NFBD1/Mdc1 Mediates ATR-Dependent DNA Damage Response

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Abstract

Budding yeast Rad9 (scRad9) plays a central role in mediating Mec1-dependent phosphorylation by recruiting its downstream substrates. The human scRad9 orthologues 53BP1 and NFBD1 associate with ionizing radiation-induced foci (IRIF) at sites of DNA repair. RNAi-based gene silencing of 53BP1 or NFBD1 has shown impaired phosphorylation of SQ/TQ [ataxia-telangiectasia mutated/ATM and Rad3-related (ATM/ATR) substrates] at IRIF, intra-S, and G2-M checkpoints and has thereby revealed essential roles for 53BP1 and NFBD1 in the DNA damage signaling pathway. Whether 53BP1 and NFBD1 are required for activation of kinases and/or for recruitment of substrates at IRIF, however, is not clear. Here we show that both 53BP1 and NFBD1 are required for recruitment of ATR to DNA damage sites, as well as for ATR-dependent phosphorylation in response to DNA damage. NFBD1 is not required for ssDNA generation at DNA damage sites and is not recruited by replication protein A (RPA; ref. 3).

Introduction

Cellular response to DNA damage is critical to maintain genomic stability. When a double-strand break (DSB) occurs, the cell activates its checkpoint machinery to halt cell cycle progression and allow proper DSB repair (1). Without such response and repair, the cell will eventually undergo apoptosis or pass on its altered DNA to daughter cells. Checkpoints are enabled through sensing DNA damage, transducing damage signals, and activating effectors. Mechanisms by which DNA damage signals are initiated have begun to be revealed (1, 2). PI-3–like kinases, including ataxia-telangiectasia mutated (ATM), ATM and Rad3-related (ATR), and DNA-PK are believed to play central roles (3). ATM is mutated (lost or inactivated) in the human genetic disorder ataxia-telangiectasia and could be the primary kinase responsible for DSB signaling transduction. Structural chromatin changes activate ATM, which then phosphorylates itself (4). Activated, autophosphorylated ATM in turn phosphorylates the checkpoint kinases Chk1 and Chk2 to arrest cell cycle progression. It also phosphorylates other proteins, including p53, BRCA1, 53BP1, NBS1, TopBP1, 53BP1, and replication protein A (RPA; ref. 3).

The molecular pathways by which ATR functions in the mammalian DNA damage response pathway have not been studied as extensively as those involving ATM. Studies using kinase dead ATR or conditional knockout strategies to mutate ATR in murine cells revealed that ATR shares some substrates with ATM and is required for optimal phosphorylation of H2AX, Rad17, Chk1, Chk2, p53, and BRCA1 in response to DSBs (3). Emerging evidence has suggested, however, that despite their structural similarities and common substrates, ATM and ATR may play different and distinct parts in the DSB response pathway. ATM and ATR may cooperate in ionizing radiation (IR)–induced G2-M checkpoint control, but ATM seems to be more dominant at the early stage of checkpoint activation and ATR seems to contribute mainly to sustained checkpoint events. The mechanisms through which ATR is regulated and exactly how ATR-dependent phosphorylation events occur in response to DSBs have not yet been delineated.

In budding yeast, recruitment of Mec1, the orthologue of ATR, into sites of cdc13-induced DNA damage or HO endonuclease–induced DSBs was studied by chromatin immunoprecipitation or through examination of IR-induced nuclear foci formation with green fluorescent protein (GFP)–tagged proteins (5, 6). These studies showed that Mec1 association with DSB sites is dependent on Ddc2/Lcd1 but not on the Rad17 complex. Similarly, in an in vitro system, the association of ATR with RPA-coated ssDNA is dependent on ATR-interacting protein, an interacting partner (7). The recruitment of ATR into DNA damage sites seems to be the major regulatory mechanism that facilitates ATR-dependent phosphorylation.

Budding yeast Rad9 (scRad9) plays central role in mediating Mec1-dependent phosphorylation by recruiting downstream Mec1 substrates (8, 9). Because of their structural similarity, 53BP1 and NFBD1 are felt to be human orthologues of scRad9. 53BP1 was identified as a protein binding to the tumor suppressor gene product p53; it associates with IR-induced foci and functions upstream of BRCA1 in the DNA damage response pathway (10). NFBD1, also called MDC1, also participates in the DNA damage response pathway; it mediates Chk2 activation and NBS1-dependent IR-induced foci (IRIF; ref. 11–15). Silencing of either 53BP1 or NFBD1 expression using RNAi-based methodology results in a similar phenotype: impaired phosphorylation of SQ/TQ (ATM/ATR substrate) at IRIF, intra-S, and G2-M checkpoints (16–19). These studies revealed the essential roles of 53BP1 and NFBD1 in mediating DNA damage signaling. Finally, RPA-coated ssDNA represents a common structure in the DNA damage response pathway and could recruit downstream factors, including ATR (7) and Rad17 complexes.

In this study, we present novel evidence to show that, in addition to ssDNA and RPA proteins, both 53BP1 and NFBD1 are required for recruitment of ATR into IR-induced foci, as well as for ATR-dependent phosphorylation in response to IR-induced DNA damage. NFBD1 is not required for ssDNA generation at DNA damage sites, however, and is not recruited by RPA-coated ssDNA.
ssDNA. Thus, the generation of ssDNA and the recruitment of NFB1 and 53BP1 by γ-H2AX are independent events, both of which are required for downstream effects in the ATR-dependent pathway.

Materials and Methods

Construction of Plasmids. The RNAi expression cassettes were generated making use of the U6 promoter and specific nucleotides sequences from NFB1 and 53BP1 as previously described (12). A similar construct with nucleotides 410 to 432 (GGGCCAGCCGCAAGTAGCTCCTC) was used to silence RPA70 expression. The NFB1 RNAi construct was integrated into pEGFP, which allowed selection of single cell clones after transfecting MCF7 cells and culturing them in the presence of G418 (250 μg/mL).

Antibodies and Western Blotting. Mouse or rabbit anti-NFB1 and anti-53BP1 were generated as previously described (11, 12). Anti-Rad17, RPA32, and RPA70 were purchased from Oncogene Research Products (Boston, MA), anti-Rad17S645 and Chk1S345 from Cell Signaling Technology (Beverly, MA), and anti-ATR and Chk1 from Santa Cruz Biotechnology (Santa Cruz, CA). Western blotting was done as previously described (20).

Immunofluorescence and Microscopy. Procedures for immunostaining were adapted from Durfee et al. (21). Briefly, cells were cultured on cover glasses, fixed in 4% formaldehyde and 0.1% Triton-X 100, and permeabilized in 0.5% saponin before immunostaining with respective antibodies. Immunofluorescence images were captured using a Zeiss Axioplan2 fluorescence microscope.

Cell Culture and Treatments. MCF7 and HeLa cell lines, were maintained in high glucose DMEM supplemented with 10% fetal bovine serum, 2 mmol/L t-glutamine, 50 units of penicillin, and 50 μg/mL of 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, San Fernando, CA). The medium was replaced immediately after irradiation. All the cells were then cultured at 37°C and harvested at the indicated time points. Cells used for chromatin association assay were treated with aphidicolin (50 μg/mL). The experiments using transient transfection of RNAi plasmids were carried out following our published protocol (12).

Chromatin-Enriched Fractionation. To isolate chromatin, cells were lysed and fractionated as described (22). Approximately 2 × 10^6 cells were washed with PBS and resuspended in 200 μL buffer A [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 1.5 mmol/L MgCl2, 0.34 mol/L sucrose, 10% glycerol, 1 mmol/L DTT, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 0.5 μg/mL pepstatin A, and 0.1 mmol/L phenylmethylsulfonyl fluoride]. Triton X-100 (0.1%) was added, then the cells were mixed gently and incubated on ice for 5 minutes. Nuclei were collected in pellet 1 (P1) by centrifugation at low speed (1,300 × g, 5 minutes, 4°C). Nuclei were then washed once in buffer A, resuspended in 200 μL buffer B (3 mmol/L EDTA, 0.2 mmol/L EGTA, 1 mmol/L DTT, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 0.5 μg/mL pepstatin A, and 0.1 mmol/L phenylmethylsulfonyl fluoride) and incubated on ice for 30 minutes. Insoluble chromatin was collected by centrifugation (1,700 × g, 5 minutes, 4°C) and washed in buffer B, followed by centrifugation at 1,700 × g for 5 minutes. The final chromatin-enriched pellet (P3) was resuspended in SDS loading buffer and subjected to brief sonication.

Results

Both 53BP1 and NFB1 Are Required for Ionizing Radiation–Induced ATR Foci Formation. ATR activation is critical for the response to DNA damage characterized by DSBs. In mammalian cells, ATR is recruited into IR-induced DSB sites by RPA-coated ssDNA, like its yeast orthologue. It is also dependent on ATR-interacting protein (7). Besides the generation and modification of ssDNA, DSBs produce other key structural features: DSB ends, which can be recognized by the Ku component of the DNA-dependent protein kinase complex (23), and phosphorylation of H2AX, which may highlight the damaged chromatin (24, 25). NFB1 and 53BP1 associate with γ-H2AX and are required for the accumulation of many proteins into IRIF (11–19). To delineate the relationship between these different stages of the DNA damage response, we analyzed the presence of ATR IRIF in cells treated with NFB1 or 53BP1 RNAi. ATR association with IRIF was reduced in cells with either 53BP1 or NFB1 expression silenced (Fig. 1A). These results suggest that NFB1 and 53BP1 may both function upstream of ATR and perhaps recruit ATR to DNA damage sites.

Both 53BP1 and NFB1 Are Required for ATR-Dependent Phosphorylation. Immunostaining experiments using phospho-SQ/TQ antibodies, which specifically recognize phosphorylated ATM/ATR substrates, have shown that these phosphorylations are increased upon IR treatment and that ATM/ATR-phosphorylated proteins colocalize at DSB sites (17). RNAi-based silencing of NFB1 or 53BP1 expression reduces these ATM/ATR–dependent phosphorylation signals at DSB sites (12–19), and therefore suggests that NFB1, 53BP1, or both affect an ATM-dependent signaling pathway.
Because we showed that NFBD1 and 53BP1 are required for ATR to associate with IRIF, we next asked whether the ATR-dependent signaling transduction in DNA damage sites is also dependent on NFBD1 and/or 53BP1. Although it is redundant in many respects with ATM, ATR has been shown to be the major responsible kinase for the phosphorylation of Rad17 at Ser635 and Ser645 in response to DNA damage. These Rad17 phosphorylation events contribute to IR-induced checkpoint activation (26, 27). Interestingly, we found that the phosphorylated Rad17S645 signal increased at DSB sites after IR treatment of control cells, but did not similarly increase when 53BP1 or NFBD1 expression was reduced significantly by RNAi (Fig. 1B). These results indicate that NFBD1 and 53BP1 are required for ATR-dependent signal transduction at DSB sites.

This reduction in phosphorylated Rad17 signal at IRIF in cells with NFBD1 and 53BP1 expression reduced is consistent with previous reports showing that NFBD1 and 53BP1 are required for ATM/ATR-dependent signal transduction at DNA damage sites (17, 19). To carefully and unambiguously determine the requirement for NFBD1 or 53BP1 in recruitment of ATR to damaged DNA sites or in ATR-dependent phosphorylation, we needed cells that reliably harbor the appropriate RNAi-cassette and that completely silence expression of either NFBD1 or 53BP1. Because cells with NFBD1 knocked down considerably were capable of forming colonies, we determined that NFBD1 is not required for cell survival. Indeed, we were able to isolate an MCF7 cell clone (M4) that stably expresses NFBD1 RNAi and reduces NFBD1 protein to an undetectable level by Western blotting analysis (Fig. 2A).

Next we asked whether NFBD1 is required for ATR-dependent phosphorylation events. Although it shares other substrates with ATM, ATR is primarily responsible for Rad17 and Chk1 phosphorylation for genotoxic stress responses (22, 26–30). We were therefore able to use antibodies recognizing specifically phosphorylated forms of Rad17 and Chk1 to look at ATR-dependent phosphorylation (Fig. 2B, 2C). In the M4 cell clone with completely silenced NFBD1 expression, IR-induced, ATR-dependent phosphorylation of Rad17 (Fig. 2B) and Chk1 (Fig. 2C) was abolished. Based on these observations, we speculated that NFBD1 might bring ATR into DNA damage sites, where it meets and phosphorylates its substrates. According to this model, failure of ATR-dependent phosphorylation upon IR treatment in cells with no NFBD1 would result from deficiency in recruitment of ATR. Alternatively, NFBD1 may be required for ATR-dependent phosphorylation in response to IR.

Figure 2. NFBD1 is required for ATR-dependent phosphorylation in response to IR. A cell clone (M4) constitutively expressing the NFBD1 RNAi construct was isolated and the expression of NFBD1 protein was examined by Western blot analysis with or without IR treatment (A). The cells with NFBD1 expression effectively silenced were treated with 20 Gy IR and harvested 3 hours later. Parental MCF7 cells served as controls. ATR-dependent phosphorylation of Rad17 on S645 (B) and phosphorylation of Chk1 on S345 (C) were detected by Western blotting with phospho-specific antibodies. Lanes 1 and 2: MCF7 cells, lanes 3 and 4: M4 cell clone stably expressing NFBD1 RNAi. Expression of Rad50 (D) served as a loading control.
for ATR kinase activity. The basal level of Rad17 phosphorylation in undamaged cells may correlate with Rad17 S phase functions. Note that this basal phosphorylation is also dependent on NFBD1 (Fig. 2B, compare lanes 1 and 3), which suggests that NFBD1 also plays a role in S phase progression, perhaps as a replication checkpoint control.

**NFBD1 Is Not Responsible for ssDNA Generation at DNA Damage Sites.** ssDNA might be a common structure in response to DNA damage, and generation of ssDNA is critical for homologous recombination repair and signal transduction. How DNA DSBs are processed to ssDNA, however, is not known. The Nbs1/Mre11/Rad50 complex is purported to have a role in ssDNA generation because of its biochemical activities, and NFBD1 associates with this NBS1 complex to mediate its recruitment into the DSBs signaling pathway. We therefore asked whether NFBD1 is required for ssDNA generation following DSBs and indispensable for downstream events in the ATR pathway.

ssDNA but not other structures recruit Rad51 and RPA into foci of damaged DNA. In response to damage, Rad51-ssDNA filaments mediate ssDNA invasion and enable homologous recombination (31). RPA coating stabilizes ssDNA and recruits the ATR and Rad17 complexes (7, 32). Thus, staining for Rad51 and RPA is a measure of ssDNA generation. To clarify the possible role of NFBD1 in ssDNA generation, we scored cells for the ATR and Rad17 complexes (7, 32). These results suggest that NFBD1 is not required for ssDNA generation from DSBs.

**Recruitment of NFBD1 into IRIF Is Independent of RPA-Coated ssDNA.** RPA stimulates the loading of both Rad17 and ATR-ATR-interacting protein complexes onto ssDNA, underlining the important role of RPA in initial damage recognition. We have described earlier that ssDNA generation and RPA recruitment are intact in the absence of NFBD1 and thereby placed these events upstream or parallel to NFBD1 recruitment. To delineate the relationship among RPA, NFBD1, Rad17, and ATR complexes in the early response to DNA damage, we reduced RPA70 subunit expression by vector-based RNAi. The vector also contains a GFP expression cassette to mark cells that have been successfully transfected with the RNAi construct. We noted that RPA70 expression inversely correlates with the presence of GFP signal (Fig. 3B). Moreover, and consistently, elimination of RPA70 impaired the association of ATR with IRIF. Elimination of RPA70 from individual cells also eliminated Rad51 from foci. Thus, although in vitro association of Rad51 with ssDNA is direct, *in vivo* accumulation of Rad51 at ssDNA sites depends on RPA. Our data also show that the association of NFBD1 with IRIF is not RPA dependent and therefore separate histone-dependent NFBD1 recruitment and ssDNA-RPA into two independent pathways.

**NFBD1 Is Required for ATR But Not Rad17 or RPA to Associate with Chromatin.** Because aberrant DNA structure by induced damage or during replication recruits DNA damage response factors, it is reasonable to suggest that DNA damage causes enhanced chromatin binding of response factors like Rad17 or RPA. We speculated that IR treatment produces ssDNA, which in turn recruits RPA, Rad17, and ATR onto chromatin. The association of chromatin with these factors should then parallel their recruitment into IRIF.

To explore this hypothesis, we isolated the chromatin-associated nuclear fractions from cells with or without IR treatment. As did previous reports (22, 33), we found increased amounts of ATR, RPA32, and Rad17 in chromatin-enriched fractions but not in whole cell extracts after IR (Fig.4). Interestingly, NFBD1 RNAi treatment abolished DNA damage-induced chromatin accumulation of ATR, but had no effect on the chromatin accumulation of RPA32 or Rad17. Elimination of NFBD1 also impaired the chromatin association of ATR in the cells treated with either UV radiation or aphidicolin. Our results therefore strongly suggest that NFBD1 is required for the association of ATR with chromatin after DNA damage or replication stress, but that it is not required

### Figure 4
NFBD1 is required for ATR, but not Rad17 or RPA, to associate with chromatin. NFBD1 RNAi expressing M4 or parental MCF7 cells were mock treated or treated with IR, UV, or aphidicolin, then harvested for whole cell extracts. In each condition, chromatin-enriched fractions were also isolated and examined. NFBD1, Rad17, RPA32, ATR, and Orc2 were detected by Western blotting and their relative abundance was quantified by densitometry. Un, untreated; APH, Aphidicolin. Lanes 1-8, whole cell extract; lanes 9-16, chromatin-enriched fraction; lanes 1-4 and 8-12, extracts from MCF7 cells; lanes 5-8 and 13-16, extracts from NFBD1 RNAi expressing clone M4.
Thus, recruitment of Rad17 and RPA to the DSBs is independent of ATR. Our results further indicate that the RPA-coated ssDNA generation is not sufficient to provoke ATR activation in vivo, and that NFBD1 is required for the ATR-chromatin association, which is critical for bringing ATR in proximity to its substrate. Moreover, our analysis also revealed that in cells without any NFBD1, phosphorylation of RPA is impaired, whereas its association with chromatin in response to DNA damage is not.

Discussion

Proteins containing tandem BRCT domains, such as scRad9, human 53BP1, NFBD1, and BRCA1, have been shown to play roles in mediating DNA damage responses. The detailed mechanisms by which these proteins mediate phosphorylation and activation of the downstream factors, however, are not well understood. The current model combining consistent findings from yeast to human cells suggests that these proteins containing BRCT domains associate with downstream factors and recruit them into DNA damage sites, where they are proximal to and phosphorylated by ATM/ATR. Data presented here, however, has shown that besides recruiting downstream factors, NFBD1 (and/or 53BP1) are responsible for the recruitment of ATR kinase into DNA damage sites. These findings reveal a new role for BRCT domain-containing proteins in transducing or coordinating the DNA damage response. Insights from structural studies will be helpful to address further questions, such as how these proteins serve as adaptors to recruit kinase and substrates, and whether the BRCT domains are directly involved.

NFBD1 and 53BP1 are potential orthologues of scRad9. In contrast to our studies in human cells, other studies in yeast have shown that Rad9 is not required for the association of Mec1, the ATR orthologue, with DNA damage sites (5). This apparent inconsistency could be produced by different experimental procedures to introduce DNA damage and to score the resultant IRIF. Alternatively, the role of Mec1/ATR may be very different in yeast when compared with its role in human cells. In yeast, Mec1, not Tel1/ATM, primarily controls the DSB responses, including Rad53 phosphorylation and checkpoint activation. In human cells, however, ATM is the major kinase in response to DSBs (1,3). ATR contributes to sustained G2-M DNA damage checkpoint activation, possibly through Chk1 phosphorylation, although it is also dominant in controlling UV-induced and replication-related DNA damage responses. Notably, NFBD1 is also reported to mediate the ATR-dependent signaling pathway in response to UV treatment (15).1

Fundamental questions about exactly how a cell senses DNA damage and initiates damage signaling have not yet been answered. Different pathways sensing DNA lesions and leading to downstream responses have been reported: Ku proteins associate with DNA DSB ends and facilitate end-joining repair (23), ATM senses chromatin conformational changes and is activated by autophosphorylation (4); histone H2AX, as it flanks DNA lesions, is phosphorylated and recruits a series of DNA damage response factors (25); and ssDNA generated from DSBs enables homologous recombination repair and recruits ATR complexes to sustain the checkpoint activation (7). Our data have clarified the DSB break sensing and repair pathway by demonstrating that recruitment of NFBD1, the downstream factor of H2AX, functions independent of ssDNA generation and RPA coating, and upstream of the ATR-dependent pathway (Fig. 5). They therefore provide novel evidence to delineate exactly where NFBD1 fits into DNA damage early responses.

It will be important to know how NFBD1 regulates ATR recruitment. Two possibilities have emerged: (1) NFBD1 may physically associates with the ATR complex and recruit it or (2)
NFBD1 is required for ATR recruitment indirectly, for instance through RPA phosphorylation. Our preliminary data support the first possibility; we have observed a potential association of NFBD1 with ATR/Claspin through coimmunoprecipitation. A more systematic examination is under way. Future studies focusing on the detailed pathways by which NFBD1 and ATR are regulated, and how they cross-talk with other key proteins in the DNA damage pathway are necessary and we are actively undertaking them.

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