, this particularly affects the lymphatic tissues, bone marrow, thymus and spleen, whereas liver, kidney and muscle are hardly affected. In vitro, null mutant murine fibroblasts can be rescued from cell death by transfer of human nibrin cDNA and, more significantly, by a cDNA carrying the 5 bp deletion. This demonstrates, for the first time, that the common human mutation is hypomorphic and that the expression of a truncated protein is sufficient to restore nibrin's vital cellular functions.

INTRODUCTION

Nijmegen breakage syndrome (NBS, MIM 251260) is a rare autosomal recessive genetic disease belonging to a group of disorders termed chromosome instability syndromes. Patients affected by NBS have a range of symptoms including microcephaly, growth retardation, radiosensitivity, immunodeficiency and an increased cancer risk, particularly for B-cell lymphoma (1,2). The gene mutated in NBS was localized to chromosome 8q21 by linkage analysis in NBS families (3) and then identified by positional cloning (4). Over 90% of all NBS patients are homozygous for a 5 bp deletion (657Δ5) in exon 6 of the NBS1 gene.

Concurrent to the identification of the NBS1 gene through positional cloning, the functional human orthologue of the yeast gene, Xrs2, was identified as the same NBS1 gene (5). In yeast cells, the product of the Xrs2 gene is part of a complex together with Rad50 and Mre11. Experimental data from the yeast Saccharomyces cerevisiae implicate the Rad50 complex in double-strand break (DSB) repair by both non-homologous end joining (NHEJ) (6) and homologous recombination (HR) (7), the two major repair pathways for DNA DSBs. In mammalian cells, MRE11, RAD50 and the NBS1 gene product, nibrin or p95, interact to form a complex (M/R/N). Although the clinical radiosensitivity of NBS patients is reflected at the cellular level and obviously implies a defect in

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DNA repair, a deficiency in repairing DSBs has not been clearly demonstrated in appropriate assays (8–11).

In addition to its potential roles in DSB repair, nibrin has been implicated in two steps of the DNA damage signal transduction pathway. First, it has been shown that nibrin is phosphorylated by ATM (12,13), the gene mutated in Ataxia telangiectasia (AT) patients. Phosphorylated nibrin facilitates the ATM-mediated phosphorylation of further target molecules, such as CHK2, CHK1 and SMC1, involved in key steps of the cell cycle, including entry into mitosis (14,15). In addition, however, the M/R/N complex, including nibrin, is required for the activation of ATM itself in response to DNA damage (16). Thus, nibrin has roles both upstream and downstream of ATM in the DNA damage response.

The relevance of nibrin’s various functions for the disease, NBS, has been difficult to analyse using patient cells as the 657Δ5 mutation and, most likely, nine other truncating mutations found in NBS1, are hypomorphic rather than null mutations. An N-terminal truncated protein of ~26 kDa is observed in cells from homozygous 657Δ5 patients. Furthermore, a 70 kDa C-terminal truncated protein is also observed in Epstein–Barr virus (EBV)-transformed NBS lymphoblastoid cells and immortalized NBS fibroblasts. It has not, however, been observed in primary patient cells. The p70 fragment is produced by alternative initiation of translation at a start codon upstream of the 5 bp deletion through which it is brought into frame (17). Similarly, truncated proteins have been observed for the 835Δ4 and 900Δ25 NBS1 mutations, although here produced presumably by initiation of translation at downstream in-frame start codons (17,18). If these truncated proteins are genuinely active in vivo, they could have a major impact on cellular and clinical phenotype. This has, however, not been demonstrated so far.

Clearly, the elucidation of the function of nibrin would be facilitated by the availability of null mutant cells. Chicken

Figure 1. Targeting the murine Nbn locus. The relevant region of the Nbn locus is shown with filled boxes representing the Nbn exons. In the drawing of the targeting vector, pTV-FLOX-Nbn, the solid line represents mouse genomic sequence with Nbn exons drawn as filled boxes. Open boxes represent vector sequences with the selection markers in grey. loxP sites are denoted by triangles. The targeted locus in ES cells shows the integration of the G418-resistance cassette and loxP sites into the murine gene. The NheI recognition sites used for Southern blot analysis and the hybridization probe (SB) located in exon 5 are indicated. After Cre recombinase mediated deletion, three alleles are produced, of which two are shown here: Nbn<sup>D6</sup>, in which exon 6 is deleted; and Nbn<sup>lox-6</sup>, in which exon 6 is flanked by loxP-sites. The genomic structure of one further Nbn allele used in these studies is also shown, Nbn<sup>ins-6</sup>, in which a neomycin-resistance cassette disrupts exon 6 (20).
DT40 cells with complete absence of nibrin have been described, and their cellular phenotype recapitulates much of the NBS phenotype (19). However, in mice, null mutation of NBS1 leads to embryonic lethality (20,21) and only animals with hypomorphic mutations survive past early embryogenesis (22,23). In order to circumvent this lethality, we have generated, for the first time, mice with an inducible null mutation in the murine Nbn gene. In this system, Cre recombinase is used to delete exon 6 of the gene by recombination of flanking loxP sites. Homozygous disruption of Nbn can therefore be induced in cells in vitro by application of Cre recombinase, or in vivo by using transgenic Cre recombinase expressed from an inducible promoter (24).

Using these null mutant cells we have been able to show, first, that absence of nibrin leads to massive spontaneous chromosome damage, reflecting its role in DNA DSB-repair. Second, when challenged with a DSB inducing treatment, null mutant cells fail to arrest in G2 but proceed into mitosis. Thus, nibrin is required for the transmission of a signal from damaged DNA to at least this cell cycle checkpoint. Third, null mutant cells have a dramatically reduced survival both in vivo and in vitro, explaining the embryonic lethality of knock-out mice. Finally, the hypomorphic nature of the major human NBS1 mutation has been demonstrated by the ability of NBS1 cDNA carrying this mutation to promote survival of null mutant mouse cells in vitro.

RESULTS

Targeted inducible disruption of the Nbn gene in mice

A targeting vector, pTV-FLOX-Nbn, was constructed which, after HR in mouse ES cells, introduces a G418-resistance cassette into the intron 5 of the Nbn locus and replaces exon 6 with a loxP-flanked copy (Fig. 1). ES clones with correctly targeted Nbn alleles were identified by PCR and Southern blot. Correctly targeted ES cells were electroporated with a Cre recombinase expression vector, and the clones that had undergone recombination at loxP sites were identified. Clones in which Cre-mediated recombination had deleted the neomycin-resistance cassette and one loxP site but left Nbn exon 6 flanked by loxP sites are denoted Nbn<sup>wt/lox-6</sup> and Nbn<sup>wt/D6</sup> (Fig. 1). In homozygous Nbn<sup>wt/lox-6</sup> and heterozygous Nbn<sup>ins-6/lox-6</sup> fibroblasts, nibrin is normally expressed. After treatment of Nbn<sup>ins-6/lox-6</sup> cells in vitro with the Cre recombinase fusion protein, HTNC, the expression of nibrin is reduced by 70%, reflecting the proportion of cells deleted to Nbn<sup>ins-6/D6</sup>.
The PCR products from the three detected alleles are indicated, the columns) and Nbn allele is not detected in this PCR. The area of each product signal as measured by the ImageQuant software is given under the fluorogram and reflects the Cre expression of Cre recombinase. (22) in contrast, the 78 kDa fragment translated from the hypomorphic Nbn allele constructed by Kang et al. (22) is readily detected in embryonic fibroblasts by western blot. As a control for effectiveness, the nibrin-specific antibodies employed here were able to detect the 70 kDa C-terminal nibrin fragment in lyses of human NBS fibroblasts (Fig. 2D). Thus, the deletion of Nbn exon 6 does indeed represent a true null mutation. After treatment of Nbnins-6/lox-6 fibroblasts in vitro with HTNC, a Cre recombinase fusion protein, the majority of cells are deleted to the Nbnins-6Δ6 genotype leading to a 70% reduction in the expression of nibrin (Fig. 2D).

Breeding amongst Nbnwt/lox-6 animals yielded Nbnlox-6/lox-6 mice indicating that the exon 6 flanked by loxP-sites remains fully functional. For experiments on induction of homozygosity in mouse fibroblasts in vitro, crosses were set up to produce Nbnwt/lox-6 and Nbnins-6/lox-6 mice. To examine the consequences of null mutation in vivo, mice were crossed with transgenic mice expressing Cre recombinase under the control of the interferon responsive promoter, Mx1 (24). For comparative purposes, both Nbnwt/lox-6 Tg-Mx1-Cre and Nbnins-6/lox-6 Tg-Mx1-Cre mice were produced. Genotyping PCRs for these mice are shown in Figure 2B.

Cre-mediated deletion through recombination at loxP sites is generally incomplete both in vitro and in vivo (24–26); therefore, a semi-quantitative assay for estimating the extent of exon 6 deletion was developed. The assay is based on the simultaneous amplification of differently sized, fluorescently labelled PCR products from the three alleles, Nbnwt , Nbnlox-6 and NbnΔ6. Under standardized conditions, the initial proportions of artificially mixed genomic DNAs were authentically reflected in the relative amounts of PCR products as measured by FluorImager analysis of polyacrylamide gels (Supplementary Material, Fig. S1).

Impaired survival of Nbn null mutant cells in vivo

Injection of Nbnwt/lox-6 Tg-Mx1-Cre mice and Nbnins-6/lox-6 Tg-Mx1-Cre mice with poly(I):poly(C) to mimic a viral

Figure 3. Recovery of the Nbn exon 6-deleted allele from mouse organs after in vivo expression of Cre recombinase. (A) Fluorogram of a representative semi-quantitative PCR analysis for deletion in the kidney of two Nbnwt/lox-6 mice and four Nbnins-6/lox-6 mice after Cre recombinase expression in vivo. The PCR products from the three detected alleles are indicated, the Nbnins-6 allele is not detected in this PCR. The area of each product signal as measured by the ImageQuant software is given under the fluorogram and reflects the Cre recombinase-mediated exon 6 deletion efficiency. (B) Recovery of the Nbn exon 6-deleted allele after Cre recombinase expression in vivo as measured by semi-quantitative PCR for eight organs in Nbnwt/lox-6 mice (white columns) and Nbnins-6/lox-6 mice (grey columns). The number of animals in each measurement is given above the columns.

Crosses amongst NbnwtΔ6 heterozygotes did not yield NbnwtΔ6 mice (18 Nbnwtwt:25 NbnwtΔ6:0 NbnΔ6Δ6 P = 0.0016 in the χ2-test) indicating that the deletion of exon 6 is embryonically lethal. Deletion of exon 6 is predicted to result in a frameshift at position 584 (serine-195) followed by 16 incorrect amino acids before termination of translation at a cryptic termination codon. The mRNA arising from the NbnΔ6 allele was examined by RT–PCR (Fig. 2C) and sequenced to confirm the theoretically expected frameshift (data not shown). Therefore, cells expressing only the NbnΔ6 allele have no authentic nibrin beyond amino acid 195.

As C-terminal nibrin fragments have been observed in NBS patient cells (17,18) and in the cells of viable Nbn knock-out mice (22,23), we looked for such products from the NbnΔ6 allele. Examination of the upstream sequence of the exon 6-deleted allele indicates the possibility of an in-frame start codon at position 438, followed, however, by a stop codon at position 465. A second potential start codon at position 486 is similarly followed by a stop codon at position 535. Thus, the production of a C-terminal nibrin fragment by the mechanism of alternative upstream initiation, as from the human NBS1 657Δ5-mutation (17), can be excluded. However, downstream start codons are potentially available at positions 754, 880, 949 and 1078; these would allow translation of C-terminal fragments of 56, 52, 49 and 45 kDa, respectively.

Western blot analysis using lysates from mouse fibroblasts (Fig. 2D), however, did not confirm that such proteins are actually produced from the Nbnlox-6 or NbnΔ6 alleles, even though they are normally transcribed (Fig. 2C). In contrast, the 78 kDa fragment translated from the hypomorphic Nbn allele constructed by Kang et al. (22) is readily detected in embryonic fibroblasts by western blot. As a control for effectiveness, the nibrin-specific antibodies employed here were able to detect the 70 kDa C-terminal nibrin fragment in lyses of human NBS fibroblasts (Fig. 2D). Thus, the deletion of Nbn exon 6 does indeed represent a true null mutation. After treatment of Nbnins-6/lox-6 fibroblasts in vitro with HTNC, a Cre recombinase fusion protein, the majority of cells are deleted to the Nbnins-6Δ6 genotype leading to a 70% reduction in the expression of nibrin (Fig. 2D).

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Impaired survival of Nbn null mutant cells in vivo

Injection of Nbnwt/lox-6 Tg-Mx1-Cre mice and Nbnins-6/lox-6 Tg-Mx1-Cre mice with poly(I):poly(C) to mimic a viral
infection, leads to interferon production and thus Cre recombinase expression in interferon-sensitive cells. DNA was extracted from the organs 15 days after induction of Cre recombinase, and the extent of exon 6 deletion was estimated using the semi-quantitative PCR assay described. Comparing deletion efficiencies in Nbn\textsuperscript{ins-6/lox-6} mice with Nbn\textsuperscript{wt/lox-6} mice indicates the surviving fraction of Nbn\textsuperscript{ins-6/Δ6} cells independently of tissue response to interferon. As shown in Figure 3, 93% of the cells recovered from the liver are deleted to heterozygosity in Nbn\textsuperscript{wt/lox-6} mice and 94% to homozygosity in Nbn\textsuperscript{ins-6/lox-6} mice. In the kidney, deletion efficiency is also comparable in Nbn\textsuperscript{wt/lox-6} mice (43%) and Nbn\textsuperscript{ins-6/lox-6} mice (40%).

In contrast, in bone marrow, only 50% of recovered cells are deleted in Nbn\textsuperscript{ins-6/lox-6} mice when compared with 100% in Nbn\textsuperscript{wt/lox-6} mice (P < 0.009 in Student’s t-test). This is a clear indication that in proliferative tissues, such as bone marrow, the homozygous mutant cells have a strong growth disadvantage and are quite rapidly lost. Even more striking is the effect in the thymus. The interferon mediated deletion efficiency is extremely high in this organ, practically 100% of Nbn\textsuperscript{wt/lox-6} cells are deleted to Nbn\textsuperscript{wt/Δ6} whereas <20% of cells in the thymus of Nbn\textsuperscript{ins-6/lox-6} mice are Nbn\textsuperscript{ins-6/Δ6} (P < 0.0005 in Student’s t-test). In spleen, the pattern is similar, with 76% of cells deleted in Nbn\textsuperscript{wt/lox-6} mice, but only 50% in Nbn\textsuperscript{ins-6/lox-6} mice (P < 0.004 in Student’s t-test).

**Increased spontaneous and DSB-induced chromosome breakage in Nbn null mutant lymphocytes**

Lymphocytes isolated from the spleens of Nbn\textsuperscript{ins-6/lox-6} mice after in vivo deletion by poly(I):poly(C) injection show increased chromosomal instability in comparison with similarly treated Nbn\textsuperscript{wt/lox-6} mice. Figure 4A shows two representative metaphase...
specific loss of breakage reflects the essential role of nibrin in maintaining each genotype. This increased spontaneous chromosome mitotic cells and damage per cell are shown for two mice of some preparation. The number of metaphases examined is given below the columns. (A) Lymphocytes isolated from the spleens of two Nbn<sup>ins-6/lox-6</sup> mice (labelled 1 and 2) were treated with HTNC (grey columns), or mock-treated (white columns), before LPS and concanavalin A stimulation of T- and B-lymphocytes. Coded slides were scored. The number of metaphases examined by applying the HTNC Cre recombinase fusion protein to the cells, before stimulation and cultivation. As shown in Figure 5A, null mutant splenic lymphocytes have a high spontaneous chromosome breakage rate; on average, 25% of the mitotic cells are aberrant, 8-fold higher than the rate in cells from the same spleens not induced to homozygosity by HTNC. Examination of chromosomal breakage in spleen lymphocytes from wild-type mice confirmed that the damage is not merely due to HTNC-treatment (data not shown).

Treatment of null mutant lymphocytes <i>in vitro</i> with the radiomimetic bleomycin, further increased the rate of chromosome breakage. Interestingly, the proportion of aberrant Nbn<sup>ins-6/Δ6</sup> mitotic cells, in contrast to Nbn<sup>wt/Δ6</sup> cells, increases only slightly with the bleomycin doses employed here (Fig. 5B). However, the damage per mitotic cell is clearly increased in the homozygous cells in a dose-dependent manner. These findings are compatible with a G2/M checkpoint defect that renders null mutant cells insensitive to the presence of damaged DNA. This was then examined more thoroughly.

**Figure 5.** Chromosomal breakage in mouse lymphocytes deleted to homozygosity <i>in vitro</i>. (A) Lymphocytes isolated from the spleens of two Nbn<sup>ins-6/lox-6</sup> mice (labelled 1 and 2) were treated with HTNC (grey columns), or mock-treated (white columns), before LPS and concanavalin A stimulation of T- and B-lymphocytes. Coded slides were scored. The number of metaphases examined is given below the columns. (B) Chromosome breakage induced by the radiomimetic bleomycin. HTNC (grey columns), or mock-treated (white columns), lymphocytes from an Nbn<sup>ins-6/lox-6</sup> mouse [labelled 2 in (A)] were treated with bleomycin at the indicated concentrations for 3 h before chromosome preparation. The number of metaphases examined is given below the columns.

spreads from Nbn<sup>ins-6/lox-6</sup> lymphocytes after deletion to Nbn<sup>ins-6/Δ6</sup> <i>in vivo</i>. In Figure 4B the quantification of aberrant mitotic cells and damage per cell are shown for two mice of each genotype. This increased spontaneous chromosome breakage reflects the essential role of nibrin in maintaining genomic integrity in dividing cells and suggests that the specific loss of Nbn<sup>ins-6/Δ6</sup> cells can be attributed to their genomic instability.

The sensitivity of null mutant lymphocytes <i>in vitro</i> was examined by applying the HTNC Cre recombinase fusion protein to the cells, before stimulation and cultivation. As shown in Figure 5A, null mutant splenic lymphocytes have a high spontaneous chromosome breakage rate; on average, 25% of the mitotic cells are aberrant, 8-fold higher than the rate in cells from the same spleens not induced to homozygosity by HTNC. Examination of chromosomal breakage in spleen lymphocytes from wild-type mice confirmed that the damage is not merely due to HTNC-treatment (data not shown).

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**The G2/M checkpoint is deficient in Nbn null mutant fibroblasts**

The mitotic index in Nbn null mutant fibroblasts after a DNA damaging treatment was determined by FACS analysis. Nbn<sup>ins-6/lox-6</sup> and Nbn<sup>wt/lox-6</sup> cells were treated with HTNC to induce deletion to Nbn<sup>ins-6/Δ6</sup> and Nbn<sup>wt/Δ6</sup> respectively. After 48 h, aliquots of the cells were exposed for 2 h to increasing concentrations of bleomycin, fixed in ethanol and then stained with a mitosis-specific antibody directed against phosphorylated Histone H3, a Cy2-conjugated secondary antibody and counter-stained with propidium iodide for DNA content. Treatment of the heterozygous, Nbn<sup>wt/Δ6</sup> cells, with bleomycin for 2 h leads to a dramatic inhibition of mitosis in comparison with mock-treated cells (Fig. 6). This inhibition is much less pronounced in Nbn<sup>ins-6/Δ6</sup> cells. At a bleomycin dose leading to 90% inhibition of mitosis in heterozygous cells, mitosis in the homozygous cells is still at 60% of untreated cells. This finding indicates that nibrin is indeed required for the G2/M checkpoint responding to damaged DNA.

**Impaired survival of Nbn null mutant fibroblasts <i>in vitro</i>**

In an attempt to isolate individual Nbn<sup>ins-6/Δ6</sup> clones, spontaneously transformed dermal fibroblasts from Nbn<sup>ins-6/lox-6</sup> mice were plated at low density 20 days after treatment <i>in vitro</i> with HTNC. Of 80 clones analysed, all were Nbn<sup>ins-6/lox-6</sup> and none were Nbn<sup>ins-6/Δ6</sup>, even though at the time of cloning, 20% of the cells were Nbn<sup>ins-6/Δ6</sup> according to the semi-quantitative PCR assay (data not shown). This suggested that Nbn<sup>ins-6/Δ6</sup> fibroblasts are specifically lost in culture, just as <i>in vivo</i>. Examination of the proportion of cells surviving with time after an HTNC treatment using the semi-quantitative PCR analysis confirmed this finding. As a control, Nbn<sup>wt/lox-6</sup> cells, which are heterozygous after Cre-mediated deletion, were also examined. Shortly after HTNC treatment, 60–70% of the treated cells were deleted from Nbn<sup>wt/lox-6</sup> to Nbn<sup>wt/Δ6</sup> or from Nbn<sup>ins-6/lox-6</sup> to Nbn<sup>ins-6/Δ6</sup> (Fig. 7). However, during cultivation there was specific loss of the Nbn<sup>Δ6</sup> allele in DNA extracted from the HTNC-treated Nbn<sup>ins-6/lox-6</sup> cells, reflecting loss of Nbn<sup>ins-6/Δ6</sup> cells from this population. In contrast, cells with this allele were stable in the HTNC-treated Nbn<sup>wt/lox-6</sup> culture. After 40 days (approximately 20 passages), 73% of the original Nbn<sup>wt/lox-6</sup> cells were Nbn<sup>wt/Δ6</sup>. Of the HTNC-treated Nbn<sup>ins-6/lox-6</sup> cells, only 6%
were Nbn\textsuperscript{ins-6/lox-6} after 40 days in culture (Fig. 7). Thus, over 90% of the homozygous null mutant Nbn cells were lost in competition with the undeleted Nbn\textsuperscript{ins-6/lox-6} cells during proliferation in vitro.

Rescue of viability in Nbn null mutant fibroblasts by human NBS1 cDNA
In order to verify and extend the earlier mentioned findings, Nbn\textsuperscript{ins-6/lox-6} fibroblasts were transduced with the wild-type human NBS1 cDNA before treatment with HTNC in an attempt to rescue them from cell death. The retroviral vector, pLXIN, was used to transfer the full-length human wild-type nibrin cDNA (27) or a cDNA, which carries the common human NBS1 mutation, 657D6. PCR with appropriate primers and immunoblots for human nibrin confirmed that the cells were successfully transduced (Supplementary Material, Fig. S2). The cells were then treated with HTNC and regularly genotyped during cultivation for up to 40 days. The initial deletion efficiency measured after 24 h was, again, 70–80%. After 40 days, in the culture transduced with LXIN, 10% of the original Nbn\textsuperscript{ins-6/lox-6} cells survived. In the culture transduced with LXIN-NBS1, >90% of the cells were still Nbn\textsuperscript{ins-6/lox-6}, indicating that expression of wild-type human nibrin was sufficient to rescue these fibroblasts from the lethal effects of their endogenous Nbn mutations (Fig. 8). Furthermore, cells transduced with a cDNA containing the 657D6 mutation were also rescued: at 10 days after Cre treatment, 100%, and at 20 days, 60% of the originally deleted cells were still Nbn\textsuperscript{ins-6/lox-6} (P < 0.004 and P < 0.003, respectively, in comparison with LXIN-transduced cells). Even after 40 days of cultivation, >40% of the cells transduced with 657D6 cDNA and deleted to Nbn\textsuperscript{ins-6/lox-6} were still alive and could clearly compete with the remaining heterozygous cells.

**DISCUSSION**

Nibrin is essential for cell viability and the major NBS1 mutation, 657D6, is hypomorphic
The lethality of Nbn mutations in the mouse was, at first, surprising, considering that patients with NBS1 mutations live, albeit with life-threatening deficiencies in their lymphoreticular system. The mouse embryos generated in those experiments died between days 3.5 and 7.5 pc owing to enhanced apoptosis and degeneration of the inner cell mass (20,21). As disruptions of RAD50 and MRE11 in the mouse also lead to embryonic lethality (28,29), the M/R/N complex clearly has an essential function in embryonic stem cell proliferation and early embryo development. A possible explanation for the viability of individuals with mutations in the human NBS1 gene was suggested by the finding that the major mutation, 657D6, can lead to an alternative initiation.
of translation and production of a 70 kDa C-terminal nibrin protein fragment (17).

In agreement with this hypothesis, mice with targeted Nbn alleles producing similar C-terminal nibrin proteins of 75 or 80 kDa also survive (22,23). All of these nibrin fragments contain the region that interacts with MRE11 (30,31) and the serines, which are phosphorylated by ATM in response to ionizing irradiation (12,13). They do not, however, contain the BRCT and FHA domains that are required for nibrin phosphorylation by ATM, the formation of nuclear foci after IR (5,32) and, indeed, correct nuclear localization of the M/R/N complex (5).

Thus, although the C-terminal nibrin fragments might be able to bind MRE11, and thus associate with RAD50, this function suffices for cellular survival but not radioresistance. Using an inducible disruption of the Nbn gene to create a null mutant has allowed us to test this hypothesis. Cre recombinase-mediated deletion of Nbn exon 6 in a cell that has an inactivating null mutation in the remaining Nbn allele leads to death in vitro and in vivo, recapitulating the lethality in mouse ES cells and early mouse embryos reported previously. Transduction of inducible knock-out cells with a retrovirus expressing wild-type human nibrin before deletion of homozygous or Nbn WT heterozygous cells (stacked grey columns) and undeleted cells (stacked white columns).

Figure 7. Survival of Nbn WT/D6 and Nbn ins-6/D6 cells after deletion in vitro. Fibroblasts with the genotypes given in the figure were treated with HTNC and returned to culture. At various time points, DNA was isolated from the cultures and subjected to semi-quantitative PCR to establish the proportion of the Nbn D6 allele, and thus the proportion of Nbn ins-6/D6 homozygous cells or Nbn WT/D6 heterozygous cells (stacked grey columns) and undeleted cells (stacked white columns).

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In agreement with this hypothesis, mice with targeted Nbn alleles producing similar C-terminal nibrin proteins of 75 or 80 kDa also survive (22,23). All of these nibrin fragments contain the region that interacts with MRE11 (30,31) and the serines, which are phosphorylated by ATM in response to ionizing irradiation (12,13). They do not, however, contain the BRCT and FHA domains that are required for nibrin phosphorylation by ATM, the formation of nuclear foci after IR (5,32) and, indeed, correct nuclear localization of the M/R/N complex (5).

Thus, although the C-terminal nibrin fragments might be able to bind MRE11, and thus associate with RAD50, this function suffices for cellular survival but not radioresistance. Using an inducible disruption of the Nbn gene to create a null mutant has allowed us to test this hypothesis. Cre recombinase-mediated deletion of Nbn exon 6 in a cell that has an inactivating null mutation in the remaining Nbn allele leads to death in vitro and in vivo, recapitulating the lethality in mouse ES cells and early mouse embryos reported previously. Transduction of inducible knock-out cells with a retrovirus expressing wild-type human nibrin before induction of homozygosity completely complemented their null mutations. Furthermore, transduction with a cDNA containing the major human mutation, 657Δ5, which produces a 70 kDa C-terminal nibrin fragment, also rescued cells from death in culture. As the N-terminal truncated human nibrin fragment theoretically expressed from this allele (amino acids 1–219) is practically identical to the N-terminal truncated mouse nibrin fragment already expressed from the targeted alleles in these cells (amino acids 1–195 from NbnD6 and 1–203 from Nbn ins-6), survival must be attributed to the C-terminal fragment. These results support the notion that NBS patients survive owing to the expression, possibly only during embryonic development, of this nibrin protein fragment.

The functions of nibrin in cell survival and radioresistance can be evidently separated by the inducible mouse model presented here. Spontaneous chromosomal instability is characteristic for NBS; however, the level of aberrant mitotic cells seen, at least in stimulated lymphocytes, is variable, ranging from 10 to 45% of cells (2,33). Unchallenged null mutant splenic lymphocytes isolated after induction of homozygosity in vivo or in vitro show a high level of spontaneous chromosome breakage: >20% of mitotic cells are chromosomally aberrant. In contrast, embryonic fibroblasts from a viable mouse model with a hypomorphic mutation leading to expression of an 80 kDa C-terminal nibrin fragment have no increased spontaneous chromosomal instability, although they do show increased breakage in comparison with wild-type cells after IR (23). Again, this implies that the p70 nibrin fragment is able to carry out some of the cellular functions of full-length nibrin in DNA-repair and/or cell cycle checkpoints.

Complete lack of nibrin, on the other hand, leads to cell death, at least during competition with heterozygous cells in vitro. Poor proliferation has been described for embryonic fibroblasts isolated from a mouse model in which a 75 kDa C-terminal nibrin fragment is produced (22). Indeed, after six passages these cells ceased to grow. In another vertebrate system, chicken DT40 cells, null mutation of the NBS1 homologue also leads to a loss of cell proliferation (19). These findings presumably reflect the accumulation of mutations with each cell cycle.
Of the organs examined after in vivo induction of homozygosity, there was no detrimental effect on cell survival in non-proliferating tissues such as liver, kidney and muscle even though nibrin is clearly expressed in these tissues (4,34). There was, however, a dramatic effect in the bone marrow: 50% of null mutant cells were lost in this tissue. This can clearly be correlated with the mitotic activity of hematopoietic stem cells and lymphocyte progenitors in this organ, which are likely to be particularly sensitive to the effects of mutation accumulation. In this respect, hematopoietic stem cells resemble embryonic stem cells during early embryogenesis, which are also particularly sensitive to nibrin null mutation (20,22).

Even more striking than bone marrow is the effect in the thymus. Few null mutant cells survive in this organ, suggesting that T-cell specific processes, such as T-cell receptor gene rearrangement, may be nibrin-dependent with abortive gene rearrangement leading to cell death. In a hypomorphic Nbn mouse model, greatly reduced thymus cellularity and a 5-fold reduction in mature CD4+ thymocytes were observed (22). Thus, the effects of null mutation and a hypomorphic mutation are essentially identical, and the truncated nibrin protein is unable to carry out the processes required for T-cell maturation, particularly T-cell receptor gene rearrangement.

A significant deficiency of nibrin null mutant cells was also found in the spleen after induction of homozygosity in vivo. As the spleen is a major reservoir for lymphocytes, the reduced recovery of homozygous cells from this organ may merely reflect their prior loss in the bone marrow. However, the process of isotype class switching in B-lymphocytes might, if nibrin were in fact involved, also contribute to the loss of Nbn null mutant cells in the spleen. Indeed, Nbn expression is particularly high in adult spleen (34,35) with an expression pattern closely resembling that of DNA-PKcs (36), an enzyme involved in NHEJ during V(D)J recombination.

The role of nibrin in DNA repair is more relevant to the disease, NBS, than its role in the G2/M checkpoint

Nibrin is clearly a protein essential for viability of proliferating cells. Cell death can be ascribed to the accumulation of massive chromosomal damage even in the absence of exogenous DNA-damaging agents. As expression of a C-terminal nibrin fragment is sufficient to ensure cellular viability, individuals with hypomorphic mutations in the human NBS1 gene survive, but are radiosensitive and cancer-prone. The relative importance of the cell cycle checkpoints and DNA repair activities of nibrin for the features of the disease have been previously addressed (9,37). A comparison of the null mutant cells described here with patient cells may help clarify this issue.

Even in the absence of exogenous DNA damaging agents, DSBs are accumulated in the cell during DNA replication and must be repaired before entry into mitosis. Analysis of SV40 transformed NBS LB1 fibroblasts, which express relatively large amounts of p70 (comparable to SV40 transformed GM7166VA7 fibroblasts; Fig. 2D) demonstrated that these cells arrest normally in response to DNA damage (38). Similarly, EBV immortalized NBS B cells, which also express truncated nibrin (17), have a functional G2/M checkpoint (38,39). This would suggest that the p70 protein fragment in patient cells is able to sustain this aspect of nibrin function. However, the p70 fragment is not phosphorylated by ATM (data not shown), presumably because the FHA/BRCT domains required for ATM-mediated phosphorylation (32)
are not present. Thus, it is unlikely that the sustained G$_2$/M checkpoint observed in patient cells is due to activity of p70 downstream of ATM.

Co-immunoprecipitation experiments have demonstrated that the p70 nibrin fragment is able to form a complex with MRE11 and RAD50 (17); this faulty complex may be sufficient to function in activating ATM. Indeed, activation of ATM does occur, although at a considerably reduced rate (16), in EBV immortalized NBS B cells and in GM7166VA7 NBS fibroblasts, which also express p70 (Fig. 2D) (40). In the complete absence of nibrin, as in the null mutant murine cells presented here, ATM is not activated by DNA damage incurred earlier in the cell cycle and, subsequently, the G$_2$/M checkpoint is not triggered. As could be expected from this, in comparison with wild-type cells, further mutagenic attack in null mutants leads to an increase in chromosome breaks per cell rather than an increase in the proportion of damaged cells (Fig. 5B).

The ability of the 657Δ5 mutant cDNA to promote cell survival of null mutant cells in vitro stems most likely from residual activation of ATM by a partially functioning M/R/N complex, and thus maintenance of the G$_2$/M checkpoint. The radiosensitivity of NBS patients would consequently be attributable more specifically to nibrin function(s) in DSB repair rather than in the control of this cell cycle checkpoint.

**MATERIALS AND METHODS**

**Nbn-Targeting vector**

The targeting vector shown in Figure 1 was generated by ligation of Nbn sequences amplified from mouse 129/Sv genomic DNA into the vector, pTV-Flox (a gift from Dr Dieter Rietmacher). Murine Nbn exon primers were used to amplify the 8 kb sequence encompassing Nbn exons 3–6 and 6–8 by long-range PCR using a high-fidelity polymerase (Expand Long Template System, Roche); the PCR products were then sequenced and the derived intronic sequences were used for designing primers to amplify fragments for the targeting vector.

A 2.4 kb fragment containing 750 bp of Nbn intron 5, the entire exon 6, intron 6, exon 7 and 811 bp of intron 7 was amplified with the primers: gctggctatgtgaagactac (forward) and BamHI-cccttgtagactctttggta (reverse). After digestion of this fragment with BamHI and Xbal (located in intron 5) the resulting 2.2 kb sequence was ligated into BamHI–Xbal-cleaved pT7T3 (Pharmacia). An unique AvaI restriction site in Nbn intron 6 was used to insert a 42 bp AvaI-flanked oligonucleotide containing a loxP sequence. A fragment containing Nbn exon 6, intron 6 and exon 7, with flanking intronic sequences was excised using BamHI and Hpal (located in intron 5) and ligated into the pTV-Flox vector at BamHI and Hpal sites. A second, 4 kb PCR product containing most of Nbn intron 5, was generated using the primers: NotI–aatacgag-gacaatgtaag (forward) and tgttttctttcatgtcgccc (reverse). This product was cut with NotI and Xbal (located in intron 5) and ligated into the prepared targeting vector at NotI/Xbal sites. The targeting vector was sequenced to verify the integrity of the Nbn coding sequences and splice junctions.

**Embryonic stem cell electroporation and blastocyst injection**

The targeting vector was linearized with NotI and then electroporated into E14.1 ES cells. After selection with 0.2 mg/ml G-418 and 2 μM gancyclovir, targeted ES clones were preliminarily identified by PCR using primers located in the neomycin resistance gene cassette and in intron 7 of Nbn. Analysis of 600 clones yielded 12 clones, with HR in the Nbn gene. Targeting was verified by Southern blot analysis after NheI digestion and hybridization with a sequence from exon 5 as a probe (Fig. 2A). One ES clone was electroporated with pMC-Cre, a Cre recombinease expression vector, plated at low density and 28 G418-sensitive clones were isolated and characterized. Two clones were identified that had undergone recombination removing the neomycin-resistance cassette and one loxP site, but leaving Nbn exon 6 flanked by loxP sites ($Nbn^{wt/lox}$). Eleven clones had both the neomycin-resistance cassette and exon 6 deleted leaving just one intact loxP site ($Nbn^{wt/Δ6}$). Both the $Nbn^{wt/lox-6}$ and $Nbn^{wt/Δ6}$ ES cell clones were microinjected into C57BL/6 blastocysts and implanted into pseudopregnant recipients. Chimeras were mated with 129/Sv mice and germline transmission was verified by PCR analysis and Southern blot as detailed earlier.

**Mouse genotyping**

The Nbn alleles with variations in exon 6 were routinely distinguished by PCR analysis on DNA isolated from tail biopsies of mice using the following primer pairs for Nbn$^{wt}$, Nbn$^{lox-6}$ and Nbn$^{Δ6}$ alleles: forward primer, gctggctatgtgaagactac, located in intron 5; and reverse primer, aatacgag-gacaatgtaag, located in intron 6. The product sizes: Nbn$^{wt}$, 1148 bp; Nbn$^{lox-6}$, 877 bp; Nbn$^{Δ6}$, 299 bp (Fig. 2B). For the $Nbn^{wt/Δ6}$ allele, the primers were: forward primer, cctgtgctcgaggtct, located in the G418-resistance cassette inserted into exon 6; and reverse primer, cctgtgctcgaggtct, located in intron 6, the specific product is 411 bp.

**Mouse breeding and induction of Nbn-homozygosity in vivo**

To induce Nbn null mutation in vivo, Nbn$^{lox-6/Δ6}$ and Nbn$^{wt/ins-6}$ mice were crossed with Tg-Mx1-Cre mice, which express Cre recombinase under the control of the interferon responsive promoter, Mx1 (24). Mice were identified by PCR genotyping of DNA isolated from tail biopsies and further crossed to produce the target animals: $Nbn^{Δ6/lox-6}$, Tg-Mx1-Cre, $Nbn^{wt/lox-6}$, Tg-Mx1-Cre and $Nbn^{wt/ins-6}$, Tg-Mx1-Cre.

Cre expression was induced in transgenic Mx1-Cre mice in vivo by intraperitoneal injection of the interferon inducer, poly(I):poly(C) (Amersham-Pharmacia). Each mouse received three 250 μl injections of a 2 mg/ml solution of poly(I):poly(C) in water every 48 h. Mice were analysed 15 days after the first injection. Organs were removed from mice after cervical dislocation and portions processed for DNA isolation and quantification of exon 6 deletion.
Semi-quantitative PCR for the estimation of Cre recombinase-mediated Nbn exon 6 deletion

Primers were selected which amplify similarly sized products from the three alleles: Nbn\textsuperscript{wt}, Nbn\textsuperscript{lox-6} and Nbn\textsuperscript{lox-6}. The two forward primers are, ataagacagtcaccac, located in intron 5 of the Nbn gene 5’ to the first loxP site and getgtgctcaagtagacg located in intron 5 downstream to the first loxP site but 5’ to exon 6. The reverse fluorescein-labelled primer is cctaggagtccagtgtt located 3’ to the second loxP site. The sizes of the amplified products are: Nbn\textsuperscript{wt}, 515 bp; Nbn\textsuperscript{lox-6}, 601 bp; Nbn\textsuperscript{lox-6/lox-6}, 554 bp. Undenatured PCR products were separated on native 12% polyacrylamide gels, which were then analysed in the ‘vistra FluorImager SI’ (Amersham Life Science) using bandpass emission filter 530 DF 30 and ImageQuaNT software (Molecular Dynamics, version 5.2).

The linearity of response was tested by amplifying Nbn\textsuperscript{lox-6/lox-6/Δ6} genomic DNA under standard conditions for 24–28 cycles. All further semi-quantitative PCRs were conducted with 27 cycles. The accuracy and variance of the semi-quantitative PCR measurements were determined by amplifying artificial mixtures of Nbn\textsuperscript{wt/wt} and Nbn\textsuperscript{lox-6/lox-6} or Nbn\textsuperscript{lox-6/6} and Nbn\textsuperscript{lox-6/lox-6} genomic DNAs. The measured ratios of Nbn\textsuperscript{lox-6/lox-6} Nbn\textsuperscript{wt} and Nbn\textsuperscript{lox-6/Δ6} alleles were compared with the actual ratios of genomic DNA in the mixtures (Supplementary Material, Fig. S1).

Induction of Nbn-homozygosity in fibroblasts in vitro

Crosses were set up between Nbn\textsuperscript{lox-6/lox-6} and Nbn\textsuperscript{wt/wt} mice (20) to produce Nbn\textsuperscript{lox-6/ins-6/lox-6} and Nbn\textsuperscript{wt/ins-6/lox-6} mice for deletion of exon 6, and generation of homozygosity, by treatment of fibroblasts and lymphocytes in vitro with Cre recombinase. Dermal fibroblasts were isolated from ear biopsies of Nbn\textsuperscript{lox-6/ins-6}, Nbn\textsuperscript{lox-6/lox-6} and wild-type mice and grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS) at 37°C, 5% CO\textsubscript{2}. Spontaneous transformation occurred regularly before passage 12, after which cells were cryopreserved.

Cre recombinase was isolated as the fusion protein, HTNC, consisting of a His-tag, the HIV-tat sequence, a nuclear localization sequence and Cre recombinase, from Escherichia coli carrying the pTriEx-HTNC expression plasmid as previously described (26). For in vitro deletion experiments, fibroblasts at 50% confluence were incubated at 37°C for 6 h in serum-free medium with 2 μM HTNC. Cells were then washed and returned to culture in DMEM with 10% FCS. Cultures were routinely split 1:5 at confluence, generally every 2 days.

Mouse lymphocytes were isolated by spleen disruption through a 80 μm cell sieve (Becton Dickinson, USA) and incubated in 1 μM HTNC in RPMI without serum for 2 h followed by washing and cultivation in RPMI with 10% FCS, 6 μg/ml concanavalin A (Sigma–Aldrich) and 40 μg/ml bacterial lipopolysaccharide (Sigma–Aldrich). Stimulated lymphocytes were harvested for chromosome preparation by standard protocols after 3 days of cultivation. For examination of chromosome breakage induced by the radiomimetic bleomycin (Medac, Germany), cultures were incubated in the drug for 3 h before harvesting and chromosome preparation.

Mitic index determination by flow cytometry

The method employed was previously described for the analysis of the G2/M checkpoint in NBS patient cells (38). Briefly, mouse fibroblasts with the genotypes Nbn\textsuperscript{lox-6/lox-6/wt} and Nbn\textsuperscript{lox-6/lox-6/wt} were treated as described with HTNC to delete the loxP-flanked exon 6. After 24 h, the cultures were split and after 48 h incubated with increasing concentrations of bleomycin (Medac) for 2 h. Cells were fixed in 75% ice-cold ethanol, and permeabilized for 10 min on ice in 0.1% Triton X-100 in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). After washing, the cells were incubated for 2 h at room temperature in 1% BSA in PBS with a polyclonal rabbit anti-phosphorylated histone H3 antibody (Upstate Biotechnology, USA) at 1:100 dilution. The cells were washed and incubated in 1% BSA in PBS with a Cy2-conjugated goat anti-rabbit antiserum (Jackson Immunoresearch) at 1:100 dilution. The cells were washed and stained with propidium iodide at 25 μg/ml in 1% BSA in PBS containing 100 μg/ml RNase A. Cytometry was performed in the FACSCalibur (Becton Dickinson), at least 20 000 cells were counted per sample. Data were analysed with the WinMDI version 2.5 software.

Western analysis

Whole cell extracts were prepared by cell lysis in RIPA buffer and were separated on 8% polyacrylamide SDS gels. Proteins were electrophoresed to nitrocellulose membranes, blocked in 5% dried milk in TBST and probed with a rabbit polyclonal anti-murine nibrin antiserum (20) or a rabbit polyclonal antihuman nibrin antiserum (Novus Biologicals) for 1 h at room temperature followed by incubation with horseradish peroxidase-conjugated secondary antibody diluted 1:1000. Chemiluminescent detection was performed using the ECL reagents (Amersham).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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