NFBD1, Like 53BP1, Is an Early and Redundant Transducer Mediating Chk2 Phosphorylation in Response to DNA Damage*

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Signaling pathways in response to DNA double strand breaks involve molecular cascades consisting of sensors, transducers, and effector proteins that activate cell cycle checkpoints and recruit repair machinery proteins. NFBD1 (a nuclear factor with BRCT domains protein 1) contains FHA (forkhead-associated), BRCT (breast cancer susceptibility gene 1 carboxyl terminus) domains, and internal repeats and is an early participant in nuclear foci in response to IR. To elucidate its role in the response pathways, small interfering RNA (siRNA) directed against NFBD1 in human cells demonstrated that its absence is associated with increased radio-sensitivity and delayed G2/M transition, but not G1 to S. NFBD1 associates with nuclear foci within minutes following IR, a property similar to histone H2AX, 53BP1, and Chk2, which are all early participants in the DNA damage signaling cascade. Temporal studies show that H2AX is required for the foci positive for NFBD1, but NFBD1 is not needed for 53BP1- and H2AX-positive foci. NFBD1, together with 53BP1, plays a partially redundant role in regulating phosphorylation of the downstream effector protein, Chk2, since abrogation of both diminishes phosphorylated Chk2 in IR-induced foci. These results place NFBD1 parallel to 53BP1 in regulating Chk2 and downstream of H2AX in the recruitment of repair and signaling proteins to sites of DNA damage.

Molecules participating in the DNA damage signal pathway can be classified as DNA damage sensors, proximal kinases, transducer kinases, and effectors (1–3). DNA damage sensors, which initiate the DNA damage signal cascade, are minimally characterized. γ-H2AX and the Rad9-Rad1-Hus1 (proliferating cell nuclear antigen-like) clamp complex were considered to be downstream of H2AX in the recruitment of Chk2 and its phosphorylation. These results place NFBD1 parallel to 53BP1 in regulating phosphorylation of the downstream effector protein, Chk2, therefore, to a property similar to histone H2AX, 53BP1, and Chk2, which are all early participants in the DNA damage signaling cascade.

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MATERIALS AND METHODS

Construction of Plasmids—The RNAi vector BS/U6 was kindly provided by Y. Shi (Department of Pathology, Harvard Medical School). Nucleotides sequences from 959 to 981 (GGGCTCAGGATTGGCT-TCAT) of NFBD1, sequence from 767 to 789 (GGGCTCAGGATTGGCT CGGCGG) of H2AX, or sequence from 1949 to 1971 (GGGTCTGAGGT-TGGAAAGATTCC) of 53BP1 were used for construction of the RNAi vectors. BS/U6:NFBDF1, BS/U6:H2AX, and BS/U6:53BP1, respectively. The RNAi expression cassette of NFBD1 was also inserted into an pcDNA3 vector that directs expression of GFP and was named pGFP/U6:NFBDF1.

Antibodies, Western Blotting, and Immunostaining—Mouse and rabbit anti-NFBD1 were generated as described previously (13). Mouse anti-53BP1 was generated against COOH-terminal 412 amino acids using standard procedures. Rabbit α-γ-H2AX was purchased from Upstate Biotechnology (Lake Placid, NY) and rabbit α-Chk2T68P was from Cell Signaling Technology (Beverly, MA). Rabbit anti-BS1 and anti-Chk2 antisera were purchased from GeneTex (San Antonio, TX). Immunoprecipitation, Western blotting, and immunostaining were performed as described previously (13, 18). Immunofluorescence images were captured using a Zeiss fluorescence microscope (Zeiss, Axiplan2).

Cell Culture and Treatments with Irradiation—MCF7, a human breast carcinoma cell line, was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine,

1 The abbreviations used are: ATR, ataxia telangiectasia and Rad3-related; ATM, ataxia telangiectasia mutated; NFBD1, a nuclear factor with BRCT domains protein 1; BRCT, breast cancer susceptibility gene 1 carboxyl terminus; siRNA, small interfering RNA; 53BP1, tumor suppressor p53 binding protein 1; DSBR, double strand break; γ-H2AX, phosphorylated H2AX at serine 139; Chk2T68P, phosphorylated Chk2 at the Thr68 residue (Thr68-P); BrdUrd, bromodeoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; IR, irradiation; IRIF, IR-induced foci; Gy, gray; GFP, green fluorescent protein.

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NFBD1 Mediates ATM-dependent Checkpoint Pathway

Fig. 1. Elimination of NFBD1 expression affects IR sensitivity and G2/M checkpoint. A, Western blot assay of the expression of NFBD1 in MCF7 cells transfected with plasmid (GFP/U6:NFBD1), which directs synthesis NFBD1 siRNA. Lane 1, MCF7; lane 2, MCF7 transfected with control vector, pCDNA3/GFP. Lanes 3 and 4, MCF7 transfected with GFP/U6:NFBD1 for 24 h (lane 3) and 48 h (lane 4). Lane 5, GFP/U6:NFBD1-transfected MCF7 cells were selected with 800 µg/ml G418 for 2 weeks and labeled as RNAi-selected in B. Lane 6, G418-resistant MCF7 cells were transfected with pBS/U6/NFBD1 and labeled as RNAi/NeoF/RNai in B. Cell extracts were separated by SDS-PAGE and then immunoassayed with antibodies to NFBD1 (upper panel) and p84 (lower panel). B, immunostaining analysis of NFBD1 expression in MCF7 cells transfected with control vector or GFP/U6: NFBD1. Cells were fixed and stained with purified rabbit anti-NFBD1. Panels identified as NFBD1 RNAi represent immunofluorescence staining with anti-NFBD1, and GFP represents GFP-expressing cells. DAPI indicates cells stained with 4',6-diamidino-2-phenylindole. C, colony formation assay for IR sensitivity. NFBD1 RNAi-transfected or empty vector-transfected cells were irradiated with the indicated doses of IR, and the surviving cells were scored as colonies. D and E, IR-induced G2/M checkpoint was examined by counting mitotic phase cells either at the indicated hours after irradiation with 12 Gy (D) or at 2 h after the cells were irradiated with indicated dose (E). F, G2/M checkpoint was done by a BrdUrd incorporation assay to monitor S phase entry using NFBD1 RNAi transfected or empty vector controls.

50 units of penicillin, and 50 µg/ml streptomycin at 37 °C with 10% CO2. Cells grown in log-phase were irradiated in a 137Cs radiation source (Mark I, model 68A Irradiator, JL Shepherd & Associates, CA). The medium was replaced immediately after irradiation. All the cells were then cultured at 37 °C and harvested at the indicated time points.

Colony Formation Assay—MCF7 cells transfected and selected with RNAi vectors or empty vector were counted and plated into 10 cm plates. After cell attachment (14 h), the cultures were treated with irradiation at the indicated doses or mock-treated (0 Gy) and then incubated for 14 days. After colony formation, cells were fixed and stained with 2% methyl blue in 50% ethanol. Colony formation was determined by counting a colony with >50 cells. Averages and S.D. values were determined from triplicates. Treated sample percentages were determined by dividing their plating efficiency by the appropriate mock-control.

G2/M Checkpoint Assay—Cells were plated on cover glasses in 35-mm plates. After 24 h, cultures were either mock-treated or irradiated with 12 Gy of IR and then returned to the incubator. After 24-h incubation, fresh medium containing 10 mM BrdUrd was added and cultured an additional 3 h. Cells were then washed once with PBS and fixed for BrdUrd immunostaining using a cell proliferation kit (Amersham Biosciences). BrdUrd-positive cells were scored by fluorescence microscopy and expressed as a fraction of the total cells.

Fig. 2. Factors recruitment in early DNA damage responses to DSB. A, kinetics of IR-induced foci formation positive for factors involved in DNA damage responses. MCF7 cells were exposed to 1 Gy IR and kept in culture for the indicated times. Treated cells were immunostained with antibodies specific for each the proteins indicated, and the percentage of the cell nuclei containing more than five foci was calculated. B–F, upper panels are mock-treated cells, and lower panels are cells treated with NFBD1 RNAi (B and D) or H2AX RNAi (C) or 53BP1 RNAi (E). The percentage of cells either RNAi-transfected or empty vector controls with more than five nuclear foci in each cell was counted and the percentage indicated in the corresponding representative photomicrograph.
RESULTS AND DISCUSSION
Elimination of NFBD1 Expression Affects IR Sensitivity and G2/M Checkpoint—Our initial work on NFBD1 (nuclear factor that contains BRCT domains 1, KIAA0170) showed that it forms IRIF within 2 min after exposure. Expression of NFBD1-derived BRCT domains compromised association of both Thr68-phosphorylated Chk2 (Chk2T68) and γ-H2AX within IRIF (13). NFBD1 phosphorylation in response to ionizing IR is mediated by ATM (14). Together, these data suggest the involvement of NFBD1 in early cellular responses to DNA damage. To investigate the precise role of NFBD1 in DNA damage response signaling pathway, we generated a vector-based RNAi construct (19) for the in vivo expression of a 22-base pair RNA duplex that targets NFBD1. The human breast cancer cell line, MCF7, transfected with the NFBD1 siRNA construct demonstrated significantly reduced expression of NFBD1 protein about 50–60 h after transfection (Fig. 1B, compare lane 1 with lanes 3 and 4). In a population of G418-selected cells expressing siRNA, NFBD1 was nearly undetectable (Fig. 1B, lanes 5 and 6), indicating effective repression by this approach. Using a GFP/NFBD1 siRNA dual expression plasmid, it is apparent that NFBD1 immunofluorescence is inversely correlated with GFP fluorescence, which is not observed using control vectors (Fig. 1B, compare rows labeled RNAi, RNAi/Neo+, and RNAi/Neo+/RNAi with vector).

Inactivation of genes essential for DNA damage signal transduction, including ATM, H2AX, BRCAP1, Chk2 and p53, results in IR sensitivity (see review in Ref. 20). Because NFBD1 is an early participant in this pathway (13), we then examined IR sensitivity in MCF7 cells expressing NFBD1 siRNA. As shown in Fig. 1C, NFBD1 RNAi-transfected cells, but not empty vector, formed significantly less colonies upon IR exposure. Similar results were also observed in human HeLa and osteosarcoma U2OS cells (data not shown). To test for the possibility

Fig. 3. Elimination of NFBD1 expression associates with reduced phosphorylation Chk2 at Thr68 in response to IR. A, NFBD1 RNAi or empty vector control MCF7 cells were irradiated with 16 Gy. Cell lysates were processed at the indicated times, separated by SDS-PAGE, and then immunoblotted with antibodies to NFBD1 (top panel), Chk2T68 (second panel), Chk2 (third panel), and p84 (bottom panel). B, Chk2 phosphorylation at Thr68 in response to different dose of IR. NFBD1 RNAi-transfected or empty vector control MCF7 cells were irradiated with the indicated doses of IR. Cell lysates were prepared 1 h post-irradiation and processed as described in the legend to A. C, Chk2 was reciprocally co-immunoprecipitated with NFBD1. MCF7 were mock-treated (−) or treated with IR (10 Gy) (+) and harvested after 2 h. Cell extracts were immunoprecipitated with preimmune sera (Pre, lane 2), anti-NFBD1 (lanes 3 and 4), or anti-Chk2 (lanes 5 and 6) antibodies and analyzed by Western blot probed with anti-NFBD1 (top panel) and anti-Chk2 (second panel) antibodies. One-tenth of the cell extracts was directly used for Western blot (lane 1), serving as references.

Fig. 4. NFBD1, like 53BP1, redundantly regulates CHK2. A, empty vector control, H2AX RNAi, NFBD1 RNAi, 53BP1 RNAi, and NFBD1–53BP1 double RNAi-transfected cells were irradiated with 8 Gy. At 2 h post-irradiation, cells were immunostained with anti-Chk2T68p antibody. The percentage of the cells with more than five foci was calculated. Arrows indicate RNAi-targeted cells. C, a model for the NFBD1 DNA damage checkpoint signaling pathway. In response to DSBs, NFBD1 and 53BP1 both play a central role in transducing DNA damage signals to Chk2.

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that IR sensitivity results from defects in checkpoint control, NFBD1 RNAi-transfected MCF7 cells were tested for G1/S or G2/M checkpoint control. In both temporal and dose-response protocols (Fig. 1, D and E, respectively), NFBD1 RNAi-transfected MCF7 cells were partially defective in G1/M checkpoint, while the G1/S checkpoint remains intact (Fig. 1F).

NFBD1 Association with IR-induced Foci Has Kinetics Similar to γ-H2AX and 53BP1—Previous studies showed that the DNA damage response factors, γ-H2AX, RAD51, 53BP1, and CHK2 form IRIF at sites of DSB (Review in 20). The kinetics of recruitment to DSBR sites is useful in exploring their functional relationship. As high dose IR treatment may mask the kinetics of early IRIF formation as reported previously (13), cells were irradiated with 1 Gy, and IR-induced nuclear foci were scored. Similar to γ-H2AX and 53BP1, the percentage of cells with NFBD1-positive IRIF increased immediately after IR treatment and diminished quickly after 60 min (Fig. 2A), implying that these proteins have nearly identical kinetics of foci association and participate at the early stages of DSB response. In contrast, CHK2T68-positive IRIF peaked within 30 min after IR, remained at high levels for 6 h, after which they slowly diminished (Fig. 2A), suggesting that phosphorylation of Chk2T68 and/or its association with IRIF is a later event compared with that of NFBD1, 53BP1, or γ-H2AX. RAD51- and BRCA1-positive foci were detected at a much later stage under conditions of low dose IR exposure (Fig. 2A).

NFBD1 Is Downstream of γ-H2AX and Parallel to 53BP1 in Early Signal Transduction Hierarchy—To determine the relationship of NFBD1, 53BP1, and γ-H2AX in early responses to DSB, cells transfected with plasmids directing expression H2AX, NFBD1, or 53BP1 siRNA were scored for IRIF positive for each protein. In the H2AX RNAi-transfected cells, NFBD1, similar to 53BP1 (16), was not detected at IRIF (Fig. 2C). In the 53BP1 RNAi-transfected cells, the presence of NFBD1 in IRIF was unchanged (Fig. 2E). Conversely, in NFBD1 RNAi-treated cells, IRIFs positive for 53BP1 and γ-H2AX were not altered (Fig. 2, B and D). These results suggest that H2AX is upstream and is required for the recruitment of NFBD1 and 53BP1 into the DSB signal transduction cascade. However, association of NFBD1 and 53BP1 with DSB may represent two independent events downstream of H2AX.

NFBD1 and 53BP1 Regulate Chk2 Redundantly—The function of NFBD1 may serve as an adaptor protein similar to the part played by yeast Rad9 for Rad53, the human homologue of Chk2. To directly test whether NFBD1 has a role in regulating Chk2 phosphorylation of Thr342 in cells transfected with NFBD1 RNAi was assayed by Western blotting using phosphorylation state-dependent antibodies. As shown in Fig. 3, A and B, the immunoreactivity detected with these antibodies was partially reduced relative to total Chk2 protein. These data are comparable with the reduced level of Chk2T28 phosphorylation observed in 53BP1 RNAi-transfected cells (15–17). Immunoprecipitation of 53BP1 could efficiently bring down Chk2 (15). This observation suggested that 53BP1 might act as an adaptor that facilitates Chk2 phosphorylation. Similarly, Chk2 can be reciprocally co-immunoprecipitated with NFBD1 (Fig. 3C). Taken together, these results suggest that NFBD1 binds to Chk2 and mediates its phosphorylation.

To delineate the signaling pathway leading to Chk2 phosphorylation, Chk2T68-positive IRIF in cells transfected with H2AX, 53BP1, and NFBD1 RNAi expression plasmids were scored. A consistent observation is that H2AX is essential for Chk2T68 association IRIF. However, in 53BP1 or NFBD1 RNAi-treated cells, Chk2T68-positive foci were only partially reduced (Fig. 4, A and B). Interestingly, in 53BP1 and NFBD1 RNAi double transfected cells, CHK2T68-positive IRIF was reduced to a level comparable with H2AX RNAi-transfected cells (Fig. 4, A and B). These results suggest that NFBD1 and 53BP1 redundantly regulate the participation of the phosphorylated Chk2T28 in IR-induced foci.

In response to DSBRs, NFBD1 and 53BP1 both appear to play a central role in transducing DNA damage signals to downstream effectors by serving perhaps as adaptor proteins connecting the upstream signal from γ-H2AX to the downstream effector Chk2 (Fig. 4C). This model is consistent with the role of Rad9 function in budding yeast. In yeast, Rad9 is an adaptor protein that plays a central role in transducing and amplifying DNA damage signals by recruiting transducer kinase Rad53 to DNA sites of double strand breaks (7, 8). Chk2 is a major target of ATM, which phosphorylates threonine 68 to activate its kinase activity in response to DNA damage. Activated Chk2, in turn, phosphorylates p53 at Ser20, CDC25A at Ser123, and CDC25C at Ser216, contributing to the G1/S, S, and G2/M checkpoint (see review in Ref. 21). The critical question of how adaptor proteins help mediate activation of Chk2 at DSBR sites remains to be solved. Our results showing redundancy of NFBD1 and 53BP1 in regulating the recruitment of Chk2T68 to DNA DSBR sites probably underscores the importance of this step in mammalian cells. Because of the dissimilarity of their structures (9, 10), it is anticipated that NFBD1 and 53BP1 may also function in different branching pathways.

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