NFBD1/KIAA0170 is a Chromatin-associated Protein Involved in DNA Damage Signaling Pathways*

Received for publication, November 7, 2002, and in revised form, December 23, 2002
Published, JBC Papers in Press, December 23, 2002, DOI 10.1074/jbc.M211392200

Xingzhi Xu and David F. Stern†
From the Department of Pathology, School of Medicine, Yale University, New Haven, Connecticut 06510

NFBD1/KIAA0170 is a nuclear factor with an N-terminal FHA (forkhead-associated) domain and a tandem repeat of BRCT (breast cancer susceptibility gene-1 C (terminus)) domains, both of which are present in a number of proteins involved in DNA repair and/or DNA damage signaling pathways. We have investigated the association of NFBD1 with DNA damage responses. We found that the NFBD1 transcript is abundant in the testis relative to other tissues. NFBD1 is a chromatin-associated protein and is modified in G2/M phase or after DNA damage. NFBD1 phosphorylation in response to ionizing radiation (IR) was ATM-dependent. NFBD1 exhibited diffuse nuclear staining in the majority of untreated cells analyzed by indirect immunofluorescence and formed discrete nuclear foci after exposure to IR, UV radiation, and hydroxyurea treatment. IR induced NFBD1 foci within 1 min. The foci colocalized with γ-H2AX foci, which have been previously shown to localize at sites of DNA double-strand breaks. IR-induced NFBD1 foci also colocalized with 53BP1 and MRE11/RAD50 foci. Taken together, these results suggest that NFBD1 is a mediator of DNA damage-dependent signaling.

The faithful duplication and segregation of genetic information to daughter cells are of primary importance for maintaining genome stability. DNA double-strand breaks (DSBs) pose serious threats to genome stability. DSBs can be induced by exogenous agents such as ionizing radiation (IR), by endogenously generated free radicals produced during normal cellular metabolic reactions, or by replication stress (1). DSBs are also naturally occurring intermediates in normal cellular processes such as meiotic recombination and V(D)J recombination (1).

A sophisticated surveillance network, including checkpoint controls, exists in all eukaryotes to detect and repair damaged DNA before a cell moves on to the next phase of the cell cycle (2). One of the earliest events in the cellular response to DSBs is the phosphorylation of a histone H2A variant, H2AX, at sites of DNA damage (3–5). H2AX is rapidly phosphorylated (within 1 min) at an evolutionarily conserved residue, Ser139, when DSBs are induced in mammalian cells, resulting in discrete phosphorylated H2AX (γ-H2AX) foci at or near the DNA damage sites. Immunofluorescence studies have revealed that γ-H2AX forms nuclear foci at the sites of DSBs (4, 5). The number of foci increases in the first 10–30 min after irradiation before they gradually decline, correlating with the number of slowly rejoining DSBs (5). γ-H2AX foci are also found at sites of V(D)J recombination-induced DSBs in developing thymocytes (6), at sites of recombinational DSBs during meiosis (7), and at sites of stalled replication forks (8). Thus, γ-H2AX foci are indicators of the presence of DSBs in vivo. Lack of H2AX causes genome instability in mice (9). H2ax−/− cells fail to recruit DNA damage signaling and repair factors such as NBS1 (Nijmegen breakage syndrome-1), 53BP1 (p53-binding protein-1), and BRCA1 (breast cancer susceptibility gene-1), but not RAD51, to irradiation-induced foci (9). Thus, H2AX is critical for facilitating the assembly of specific DNA repair complexes on damaged DNA.

DNA damage activates a cascade of protein kinases that relay the signal to downstream effectors to halt the cell cycle and that facilitate repair of the damage (2). In budding yeast, the serine/threonine kinase Mec1 is a master regulator of cellular responses to DNA damage. In response to DNA damage, Rad9 is phosphorylated in a MEC1-dependent manner (10–12); and in turn, phosphorylated Rad9 apparently recruits another serine/threonine kinase, Rad53, to the Mec1 complex for activation (13, 14). (Note that budding yeast Rad9 is unrelated to human RAD9.) Alternatively, phosphorylated Rad9 oligomers may act as a scaffold to bring Rad53 molecules into close proximity to each other, facilitating cross-phosphorylation between Rad53 molecules and subsequent release of activated Rad53 (15). Rad9 also regulates activation of Chk1, another important effector kinase of Mec1, in the G2/M checkpoint (16).

Mec1 belongs to the family of phosphatidylinositol 3′-kinase-related kinases (PIKKs). In mammals, these kinases include ATM (ataxia-telangiectasia mutated) and ATR (ATM- and Rad3-related). ATM, which is not essential for development, mediates the early response to DNA DSBs, and its inactivation in patients with ataxia-telangiectasia leads to checkpoint defects and genome instability (17). Rad53 is an FHA (forkhead-associated) domain-containing kinase, and its human ortholog is CHK2. CHK2 mutations have been found in some Li-Fraumeni syndrome kindreds that do not have p53 mutations (18, 19) and in a variety of sporadic cancers (20–23). IR-dependent CHK2 activation requires that its FHA domain be functional (24) and is ATM-dependent (25). The Rad9 checkpoint protein in Saccharomyces cerevisiae and its ortholog Crb2/Rhp9 in Schizosaccharomyces pombe share sequence similarity primarily within their tandem repeat of C-terminal BRCT (BRCA1 C

*This work was supported by United States Army Research and Material Command Grants DAMD 17-98-1-2872 (to D. F. S.) and DAMD 17-01-1-0485 (to X. X.), United States Public Health Service Grant RO1CA82257 (to D. F. S.), and a Leslie H. Warner fellowship from the Yale Comprehensive Cancer Center (to X. X.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Pathology, School of Medicine, Yale University, 310 Cedar St., BML 342, New Haven, CT 06510. Tel.: 203-785-4832; Fax: 203-785-7467; E-mail: Df.stern@yale.edu.

‡ The abbreviations used are: DSBs, double-strand breaks; IR, ionizing radiation; PIKK, phosphatidylinositol 3′-kinase-related kinase; GST, glutathione S-transferase; HA, hemagglutinin; siRNA, small interfering RNA; Gy, gray; PBS, phosphate-buffered saline.
Gene) were probed using a Smal-XbaI fragment from pcDNA-HANFBD1 labeled with 32PdCTP using a random-primer DNA labeling kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. A probe for mouse Nfd1 was derived from a mouse expressed sequence tag clone (GenBankTM/EBI accession number AW106340) encoding putative mouse NFD1. The sequence of this expressed sequence tag clone and similarity to a portion of human NFD1 were verified by end-to-end sequencing. A mouse tissue mRNA blot (Clontech) was probed with an EcoRI-SacI fragment encoding the first 490 amino acids derived from this cDNA.

**Cell Culture and Transfection—**ATM-deficient SV40-transformed human GM5849C fibroblasts were obtained from the Corell Institute for Medical Research (Camden, NJ). Other cell lines were obtained from the American Type Culture Collection. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mm glutamine, 50 units/ml penicillin, and 50 mg/ml streptomycin.

Transfection was performed with FuGENE 6 (Roche Molecular Biochemicals) at a ratio of 1 µl of plasmid to 2 µl of FuGENE 6. The small interfering RNA (siRNA) duplex targeting NFD1 was prepared by annealing two 21-ribonucleotide oligonucleotides, 5’-GCC UGA CAC CUC CUU AGG UTT-3’ and 5’-ACC UUA GGA GGU CUG ATT-3’ (Dharmacon Research). The scrambled siRNA duplex sequences were 5’-GUU CAC UGA UCG AGC CTT-3’ and 5’-GGC UCG AUG AGU GAA GAT-3’. HeLa cells were transfected with the siRNA duplex using OligofectAMINE (Invitrogen) and analyzed 60 h after transfection. Control cells were transfected with an empty vector (pCMV6-Neo). Two－4 days after transfection, cells were collected by trypsinization and cells were harvested in a Shepherd Mark 1 127Cc irradiator at a dose rate of 1.75 gray (Gy)/min. Cells were UV-irradiated at a dose of 50 J/m2 with a Stratagen cross-linker 48－60 h after transfection. 48－60 h after transfection, cells were treated with 1 µg hydroxyurea (Sigma) for 24 h.

**Synchronization of HeLa Cells—**HeLa cells were grown in the presence of 2 µM thymidine (Sigma) for 18 h, washed with phosphate-buffered saline (PBS), and grown in fresh medium without thymidine for 8 h. Thymidine was added again to 2 µM to block cells at G1/S. After another 18 h, cells were transferred to fresh medium, and samples were harvested every 2 h for a period of 16 h. To arrest cells in mitosis, cells were first treated with 2 µM thymidine, released into fresh medium for 3－4 h, then blocked with 100 µM nocodazole for 16 h. Floating cells were collected, washed with PBS, and seeded into fresh medium. Synchronization and cell cycle state were examined by propidium iodide staining and fluorescence-activated cell sorter analysis.

**Chromatin Fractionation—**Chromatin fractionation was performed essentially as described (37). Briefly, 3×106 cells were washed with PBS and resuspended in 200 µl of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 0.34 mM sucrose, 10% glycerol, 1 mM dithiothreitol, and protease inhibitor mixture (Roche Molecular Biochemicals)). Triton X-100 was added to a final concentration of 0.1%, and the cells were incubated for 5 min on ice. Nuclei were collected by pelleting (P1) by low speed centrifugation (1500 g, 4 min, 4 °C). The supernatant (S1) was further clarified by high speed centrifugation (13,000 × g, 10 min, 4 °C) to remove cell debris and insoluble aggregates. The supernatant was designated S2. Nuclei were washed once with buffer A and then lysed in 200 µl of buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, and protease inhibitor mixture). After a 10-min incubation on ice, soluble nuclear proteins (S0) were separated from chromatin by centrifugation (2000 × g, 4 min). Isolated chromatin (P3) was washed once with buffer B and spun down at high speed (13,000 × g, 1 min).

**Immunoprecipitation and Immunoblotting—**Cell lysate was harvested after treatment in high salt buffer containing 20 mM Tris-HCl (pH 7.5), 0.4 M NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and protease inhibitor mixture. 2 µg of antibodies was used for immunoprecipitation from 400 to 500 µg of total lysate plus an equal volume of buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% Nonidet P-40, and protease inhibitor mixture at 4 °C overnight. Precipitates were washed with 20 mM Tris-HCl (pH 8.0), 0.2 M NaCl, 1 mM EDTA, and 0.5% Nonidet P-40. Immunoblot on nitrocellulose were blocked with 5% nonfat milk in PBST (PBS with 0.5% Tween 20) and washed with PBST. Primary antibodies were incubated with 5% bovine serum albumin (BSA) in PBST. Secondary antibodies were incubated with 5% nonfat milk in PBST.

**Phosphatase Treatment—**Immunoprecipitates of endogenous NFD1 from HEK293 and HeLa cells before and after exposure to γ-irradiation or from HeLa cells treated with 250 µM/ml nocodazole for 18 h were incubated with 1000 units of λ-phosphatase (New England Biolabs Inc.) in the presence of 2 mM MnCl2 for 1－2 h at 30 °C in a 50-µl reaction volume.

**Experimental Procedures**

**Plasmids—**A clone in the HUGE Database (GenBank™/EBI accession number D79992) containing the entire coding sequence of NFD1 (KIAA0170) was obtained from Takahiro Nagase (Kazusa DNA Research Institute, Chiba, Japan). For expression in mammalian cells, NFD1-coding sequences were amplified by PCR and cloned into the pcDNA3xHA-Neo, resulting in pcDNA-HANFBD1. Internal deletion mutants were generated from pcDNA-HANFBD1 by subcloning using appropriate restriction enzymes. Plasmid constructs were verified by sequence analysis.

**Antibodies—**A Smal-XbaI fragment encoding a polypeptide from residues 142 to 568 was released from pcDNA-HANFBD1 and subcloned into the glutathione S-transferase (GST) vector pGEX4T-3. The resulting construct was used to produce GST fusion protein in Escherichia coli. The GST fusion protein, purified as described (26), was used to immunize rabbits for antibody production (Yale Animal Resources Center, New Haven). Antibodies were purified from crude sera on a protein G/protein A-agarose column (Calbiochem) and then an affinity column conjugated to the antigenic GST fusion protein. The final concentration of the purified antibodies was adjusted to 1 mg/ml. Mouse anti-S3BP1 monoclonal antibody was a kind gift of Thanos D. Halazonetis (Wistar Institute, Philadelphia).

**Immunoprecipitation—**A cell lysate was harvested after treatment in high salt buffer containing 20 mM Tris-HCl (pH 7.5), 0.4 M NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and protease inhibitor mixture. 2 µg of antibodies was used for immunoprecipitation from 400 to 500 µg of total lysate plus an equal volume of buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% Nonidet P-40, and protease inhibitor mixture at 4 °C overnight. Precipitates were washed with 20 mM Tris-HCl (pH 8.0), 0.2 M NaCl, 1 mM EDTA, and 0.5% Nonidet P-40. Immunoblot on nitrocellulose were blocked with 5% nonfat milk in PBST (PBS with 0.5% Tween 20) and washed with PBST. Primary antibodies were incubated with 5% bovine serum albumin (BSA) in PBST. Secondary antibodies were incubated with 5% nonfat milk in PBST.

**Phosphatase Treatment—**Immunoprecipitates of endogenous NFD1 from HEK293 and HeLa cells before and after exposure to γ-irradiation or from HeLa cells treated with 250 µM/ml nocodazole for 18 h were incubated with 1000 units of λ-phosphatase (New England Biolabs Inc.) in the presence of 2 mM MnCl2 for 1－2 h at 30 °C in a 50-µl reaction volume.

**Northern Blotting—**Human tissue mRNA blots (Clontech and Ori...
NFBD1/KIAA0170 in DNA Damage Signaling

Indirect Immunofluorescence—Cells grown on poly-D-lysine-coated eight-chamber slides were either mock-treated or exposed to 50 J/m² UV light or 1 mCi hydroxyurea for 24 h or the specified dose of IR (0.1–5 Gy from a 137Cs source). In some experiments, 10 or 50 μM wortmannin was added to the cells 1 h before irradiation. At the indicated time points, cells were fixed in 4% paraformaldehyde in PBS for 15 min, followed by permeabilization for 15 min in 0.5% Triton X-100 in PBS. Slides were blocked with 5% bovine serum albumin in PBST for 30 min at 37 °C, incubated with primary antibody for 30 min at 37 °C, washed with PBST, and incubated with secondary antibody (rhodamine-conjugated donkey anti-mouse IgG (1:1000) or fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:200)) for 30 min at 37 °C. Dilutions of primary antibodies were 1:1000 for anti-NFBD1; 1:500 for anti-γ-H2AX, anti-MRE11, and anti-HA (16B12); 1:15 for anti-53BP1; and 1:50 for anti-BRCA1. After washing, the slides were mounted with coverslips with mounting medium containing 4,6-diamidino-2-phenylindole (Vector Labs, Inc.). Images were acquired using a Nikon Microphot-FX microscope with a ×40 or ×60 objective and a SPOT digital camera.

RESULTS
NFBD1 Is a Chromatin-associated Nuclear Protein—Human and mouse tissue mRNA blots were probed with NFBD1 cDNA sequences to determine the tissue-specific distribution of NFBD1 transcripts. Both human and mouse blots revealed an abundant transcript at a size of ~7.5 kb in testis and low levels in placenta, kidney, brain, heart, liver, thymus, smooth muscle, lung, colon, and peripheral blood leukocytes (data not shown). Although the high expression in testis could be connected with meiotic functions such as recombination, the significance is not clear because of the strong transcriptional induction of many genes during spermatogenesis.

The human NFBD1 clone includes an open reading frame of 6270 nucleotides that would encode a polypeptide of 2089 amino acids with a predicted molecular mass of 226 kDa. This polypeptide has a C-terminal tandem repeat of BRCT domains and an N-terminal FHA domain (Fig. 1A). In the middle of the polypeptide is a tandem array of nine serine- and threonine-rich 24-amino acid repeats interspersed with other sequences (Fig. 1B). Data mining did not reveal other proteins with significant homology to these repeats.

We produced and affinity-purified rabbit polyclonal antibodies against the N terminus of the putative polypeptide. This antibody preparation recognized three bands with a slower electrophoretic mobility than that of the 250-kDa protein marker in immunoblots of a variety of cell lines, including HEK293, U2OS osteosarcoma cells, normal human WI38 fibroblasts, ATM-deficient human GM05849C fibroblasts, and HeLa cells (Fig. 1C and data not shown). These three bands detected in HeLa cells were dramatically diminished after transfection with a pair of siRNA oligonucleotides specific for the NFBD1-coding region, but not after transfection with scrambled siRNA oligonucleotides (Fig. 1D). These three bands were probably derived from translation of alternatively spliced transcripts or alternative transcriptional initiation sites because expression of HA-tagged NFBD1 cDNA yielded only a single band (Fig. 1C, lanes 2, 5, and 9). HA-NFBD1 cotransfected with the largest endogenous polypeptide recognized by anti-NFBD1 antibody and was recognized as well by anti-HA antibody (Fig. 1C). This also indicates that the cDNA clone contains, at a minimum, most of the coding sequence for NFBD1.

Indirect immunofluorescence of cells transfected with a plasmid encoding HA-NFBD1 with anti-NFBD1 antibody revealed diffuse nuclear staining (Fig. 1E). The staining intensity was increased in the subset of cells expressing HA-NFBD1 (Fig. 1E, compare HA and NFBD1 panels). The staining was blocked if the antibody was preincubated with the antigenic GST fusion protein, but not with GST fusion proteins derived from other NFBD1 fragments (data not shown). The exclusively nuclear staining indicates that NFBD1 encodes a nuclear protein, which is also supported by a previous report using green fluorescent protein-tagged NFBD1 (38).

To further verify the antibody specificity and to identify domains required for nuclear localization, we generated a series of overlapping deletion mutants with three copies of the HA epitope tag at their N termini (Fig. 1A). Transiently transfected HEK293 cells were co-immunostained with anti-NFBD1 and anti-HA antibodies. In cells transfected with expression constructs with sequences encoding the antigenic polypeptide used to produce anti-NFBD1 antibody (Fig. 1F, N2 and N3 panels), but not with other deletion mutants (panels N1, C1, and C2), the subset of cells that reacted with anti-HA antibody also showed increased intensity with anti-NFBD1 antibody. All of the deletion mutants except the N1 fragment showed an exclusively nuclear staining pattern with anti-HA antibody (Fig. 1F). The N1 fragment contains the FHA core sequence homology domain, which apparently is not sufficient for nuclear localization. The N2 fragment is an extension of the N1 fragment and was sufficient for nuclear localization. Thus, a nuclear localization signal may be present between residues 145 and 568, consistent with previous observations (38). The C1 fragment was also sufficient for nuclear localization (Fig. 1F). The earlier report (38) did not identify any nuclear localization signal within this fragment because only progressive deletions from the C terminus were analyzed. Canonical nuclear localization signals are characterized by short, single, or bipartite stretches of basic amino acids separated by a flexible spacer with the relative consensus sequence K(K/R)(K/R) (39). This fragment contains at least two stretches of basic residues within a highly basic region (1860KPGKRKRDQAEEEPNRIIPSRSLRKK1885). Thus, NFBD1 may have a second nuclear targeting signal near its C terminus.

We performed nuclear fractionation to determine whether NFBD1 is associated with chromatin. NFBD1, like chromatin-bound ORC2, but not the cytoplasmic protein GRB2, was present exclusively in the chromatin-enriched P3 fraction (Fig. 1G). This association did not change dramatically after γ-irradiation, UV radiation, or hydroxyurea treatment (data not shown). Thus, NFBD1 may constitutively bind to chromatin.

NFBD1 Is Modified in G2/M Phase—NFBD1 contains FHA and BRCT domains, both of which are present in a variety of genes involved in cell cycle controls. NFBD1/KIAA0170 has been flagged as a G2/M-regulated transcript in the genome-wide microarray analysis of cell cycle-regulated transcription in HeLa S3 cells (40). Using cells synchronized by double thymidine block, we found that NFBD1 protein levels were low in S phase and higher in cell populations enriched for G2/M and G1 (Fig. 2A), consistent with the transcriptional profile.

A number of DNA damage-response proteins undergo regulated phosphorylation. Therefore, we sought to determine whether NFBD1 is phosphorylated during the cell cycle. HeLa cells were synchronized at the beginning of S phase by using a double thymidine block, and cell synchrony was monitored by flow cytometry of propidium iodide-stained cells. Mobility shift of NFBD1, consistent with phosphorylation, occurred 8 h after release from the thymidine block, coincident with a large subpopulation of G2/M cells, and was maintained until a shift to more rapid mobility 12 h after release, at which time G1 cells predominated, and continued in this form through S phase (Fig. 2A). Thus, NFBD1 is enriched and modified in G2/M cells.

NFBD1 Is Hyperphosphorylated in Response to the Spindle Checkpoint Activated by Nocodazole—Nocodazole is an antimotic agent that disrupts mitotic spindle function and arrests the cell cycle at G2/M (41). HeLa cells were arrested in mitosis by consecutive incubation with thymidine and nocodazole. Nocodazole blockade resulted in a supershift of NFBD1 to a form
**FIG. 1.** **NFBD1 is a chromatin-associated nuclear protein.** A, schematic diagram of NFBD1 structure and deletion mutants. The known structural domains, FHA and BRCT domains, are indicated by the striped and filled boxes, respectively. Solid bars mark repeats shown in Fig. 1B. Deletion mutants N1–N3, C1, and C2 were produced by restriction enzyme digestions. Restriction enzymes used were EcoRI and ApeI for N1, EcoRI and XhoI for N2, EcoRI and XbaI for N3, EcoRI and XhoI for C1, and BamHI and XbaI for C2. B, tandem repeats in NFBD1. Each repeat is shown as an individual solid bar in A. C, antibody specificity evaluated by immunoprecipitation (IP) and/or immunoblotting (IB). Cell lysates were extracted from HEK293 cells (lanes 1, 3, 4, 6, 8, 10, 11, and 13) or from HER293 cells transfected with HA-NFBD1 (lanes 2, 5, 7, 9, 12, and 14). Total lysates, not immunoprecipitates, are analyzed in lanes 6, 7, 13, and 14. The remaining lanes depict immunoprecipitations with the antibodies indicated. D, antibody specificity by immunoblotting after siRNA depletion of NFBD1. HEK cells were non-transfected (Untreated) or transfected with siRNA duplex against NFBD1 (siRNA) or with scrambled siRNA duplex (Scrambled) using OligofectAMINE. Transfectants were exposed to 5 Gy of irradiation 60 h after transfection. Non-transfected cells were treated with the same dose of γ-irradiation. Lysates were extracted at different time points after irradiation as indicated and analyzed by immunoblotting for NFBD1 and β-actin. E, antibody specificity in immunofluorescence. HEK293 cells were transfected with HA-NFBD1. Transfectants were seeded on poly-O-lysine-coated eight-chamber slides. F, nuclear localization of NFBD1. The experiment was performed as described for E, except that HEK293 cells were transfected with the deletion mutants of NFBD1/NFBD1/KIAA0170 in DNA Damage Signaling.
NFBD1 is modified in G2/M phase. A, HeLa cells were synchronized as described under “Experimental Procedures.” Cells were stained with propidium iodide. Cell cycle profiles were determined by flow cytometry. B, NFBD1 is hyperphosphorylated after IR or nocodazole block. Cell lysates were extracted from HeLa cells 1 h after 10 Gy of irradiation or nocodazole treatment for 18 h. Total lysates, not immunoprecipitates, are analyzed in lanes 5, 6, 7, and 12. Affinity-purified immunocomplexes using anti-NFBD1 antibody from mock-treated cells, irradiated cells, and nocodazole-treated cells were left untreated (lanes 1, 2, 8, and 9) or were mock-treated (lanes 3 and 10) or incubated with protein phosphatase (PPase; lanes 4 and 11). IB, immunoblotting; IP, immunoprecipitation; Noc, nocodazole; Asn, asynchronous cells; DT, double thymidine block.

A.

|   | 0 | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|
| Ch | G1 | G2 |
| 0  | 25.7 | 31.1 |
| 1  | 98.0 | 1.5 |
| 2  | 97.2 | 1.9 |
| 3  | 83.6 | 12.3 |
| 4  | 25.0 | 66.8 |
| 5  | 34.5 | 71.1 |
| 6  | 32.0 | 21.5 |
| 7  | 61.0 | 3.7 |
| 8  | 30.2 | 20.3 |
| 9  | 83.8 | 4.7 |
| 10 | 9.2 | 3.4 |
| 11 | 52.2 | 6.3 |
| 12 | 30.7 | 5.3 |
| 13 | 27.2 | 11.9 |
| 14 | 60.1 | 6.1 |

In ATM-deficient fibroblasts, IR-dependent retardation of HA-NFBD1 occurred with expression of wild-type, but not kinase-defective (ATMKD), ATM (Fig. 3B, lanes 6 and 8). When expressed in HEK293 cells and exposed to γ-irradiation, the HA-tagged N3 fragment, but not the other deletion mutants, was detected with anti-phospho-(S/T)Q antibody, which recognizes the phosphorylated (S/T)Q motif present in the ATM/ATR substrates (data not shown). These results indicate that NFBD1 is likely a substrate of ATM.

NFBD1 Forms Nuclear Foci in Response to DNA Damage or Replication Block—A number of proteins involved in DNA damage signaling and repair, including 53BP1 (28–30), BRCA1 (42), MRE11/RAD50/NBS1 (43), γ-H2AX (3, 5), and phospho-Thr68 CHK2 (44), localize and form subnuclear foci in the cells in response to DNA damage or replication blocks. In untreated HEK293 cells, HeLa cells, U2OS cells, and WI38 fibroblasts, anti-NFBD1 antibody staining yielded diffuse nuclear staining and few nuclear foci (Fig. 4 and data not shown). Numerous nuclear foci were apparent in all cells 2 h after irradiation at 5 Gy (Fig. 4). NFBD1 foci were also evident in HEK293 and HeLa cells 2 h after exposure to UV light (50 J/m2) or 24 h after hydroxyurea treatment (Fig. 4 and data not shown).

NFBD1 Colocalizes with H2AX, MRE11, and 53BP1—Histone H2AX becomes hyperphosphorylated in response to IR and forms discrete nuclear foci on sites of DSBs within 1 min (5). Several essential DNA repair factors, including BRCA1 (5, 8) and MRE11/RAD50 (5, 45), form foci that colocalize with that migrated significantly more slowly than the G2/M form detected with double thymidine block/release (Fig. 2A). NFBD1 returned to normal mobility 8 h after release from the nocodazole block (data not shown). Nocodazole-induced supershift of NFBD1 was due to phosphorylation because it was eliminated by treatment of NFBD1 with protein phosphatase (Fig. 2B, compare lanes 6, 7, and 9–11). Thus, NFBD1 is hyperphosphorylated in response to nocodazole blockade.

ATM Is Required for IR-induced Hyperphosphorylation of NFBD1—DNA damage activates a cascade of protein kinases and induces phosphorylation of many DNA damage-response and repair proteins (2, 17). Similarly, we found that endogenous NFBD1 in γ-irradiated HeLa and HEK293 cells had reduced electrophoretic mobility (Figs. 2B, lanes 2, 3, and 5; and 3A, lanes 2, 3, and 6). This retardation was due to phosphorylation because it was eliminated by treatment of immunoprecipitated NFBD1 with protein phosphatase (Figs. 2B, lane 4; and 3A, lane 4). It is noteworthy that phosphatase treatment of the hyperphosphorylated forms of NFBD1 induced by IR or nocodazole resulted in faster migration forms than in untreated cells (Figs. 2B, compare lanes 4 and 5 and lanes 11 and 12; and 3A, compare lanes 4 and 5). These results suggest that NFBD1 is basally phosphorylated. Ectopically expressed HA-NFBD1 in HEK293 cells was also phosphorylated after IR (data not shown).

The PIKK ATM is a central signaling protein in the response to IR and other sources of DSBs. We sought to determine whether NFBD1 phosphorylation after IR is ATM-dependent.

The resultant fractions were resolved by SDS-PAGE. S2, cytoplasmic fraction; S3, nuclear soluble proteins; P3, chromatin-enriched pellet.
NFBD1/KIAA0170 in DNA Damage Signaling

γ-H2AX. 53BP1, a mediator of the DNA damage checkpoint that is required for CHK2 activation in response to IR (31), also colocalizes with γ-H2AX. HEK293 cells induced NFBD1 foci that colocalized with H2AX foci within 2 h after IR (Fig. 5). This colocalization was also observed in HeLa and U2OS cells (data not shown). In BRCA1-deficient HCC1937 cells, spontaneous NFBD1 and H2AX foci were evident and colocalized (data not shown). As shown in Fig. 5, IR-induced NFBD1 foci colocalized with MRE11 and 53BP1 and partially with BRCA1. NFBD1 foci also colocalized with γ-H2AX after UV radiation or replication block by hydroxyurea treatment (Fig. 4).

**Dose Dependence and Time Course of NFBD1 Focus Formation**—H2AX focus formation on the DSB sites is one of the earliest events in response to forms of DNA damage that induce DSBs (3). The colocalization between the NFBD1 and H2AX foci suggested that NFBD1 is an early participant involved in DSB sensing and damage signaling. We determined the time course of NFBD1 focus formation in HEK293 cells in response to 5 Gy of irradiation (Fig. 6, A and B). Within 1 min, NFBD1 foci were visible and colocalized with the H2AX foci. NFBD1 foci peaked at 30 min and lasted at least 2 h after IR. NFBD1 foci significantly declined 4 h after IR. Although the majority of cells did not have a significant number of nuclear foci 12 h after irradiation, a small population of the cells (<20%) retained ~10–20 nuclear foci/cell. Throughout this time course, the number of H2AX foci was similar to that of NFBD1 foci, and the two foci were always colocalized. Similar kinetics of NFBD1 focus formation in response to γ-irradiation were observed in HeLa and U2OS cells.

Rapid focus formation and colocalization with H2AX foci...
suggest that NFBD1 may relocalize to the same DSB sites as H2AX after DNA damage. If this is the case, the number of NFBD1 foci should correlate with the dose of γ-irradiation. Indeed, as shown in Fig. 6 (C and D), with increased doses of γ-irradiation from 0.1 to 5 Gy, NFBD1 foci increased from several foci to >40 foci/cell. NFBD1 foci also colocalized with H2AX foci at all doses.

ATM Is Involved in NFBD1 Focus Formation—Having found that NFBD1 underwent ATM-dependent phosphorylation and formed discrete nuclear foci after IR, we determined whether NFBD1 focus formation requires ATM. The fungal phosphatidylinositol 3'-kinase inhibitor wortmannin inhibits the kinase activities of ATM and DNA-dependent protein kinase in intact cells, with half-maximal inhibition at concentrations of ∼5 μM. The kinase activity of ATR is significantly more resistant to this drug, with half-maximal inhibition at concentrations >100 μM (46). HEK293 cells were pretreated with various concentrations of wortmannin 1 h prior to exposure to 5 Gy of γ-irradiation. Cells were fixed and co-immunostained with anti-NFBD1 and anti-phospho-Ser139 H2AX antibodies 1 h after irradiation. Formation of both NFBD1 and H2AX foci was greatly impaired in the presence of 10 μM wortmannin, and this impairment was more severe with 50 μM wortmannin (Fig. 7). This is consistent with previous reports that wortmannin treatment inhibits H2AX focus formation in response to IR (5). We also observed that NFBD1 focus formation in DNA-dependent protein kinase-deficient cells was intact after γ-irradiation (data not shown). These results indicate that ATM is involved in NFBD1 focus formation in response to γ-irradiation.

DISCUSSION

We provide here the first identification of endogenous NFBD1 protein. We found that NFBD1 is heterogeneously expressed and modified during the cell cycle and that NFBD1 is phosphorylated in response to IR. Like many DNA damage-response proteins, NFBD1 relocates to sites of DNA damage upon irradiation. The phosphorylation and relocalization of NFBD1 in response to IR require ATM.

NFBD1 is modified, probably by phosphorylation, in the G2/M phase in a normal cell cycle and is hyperphosphorylated indicated times after irradiation; B, average number of NFBD1 foci/cell calculated by counting at least 40 cells/time point; C, immunofluorescence images of HEK293 cells exposed to 5 Gy of irradiation and co-immunostained with anti-NFBD1 and anti-phospho-Ser139 H2AX antibodies at the indicated times after irradiation; D, average number of NFBD1 foci/cell calculated by counting at least 40 cells/dose point.
after nocodazole treatment, which activates the spindle checkpoint (41). We speculate that NFBD1 may be involved in G_{2}/M checkpoint control, and it will be of interest to determine whether protein kinases activated by the spindle checkpoint are involved in NFBD1 regulation.

Central to all DNA damage-induced checkpoint responses is a pair of PIKKs: the ATM and ATR kinases (17, 47). The deployment of ATM _versus_ ATR depends upon the initiating DNA lesion. Although ATR is more important for the response to UV light, alkylating agents, and replication inhibitors, ATM is foremost in the response to IR. Proteins regulated directly by ATM are important in control of the G1, p53, CHK2, and MDM2 (25, 48–51), S (NBS1 and CHK2) (52–55), and G2 (BRCA1 and human RAD17) (56–58) checkpoint pathways. Identification of additional substrates of ATM will be critical for understanding checkpoint controls.

Taken together, our results strongly suggest that NFBD1 is a substrate of ATM. Phosphorylation and relocation of NFBD1 in response to IR depend upon ATM. NFBD1 was phosphorylated in response to IR (Figs. 2B and 3A). This phosphorylation was _ATM_-dependent (Fig. 3B). The PIKKs prefer to phosphorylate substrates at the (S/T)Q motifs (59, 60). There are 32 (S/T/Q) motifs present in NFBD1. However, we cannot at present rule out the possibility that _ATM_-dependent phosphorylation of NFBD1 is mediated indirectly by ATM-regulated kinases, including CHK1 and CHK2.

Recent studies have shed light on how damaged DNA is detected by the sensor complexes (47). In response to DNA damage, both the ATR-TRIP (ATR-interacting protein) kinase complex and the proliferating cell nuclear antigen-related RAD9-RAD1-HUS1 complex are independently loaded onto the chromatin at or near the sites of DNA damage (61–64). Loading of the RAD9-RAD1-HUS1 complex is RAD17-dependent (61). The interaction between RAD17 and ATM/ATR is DNA damage-inducible (57). Phosphorylation and relocation of H2AX are two of the earliest events after DNA damage (3–5) and are _ATM_-dependent (8, 65). Thus, it is plausible that H2AX is recruited by the ATR-TRIP and/or RAD9-RAD1-HUS1 complex to ATM/ATR kinases for phosphorylation. In turn, phosphorylated H2AX may mark the region of chromatin targeted for further assembly of signaling machinery and DNA repair. H2AX is critical in facilitating recruitment of signaling and repair factors such as 53BP1, MRE11/RAD50, and BRCA1 to the sites of DNA damage (9).

We have provided evidence that NFBD1, together with H2AX, relocates to DSBs after IR. First, NFBD1 is a nuclear protein that constitutively bound to chromatin (Fig. 1). Second, NFBD1 formed discrete nuclear foci after IR (Figs. 4–6), and the number of foci correlated with the dose of γ-irradiation (Fig. 6C). Third, NFBD1 foci colocalized with γ-H2AX and MRE11/RAD50 (Fig. 5), known DNA repair and damage signaling factors bound to DSBs after DNA damage. Fourth, NFBD1 focus formation occurred very rapidly (within 1 min) and peaked 30 min after IR (Fig. 6A). These kinetics are similar to those of γ-H2AX focus formation. Whether NFBD1 recolonization and focus formation, like 53BP1 (9), are dependent on the phosphorylation and relocation of H2AX is unclear. Knockdown of NFBD1 by siRNA in HeLa cells did not prevent or delay H2AX focus formation in response to IR (data not shown). Thus, H2AX functions either in parallel to or upstream of NFBD1.

Once NFBD1 is recruited to the sites of damaged DNA and is phosphorylated by upstream kinases such as ATM, NFBD1 may, in turn, recruit downstream effectors for activation. Such effectors may include CHK1 and CHK2. H2AX and 53BP1 foci colocalize with phospho-Thr68 CHK2 foci after DNA damage (44). This suggests that phospho-Thr68 CHK2 relocates to and binds to DSBs. Recently, 53BP1 has been shown to interact with CHK2 and to be required for CHK2 activation (31). A bacterially produced GST fusion of the first 337 residues of NFBD1 containing the FHA domain was capable of binding to bacterially produced wild-type CHK2 (which undergoes auto-phosphorylation (26)), but not to kinase-defective CHK2 (data not shown). These results suggest a link between NFBD1 and CHK2. Our preliminary results indicate that depletion of endogenous NFBD1 in HeLa cells by siRNA may reduce early phospho-Thr68 CHK2 after IR (data not shown). And, ectopic overexpression of the NFBD1 BRCT domains compromises production of phospho-Thr68 CHK2 foci after IR (66). Thus, timely IR-dependent CHK2 activation may be dependent on NFBD1.

In budding yeast, in response to DNA damage, CHK1 and CHK2 homologs are coupled to PIKK through the intermediary Rad9. Orthologs of Rad9 should ideally fulfill criteria of sequence homology, regulation through phosphorylation by PIKKs, and physical interaction with CHK proteins. These criteria are fulfilled by fission yeast Crb2/Rhp9, where the only extended sequence homology is in the C-terminal tandem BRCT domains. There are only three genes in the human genome encoding C-terminal tandem BRCT domains, which are BRCA1, 53BP1, and NFBD1. Both 53BP1 (28, 29) and NFBD1 (this study) are probable substrates of ATM. BRCA1 is a substrate of both ATM and ATR (58, 67). 53BP1 is required for CHK2 activation and is involved in S and G2/M checkpoint controls (31). BRCA1 regulates CHK1 activation in the G2/M checkpoint. BRCA1 is also required for the S phase checkpoint (42). Therefore, all three of these BRCT domain-containing proteins may serve overlapping functions analogous to those of budding yeast Rad9. However, information is still scant on the means through which these proteins affect CHK activity.

In summary, our data demonstrate that NFBD1 is a component of the early ATM/ATR response to DNA damage and localizes, along with several DNA damage-response proteins, to DNA damage foci. With the importance of DNA damage signaling and repair factors in genome stability and the intriguing chromosomal location of this gene, it will be interesting to determine whether NFBD1 is mutated in cancer, particularly cervical carcinoma.

Acknowledgments—We thank Michael Kastan and Takashiro Nagase for plasmids, Thanos D. Halazonetis for mouse monoclonal antibody against 53BP1, and JoAnn Falato for secretarial assistance. We thank Soo-Jung Lee for critically reading the manuscript and other members of the Stern laboratory for helpful comments.

REFERENCES

1. van Gent, D. C., Hoeijmakers, J. H., and Kanaar, R. (2001) _Nat. Rev. Genet._ 2, 196–206
2. Zhou, B. B., and Elledge, S. J. (2000) _Curr. Biol._ 10, 886–895
3. Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S., and Bonner, W. M. (1998) _J. Biol. Chem._ 273, 5658–5668
4. Chen, H. T., Bhandoola, A., Filippantoni, M. J., Zhu, J., Brown, M. J., Tai, X., Rogakou, E. P., Bresta, T. M., Bonner, W. M., Ried, T., and Nussenzweig, A. (2000) _Curr. Biol._ 10, 905–916
5. Feng, T. T., Rogakou, E. P., Yamanaki, V., Kirchgesner, C. U., Gellert, M., and Bonner, W. M. (2000) _Curr. Biol._ 10, 886–895
6. Camerini-Otero, R. D., Tessarollo, L., Livak, F., Manova, K., Bonner, W. M., and Nussenzweig, M. C. (2002) _Science_ 296, 922–927
7. Emili, A. (1998) _Cold. Cell_ 2, 183–189
8. Sanchez, Y., Desany, B. A., Jones, W. J., Liu, Q., Wang, B., and Elledge, S. J. (1996) _Science_ 271, 357–360
9. Sun, Z., Hsiao, J., Fay, D. S., and Stern, D. F. (1998) _Science_ 281, 272–274
