

Review

NFBD1/MDC1: DNA damage response, cell cycle regulation and carcinogenesis

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Abstract

The cellular genome is constantly exposed to the endogenous as well as the exogenous DNA damages. Thus, the proper cellular response to DNA damage plays a critical role to maintain genomic integrity and acts as a molecular barrier against carcinogenesis. Without such DNA damage response, cells eventually pass on the damaged DNA to daughter cells, leading to the malignant transformation. When DNA damage is excessive, cells undergo apoptotic cell death. A growing body of evidence strongly suggests that one of the earliest events which takes place at chromatin surrounding the DNA damage is the phosphorylation of the histone H2A variant H2AX at Ser-139 (γ -H2AX) to generate nuclear foci. γ -H2AX acts as a docking site for DNA damage response/DNA repair proteins. NFBD1/MDC1 interacts with γ -H2AX through its BRCT domain and promotes the efficient recruitment of these proteins at the sites of DNA damage. Since NFBD1-deficient mice displayed the remarkable DNA repair defect and genomic instability, it is likely that NFBD1 plays an important role in the regulation of DNA damage response. In addition to DNA damage response, NFBD1 is closely involved in the regulation of mitotic progression. Recent studies demonstrated that NFBD1 interacts with the mitotic regulator APC/C and enhances its E3 ubiquitin protein ligase activity. In the present review article, we describe the functional significance of NFBD1 in the regulation of DNA damage response, cell cycle progression and carcinogenesis.

Keywords: ATM, cell cycle, DNA damage, γ -H2AX, NFBD1/MDC1

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The abbreviations used are: ADR, adriamycin; APC/C, anaphase promoting complex/cyclosome; ATM, ataxia-telangiectasia-mutated; ATR, ataxia telangiectasia mutated and Rad3-related; BRCA1, breast cancer susceptibility gene 1; BRCT, BRCA1 C-terminus; CBP, CREB-binding protein; CDDP, cisplatin; CDK, cyclin-dependent kinase; DNAPK, DNA-dependent protein kinase; Emi1, early mitotic inhibitor 1; EMT, epithelial-mesenchymal transition; FHA, forkhead-associated; 53BP1, p53-binding protein 1; MDC1, mediator of DNA damage checkpoint protein 1; MRE11, meiotic recombination 11; MRN, MRE11/RAD50/NBS1; NBS1, nijmegen breakage syndrome 1; NFBD1, nuclear factor with BRCT domains protein 1; PIKK, phosphatidylinositol 3-kinase-related kinase; Plk-1, polo-like kinase 1; PP4, protein phosphatase 4; PST, proline/serine/threonine-rich repeat; RNF8, ring finger protein 8; Sp1, specificity protein 1; Wip1, wild-type p53-induced phosphatase 1.

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Introduction

NFBD1 (nuclear factor with BRCT domains protein 1)/MDC1 (mediator of DNA damage checkpoint protein 1) (henceforth NFBD1) has been initially identified by a random screening of a cDNA library derived from a human immature myeloid cell line, KG-1 (1). According to their results, *NFBD1* was mapped at human chromosome 6p21 and encodes a novel protein of 2089 amino acid residues, which shared no significant structural similarity to known proteins filed in the data bases. Koonin *et al.* found that NFBD1 contains a conserved repeated motif termed BRCT (BRCA1 C-terminus) domain at its COOH terminus (2). The BRCT domain was initially detected in the 202 amino acid residues COOH-terminal globular domain of breast cancer suppressor BRCA1, and the structurally related motif was also found at the COOH-terminal region of tumor suppressor p53-binding protein 53BP1 and cell cycle checkpoint protein RAD9. Therefore, NFBD1 is a member of BRCT superfamily including BRCA1. Previously, we have found for the first time that NFBD1 is a nuclear DNA-binding protein and also has an anti-apoptotic function as examined by *in vitro* DNA-binding assay and colony formation assay, respectively (3).

The cellular genome is continuously exposed to the endogenous DNA damage which occurs during normal DNA replication as well as the exogenous DNA damage insults caused by a variety of genotoxic agents such as ionizing radiation [4-6]. To maintain the genomic integrity is particularly important for cell survival. Without proper DNA damage response, DNA damage often results in the induction of genomic instability, which contributes to the development malignancies (7, 8). To avoid DNA damage-mediated genomic alterations and also transfer damaged DNA to daughter cells, cells have developed the complicated signaling pathway which delays or arrests cell cycle progression and coordinates DNA repair in response to DNA damage (9, 10). When DNA damage is highly toxic, cells with unrepaired or incorrectly repaired genome DNA undergo apoptotic cell death. This DNA damage response pathway is a highly conserved cellular process throughout evolution (11). Alternatively, when cells fail to repair damaged DNA correctly due to the premature termination of DNA replication forks as well as DNA double-strand breaks, cells undergo cellular senescence to protect them from initiation and/or progression of cancer (12). Although p53-dependent induction of CDK inhibitor p21^{WAF1} contributes to the promotion of cell cycle arrest and/or cellular senescence and NFBD1 inhibits pro-apoptotic

activity of p53 (13, 14), it has remained unclear how cells choose their cell fate in response to DNA damage.

The initial event in response to DNA damage is the activation of sensor kinases of the phosphatidylinositol 3-kinase-related kinase (PIKK) family including ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia mutated and Rad3-related) and DNAPK (DNA-dependent protein kinase) (15-17). These protein kinases which are activated by a variety of DNA damage such as ionizing radiation, UV exposure, anti-cancer drug treatment and replication stress, phosphorylate multiple substrate proteins, and thereby leading to a proper DNA damage response. Considering that ATM plays a pivotal role in DNA damage response, loss of function mutations of *ATM* and/or genes involved in the regulation of ATM catalytic activity cause severe genetic disorders manifested by genomic instability, radiation sensitivity and cancer predisposition (18). Upon DNA damage, the histone variant H2AX at the sites of DNA damage is phosphorylated at Ser-139 (γ -H2AX) by the above-mentioned sensor kinases to form nuclear foci (16, 19, 20). Cells prepared from *H2AX*-deficient mice exhibit radiation-induced genomic aberrations, suggesting that H2AX is tightly involved in proper DNA damage response (21, 22). Intriguingly, it has been shown that NFBD1 is phosphorylated in response to DNA damage in an ATM-dependent manner and recruits DNA repair machinery including MRN (MRE11/RAD50/NBS1) complex onto the sites of DNA damage marked by γ -H2AX (23-25). The MRN complex in turn further stimulates ATM activity, which results in rapid spreading of γ -H2AX around the sites of DNA damage, and thereby amplifying DNA damage response signal. Since *NFBD1*-deficient mice display the remarkable DNA repair defect and genomic instability, NFBD1 also participates in the regulation of DNA damage response (26).

In addition to the regulation of DNA damage response, NFBD1 is closely involved in the regulation of cell cycle progression. It has been well-established that a multi-protein complex termed APC/C (anaphase promoting complex/cyclosome) with an E3 ubiquitin protein ligase activity is a major regulator of the cell cycle progression. APC/C is required to induce progression and exit from mitosis by stimulating the proteolytic degradation of its substrates such as mitotic seculin and cyclin B (27-29). The activity of APC/C is regulated during mitosis by two independent activators, Cdc20 and Cdh1. Recently, it has been shown that NFBD1 binds to and activates APC/C



Fig. 1 Structure of NFB1. An NH₂-terminal FHA, a central PST and a COOH-terminal tandem repeat of BRCT domains are indicated.

through the interaction with Cdc20 (30). Indeed, we have demonstrated that knocking down of *NFB1* results in the induction of G2 arrest (31). In the present review article, we describe the functional importance of NFB1 in the regulation of DNA damage response, cell cycle progression and carcinogenesis.

Structure of NFB1

NFB1 is a large nuclear adaptor protein (2089 amino acid residues) with an NH₂-terminal FHA (forkhead-associated) domain (amino acid residues 2-141), a large central PST (proline/serine/threonine-rich repeat) domain (amino acid residues 788-1645) and COOH-terminal two BRCT (BRCA carboxy-terminal) domains (amino acid residues 1858-2068) (Fig. 1) (3, 23-25).

These modular domains (FHA and BRCT domains) are often found in cellular proteins implicated in DNA damage response and/or cell cycle control (32, 33). On the other hand, PST domain has no significant homology to any other cellular proteins (25). FHA domain has been considered to be a phospho-protein binding module commonly found in signaling proteins (34-37). Liu *et al.* found that FHA domain recognizes NH₂-terminal phospho-threonine of NFB1 and promotes its dimerization (38). Like FHA domain, it has been shown that the tandem BRCT domains act as phospho-peptide binding motif which mediates protein-protein interaction (39-41). For example, the phosphorylated form of histone H2A variant H2AX (γ -H2AX) binds to the BRCT domains of NFB1 (42). As described previously (43), the BRCT domains of the tumor suppressor BRCA1 contained a transactivation potential. According to their results, the GAL4 DNA-binding domain was fused to the BRCT domain of BRCA1 and the authors detected the activation of *HIS3* reporter gene in yeast. Additionally, GAL4-BRCT domain fusion protein enhanced the luciferase activity in mammalian cells. Consistent with this notion, BRCA1 was a component of RNA

polymerase II holoenzyme (44), and the point mutations detected within the BRCT domains of BRCA1 significantly inhibited its transcriptional activity (43). In addition, BRCT domains of BRCA1 were associated with histone acetyltransferase CBP and thereby BRCA1 enhanced p53-dependent transcription (45). Pao *et al.* demonstrated that p300/CBP interacts with BRCA1 and acts as transcriptional co-activators for BRCA1 (46). In contrast to BRCA1, the BRCT domains of NFB1 did not have the transcriptional potential (3). Therefore, the transactivation potential might not be a common functional feature of the BRCT domain.

Initiation of DNA damage response

The early molecular event which occurs in response to DNA damage, is a DNA damage-induced auto-phosphorylation of ATM at Ser-1981 (16). Phosphorylated form of ATM (p-ATM) is an active one. p-ATM then phosphorylates histone variant H2AX at Ser-139 to form γ -H2AX flanking the sites of damaged DNA (16, 19, 20). H2AX is a component of the nucleosome core structure which comprises 10-15% of total cellular H2A (47). These local accumulations of γ -H2AX are visualized as the discrete nuclear regions termed nuclear foci. Since nuclear foci are highly dynamic structure, multiple proteins implicated in DNA damage response are recruited, retained and disassembled in a distinct order within nuclear foci (6, 20). DNA damage response proteins assemble in multiprotein complexes and are regulated by protein-protein interactions in a spatiotemporal manner. Thus, ATM-dependent phosphorylation of H2AX around the sites of DNA damage is one of the early events of DNA damage response and is followed by recruitment of DNA damage response proteins.

It has been demonstrated that NFB1 collaborates with γ -H2AX to promote the efficient recruitment of DNA repair machinery such as MRN complex (MRE11,

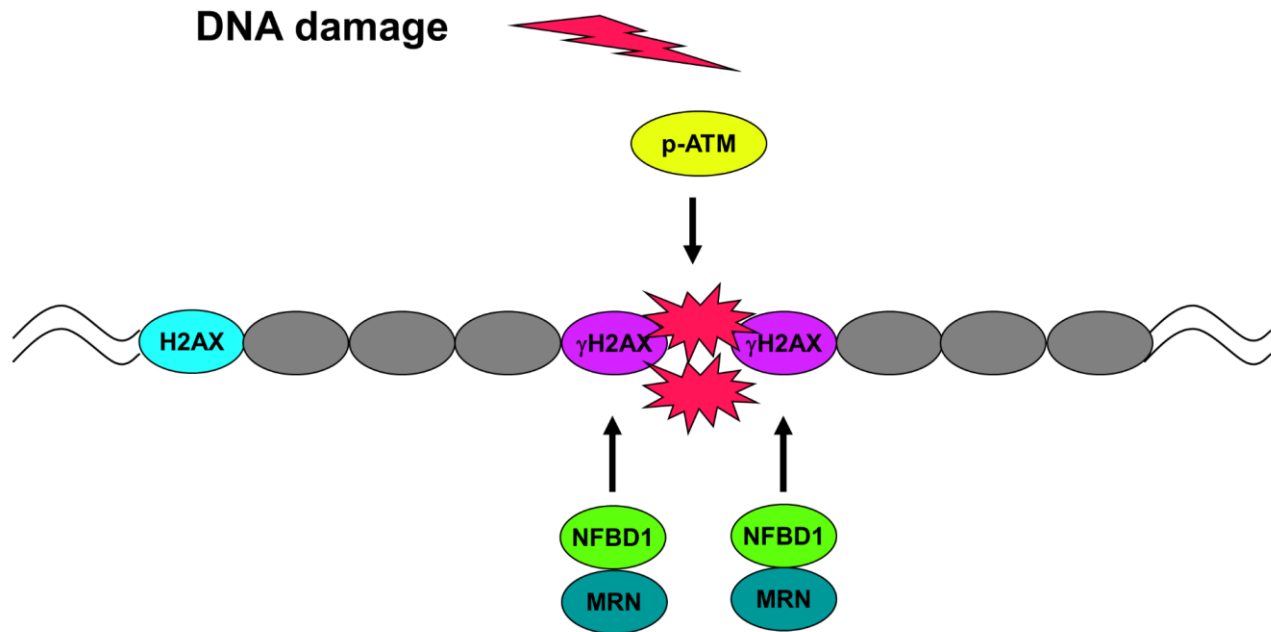


Fig. 2 NFB1 participates in the early DNA damage response. Upon DNA damage, p-ATM immediately phosphorylates H2AX (γ -H2AX) adjacent to the sites of damaged DNA to generate nuclear foci. NFB1 is also phosphorylated by p-ATM and recruits MRN complex onto the sites of DNA damage.

RAD50 and NBS1) to the sites of the damaged DNA (Fig. 2) (23-25).

According to their results, NFB1 was highly phosphorylated by p-ATM and co-localized with nuclear foci containing γ -H2AX, MRN complex and 53BP1 in response to DNA damage. Lee *et al.* described that BRCT domains of NFB1 directly binds to γ -H2AX tail (48). Xu and Stern found that FHA domain of NFB1 is responsible for the interaction with MRN complex (49). Of note, down-regulation of H2AX and NFB1 resulted in a significant abolishment of NFB1 foci formation and a partial decrease in γ -H2AX foci formation in response to DNA damage, respectively (50, 51). These observations suggest H2AX phosphorylation and NFB1 foci formation depend on each other. Collectively, NFB1 together with γ -H2AX plays a critical role in the regulation of the early DNA damage response signaling.

As mentioned above, NFB1 is one of the substrate proteins of ATM. Huen *et al.* revealed that a FHA/RING domain-containing protein termed RNF8 also accumulates at γ -H2AX containing nuclear foci including NFB1, MRN complex, BRCA1 and p-ATM in response

to DNA damage (52). According to their results, RNF8 foci formation was abrogated in *H2AX*-deficient cells and also in *NFB1*-deficient cells, indicating that RNF8 functions downstream of H2AX and NFB1 following DNA damage. Additionally, silencing of *RNF8* prohibited DNA damage-mediated recruitment of 53BP1 and BRCA1 onto the sites of DNA damage, suggesting that RNF8 acts upstream of them. Notably, ATM-dependent phosphorylation of NFB1 was required for the interaction with RNF8 and also responsible for NFB1-mediated efficient recruitment of RNF8 onto the sites of DNA damage. 53BP1 has been considered to be a critical component of genome surveillance network during DNA damage response. As described previously (53), 53BP1 was initially identified as one of p53-binding partner. Subsequent study demonstrated that 53BP1 is localized at the sites of DNA damage in response to ionizing radiation (54, 55). γ -H2AX has been shown to be required for the retention of 53BP1 at the sites of DNA damage (56). Bekker-Jensen *et al.* found that the interaction between 53BP1 and NFB1 is prerequisite for the access of 53BP1 to the DNA lesions (57). Recently, Ferrari *et al.* described that 53BP1 has an ability to protect genomic integrity from extensive DNA

degradation and rearrangements during DNA repair process (58).

Interaction between NFBD1 and p53

Since knocking down of *NFBD1* led to an increase in a sensitivity to ionizing radiation and anti-cancer drug camptothecin, NFBD1 was required for cell survival in response to DNA damage (49). In support with this notion, Yang *et al.* described that depletion of *NFBD1* sensitizes human esophageal cancer cells to chemotherapeutic drugs such as adriamycin (ADR) and cisplatin (CDDP) (59). Intriguingly, Xu and Stern found that NFBD1 is associated with tumor suppressor p53, however, the functional significance of this interaction remained elusive. Previously, we have described that NFBD1 interacts with NH₂-terminal transactivation domain of p53 and inhibits its pro-apoptotic activity (14). Recently, Shahar *et al.* found that DNA damage-induced phosphorylation at Ser-392 and acetylation at Lys-382 of p53 enhance the complex formation between p53 and NFBD1 (60). Based on our previous results, knockdown of *NFBD1* in human lung cancer A549 cells bearing wild-type *p53* resulted in an increase in a sensitivity to a variety of anti-cancer drugs, indicating that NFBD1 might attenuate DNA damage-induced pro-apoptotic pathway mediated by p53 (14). During the early phase of DNA damage response, NFBD1 inhibited ATM-dependent phosphorylation of p53 at Ser-15 and thereby allowing cells to repair damaged DNA. During the late

phase of DNA damage response, the expression level of NFBD1 sharply reduced and p53 dissociated from NFBD1/p53 complex to exert its pro-apoptotic activity. Thus, the presence or absence of NFBD1/p53 complex plays a pivotal role in the cell fate determination in response to DNA damage. According to our previous observations, expression level of NFBD1 was sharply decreased in A549 cells exposed to ADR as examined by RT-PCR and immunoblotting (14). Therefore, DNA damage-mediated down-regulation of NFBD1 was regulated at least in part at transcriptional level. We have also found that the general transcription factor Sp1 acts as a transcriptional activator for *NFBD1* (61).

Termination of DNA damage response

As mentioned above, NFBD1 together with γ -H2AX plays an essential role in the regulation of the early onset of DNA damage response, however, the precise molecular mechanisms behind the termination of DNA damage checkpoint signaling (checkpoint recovery) remain unclear. After DNA repair completes, the local accumulation of DNA repair protein complexes and chromatin modifications such as the presence of γ -H2AX are removed from the sites of DNA damage and thereby the checkpoint is switched off to allow re-entry into the normal cell cycle. Recently, Nakada *et al.* demonstrated that PP4 phosphatase catalytic subunit (PP4C) contributes to the dephosphorylation of γ -H2AX at the sites of DNA damage (62). According to their results,

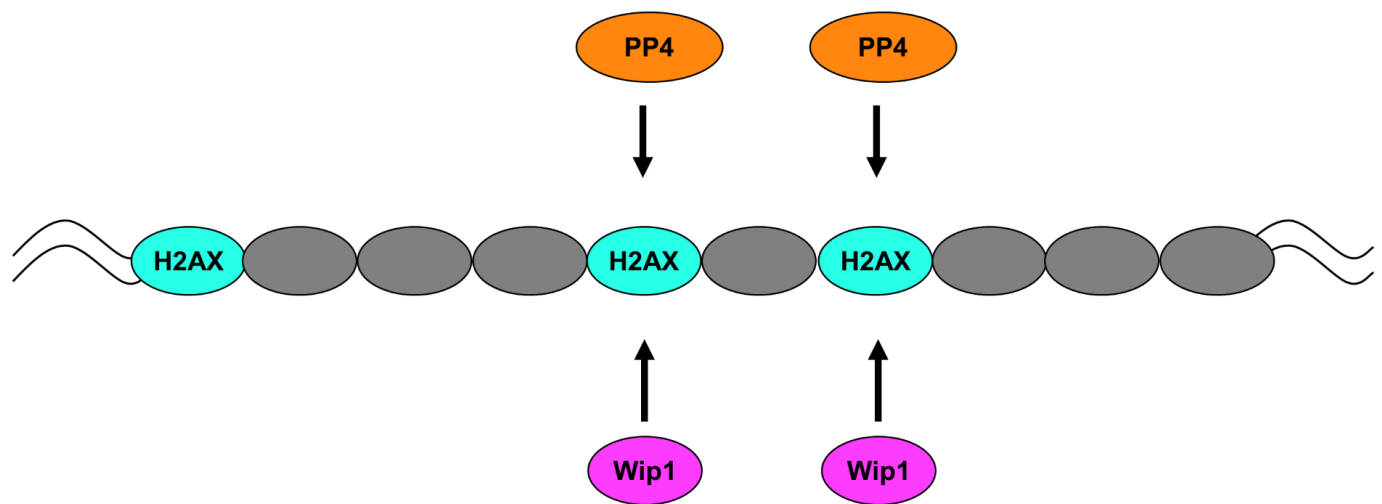


Fig. 3 Termination of DNA damage response. At the end of DNA damage response, γ -H2AX is dephosphorylated by various protein phosphatases including PP4 and Wip1 and cells with repaired DNA re-enter into the normal cell cycle.

depletion of *PP4C* results in increased steady-state levels of γ -H2AX. Previously, Fiscella *et al.* found that Wip1 (wild-type p53-induced phosphatase 1) which has an intrinsic phosphatase activity, is induced in response to DNA damage in a p53-dependent manner (63). Following DNA damage, Wip1 co-localized with NFB1 and γ -H2AX in nuclear foci, however, the timing of its nuclear re-distribution to these nuclear foci was slower than that of NFB1. Notably, Wip1 had an ability to dephosphorylate γ -H2AX directly and knocking down of *Wip1* resulted in the sustained high levels of γ -H2AX after DNA damage (64), indicating that Wip1 is required for the efficient removal of γ -H2AX from chromatin. Consistent with these results, forced expression of Wip1 reduced the formation of nuclear foci in response to DNA damage, and inhibited the efficient recruitment of NFB1 and 53BP1 to nuclear foci (65). Thus, it is likely that the restoration of the cells to the pre-stress state is associated with the dephosphorylation of γ -H2AX mediated by various phosphatases such as *PP4C* and Wip1 (Fig. 3).

Alternatively, the disassembly of nuclear foci is mediated by ubiquitin/proteasome-dependent protein degradation system. Shi *et al.* found that ubiquitylation targets NFB1 for proteasome-dependent degradation, and blocking NFB1 degradation by proteasome inhibitors results in a persistence of nuclear foci (66). Unfortunately, the E3 ubiquitin protein ligase acting on NFB1 remains to be identified. Since γ -H2AX serves as a docking site for various DNA damage/repair proteins whose local accumulation is mediated by NFB1, it is possible that proteasome-dependent degradation of NFB1 might promote the disassembly of DNA repair complex.

Regulatory role in the mitotic progression

Progression through mitosis is regulated by the sequential proteolytic degradation of mitotic proteins such as Plk-1, cyclin B and securin, which is mediated by ubiquitin/proteasome pathway. It has been well-established that a large protein complex termed APC/C containing the intrinsic E3 ubiquitin protein ligase activity, controls the ubiquitin-dependent proteolytic degradation of the above-mentioned mitotic proteins, and is essential for proper cell cycle progression (27-29). APC/C is composed of at least 12 subunits and is active from early mitosis through late G1 phase of the cell cycle (67, 68). At the end of G1 phase, APC/C becomes inactive to allow the accumulation of essential cell cycle regulators in cells (29). Previous studies suggest that

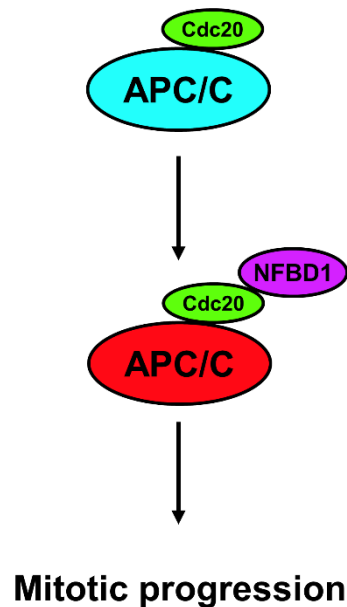
APC/C-mediated degradation of substrate proteins (cyclin B1, Plk-1 and phosphorylated histone H3 at Ser-10) requires CBP, which acts as an E4 ubiquitin protein ligase (69). E4 ubiquitin ligase catalyzes the elongation of polyubiquitinated chain to allow the targeting of the polyubiquitinated substrate proteins for degradation by proteasome (70). Catalytic activity and substrate specificity of APC/C are regulated during mitosis by one of two closely related activator proteins such as Cdc20 and Cdh1 (71, 72). It has been shown that Cdc20 and Cdh1 recognize the destruction box (D box), and Cdh1 also recognizes the KEN box (73-75). On the other hand, a F-box protein termed Emi1 acts as an inhibitor for APC/C (76). Emi1 inhibits APC/C by direct binding to Cdc20 and Cdh1 and thereby blocking the interaction between APC/C and its substrates.

Uncontrolled proteolysis of the cell cycle regulators which might be due to the dysfunction of APC/C, is tightly involved in the induction of genomic instability and subsequent carcinogenesis. For example, inhibition of APC/C-mediated proteolytic degradation of securin or cyclin B resulted in genomic instability (77, 78). When Emi1 escapes from the proteolytic degradation, abnormal accumulation of substrates of APC/C takes place and cells undergo mitotic catastrophe (79, 80). Indeed, various cancers overexpress Emi1 (81). In addition, securin is frequently overexpressed in hematopoietic neoplasms, and the high expression levels of cyclin B are detectable in colon and lung cancers (82-86). These observations suggest that the proper mitotic checkpoint activation pathway is disrupted in certain cancers.

Recently, Coster *et al.* described that NFB1 directly interacts with APC/C (87). According to their results, this interaction was mediated by BRCT domain of NFB1 and COOH-terminus of Cdc27 (one of subunits of APC/C) in a phosphorylation-dependent manner. Phosphorylated form of Cdc27 but not unphosphorylated form of Cdc27 bound to NFB1. Under their experimental conditions, 53BP1 which acts as a mediator of DNA damage response, was not associated with APC/C. Although the expression levels of the endogenous NFB1 appeared to be constant after DNA damage induction, the amounts of complex containing NFB1 and APC/C increased, indicating that NFB1 is not a substrate of APC/C. However, the functional significance of the interaction between NFB1 and APC/C remains elusive. Subsequent studies demonstrated that, in addition to Cdc27, NFB1 binds directly to Cdc20 as well as CBP and regulates catalytic activity of APC/C (87). Based on their results, NFB1

acts as a molecular bridge between Cdc20 and APC/C and thereby promoting efficient APC/C activation during mitotic progression. In support with this notion, knocking down of the endogenous *NFBD1* resulted in a mitotic arrest, which might be due to the decreased recruitment of Cdc20 to APC/C mediated by NFBD1 (Fig. 4). Recently, we have found that Plk-1 interacts with NFBD1 and phosphorylates its PST domain (88). Based on our observations, Plk-1-mediated phosphorylation of NFBD1 was required for M phase entry. Taken together, in addition to DNA damage signaling pathway, NFBD1 is involved in the regulation of mitotic progression.

Fig. 4 NFBD1 is required for mitotic progression. NFBD1 has an ability to enhance E3 ubiquitin protein ligase activity of APC/C complex through the interaction with Cdc20.



Functional implications in carcinogenesis

Since the constitutive activation of DNA damage response pathway has been shown to take place in the early precursor lesions of several types of human cancers (89), it is highly likely that the activated DNA damage checkpoints triggered at least in part by NFBD1 provide an early biological barrier against the genomic instability and the subsequent progression of cancers. Consistent with this notion, *NFBD1*-deficient mice exhibited growth retardation, male infertility, immune defects, chromosome instability, DNA repair defects and radiation sensitivity (26). In a good agreement with these observations, Bartkova *et al.* described that NFBD1 is aberrantly down-regulated in subsets of lung and breast cancers (90). In contrast, Yuan *et al.* described that *NFBD1* is expressed higher in human cervical cancer tissues than in matched normal ones, and knockdown of

NFBD1 inhibits tumor growth in nude mice, suggesting that NFBD1 has an oncogenic potential (91). In accordance with these observations, Liu *et al.* revealed that NFBD1 promotes ovarian cancer metastasis through the induction of epithelial-mesenchymal transition (EMT) (92). Since number of the expression studies regarding the expression level of *NFBD1* in a variety of cancers is very small, it has been still elusive whether the expression level of *NFBD1* could be associated with carcinogenesis and/or prognosis of the patients. Recently, it has been shown that NFBD1 is cleaved by caspase-3 at position 173 between FHA and BRCT domains (93). Structural integrity of NFBD1 is critical for the efficient DNA repair as well as DNA damage response. γ -H2AX and ATM are associated with BRCT and FHA domains, respectively. Thus, caspase-3-mediated cleavage disrupts their interaction, aborts a full activation of DNA damage response and thereby accumulation of DNA damage in cells. Indeed, inactivation of NFBD1 has been found in a large number of human breast and lung cancers, which might have a significant impact on carcinogenesis (89, 94).

Conclusions

Upon DNA damage, NFBD1 acts as an adaptor protein to recruit DNA repair machinery to the sites of DNA damage marked by γ -H2AX. NFBD1 also contributes to the proper mitotic progression through the regulation of the activity of APC/C complex. Since *NFBD1*-deficient mice displayed the remarkable DNA repair defect and genomic instability, dysfunction of NFBD1 might accumulate cells with serious DNA damages and thereby leading to the genesis and/or development of cancers. Thus, it is quite important to check the genomic status and expression level of *NFBD1* might contribute to the early detection of precancers and/or cancers.

Acknowledgments

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