Inactivation of the Nijmegen Breakage Syndrome Gene Leads to Excess Centrosome Duplication via the ATR/BRCA1 Pathway

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Abstract

Nijmegen breakage syndrome is characterized by genomic instability and a predisposition for lymphoma and solid tumors. Nijmegen breakage syndrome 1 (NBS1), the protein which is mutated in these patients, functions in association with BRCA1 and ATR as part of the cellular response to DNA double-strand breaks. We show here that NBS1 forms foci at the centrosomes via an interaction with γ-tubulin. Down-regulation of NBS1 by small interfering RNA induces supernumerary centrosomes, and this was confirmed with experiments using Nbs1 knockout mouse cells; the introduction of wild-type NBS1 (wt-NBS1) cDNA into these knockout mouse cells reduced the number of supernumerary centrosomes to normal levels. This phenotype in NBS1-deficient cells is caused by both centrosome duplication and impaired separation of centrioles, which have been observed in BRCA1-inhibited cells. In fact, supernumerary centrosomes were observed in Brca1 knockout mouse cells, and the frequency was not affected by NBS1 down-regulation, suggesting that NBS1 maintains centrosomes via a common pathway with BRCA1. This is consistent with findings that NBS1 physically interacts with BRCA1 at the centrosomes and is required for BRCA1-mediated ubiquitination of γ-tubulin. Moreover, the ubiquitination of γ-tubulin is compromised by either ATR depletion or an NBS1 mutation in the ATR interacting (FHA) domain, which is essential for ATR activation. These results suggest that, although centrosomes lack DNA, the NBS1/ATR/BRCA1 repair machinery affects centrosome behavior, and this might be a crucial role in the prevention of malignancies.

Introduction

In contrast to normal cells, which have a defined and constant number of chromosomes, the presence of an abnormal number of chromosomes, a condition called aneuploidy, is consistently observed in virtually all human cancers (1, 2). A growing body of evidence suggests that aneuploidy is often caused by chromosomal instability during mitosis (3, 4), which may result from an improper duplication of centrosomes. The centrosome organizes the spindles for the separation of chromosomes during mitosis, and the presence of more than two centrosomes in a cell can result in lost chromosomes or in an excess number of chromosomes (2, 5). The occurrence of excess or supernumerary centrosomes is associated with problems in cell cycle regulation, because centrosomes can continue to duplicate when DNA replication is halted, for example, to repair damage (5–8). Therefore, when replication forks are stalled in response to exposure to hydroxyurea or radiation, the frequency of supernumerary centrosomes increases in checkpoint-efficient cells because DNA replication is delayed (9, 10). However, the disruption of BRCA1 also induces supernumerary centrosomes in the absence or presence of hydroxyurea, although BRCA1-deficient cells continue DNA replication in the presence of DNA damage (11). BRCA1 is directly involved in the maintenance of centrosome duplication through the ubiquitination of γ-tubulin, the main component of centrosomes, and the disruption of ubiquitination sites results in an excess number of centrosomes (12–14). Indeed, supernumerary centrosomes have been frequently observed in the early stages of breast cancer (15, 16).

Nijmegen breakage syndrome (NBS; MIM 251260) is characterized by microcephaly, chromosome instability, and a predisposition for lymphoid malignancies (17) and solid tumors. NBS1 mutant carriers also have a high risk for solid tumors, including breast cancer (18–20). Cells derived from such patients show defects in cellular responses to the presence of DNA double-strand breaks (DSB), such as homologous recombination repair (21, 22) and an abnormal regulation of checkpoints (23–25). DSBs are spontaneously generated in lymphoblastoid cells during VDJ recombination, and an impaired response to DSBs presumably contributes to the etiology of lymphoma in patients. This conclusion is supported by observations showing that lymphocytes from patients have a high frequency of translocations at chromosomes 7 and 14, wherein VDJ recombination segments are localized. However, this type of DSB formation is restricted to lymphoblastoid cells, and possibly other pathways are involved in the development of solid tumors.

In the presence of BRCA1, NBS1 regulates the phosphorylation of SMC1 by either ATR or ATM in response to DSBs (26). In this pathway, NBS1 physically interacts with BRCA1 and SMC1 in the nucleus, and disruption of either protein leads to abnormal checkpoint regulation and genomic instability (27, 28). In responding to DSBs, NBS1 functions as a regulatory protein for ATR activation to transduce the checkpoint signal (29, 30). On the other hand, recent studies have indicated that cells from patients with pericentric (PCNT)-Seckel syndrome, which have mutations in PCNT, also have defects in the ATR pathway (31). PCNT, which has both structural and regulatory functions, anchors at the centrosomes and is presumed to regulate centrosome duplication through ATR signaling at the centrosomes. This model is supported by evidence showing that ATR localizes in the centrosomes (32) and that the hypomorphic mutation of ATR, known as ATR Seckel, results in the production of supernumerary centrosomes (31). Because NBS1 is involved in ATR activation, the aim of the work described here was to examine the association of NBS1 with ATR and BRCA1 in centrosome maintenance, because this could play a role in tumorigenesis.
Materials and Methods

Cell culture and transfection. A31-1 cells (Nbs1<sup>+/−</sup>) and OCN-01 cells (Nbs1<sup>−/−</sup>) were established from lung fibroblasts of Nbs1 knockout (chimera) mouse (23). The NBS cell lines 2016JSV and 2083HSV were established from the fibroblasts of Polish patients with NBS. The NBS cell line EUFA1020 was generously provided by Dr. H. Joenje. Brca1<sup>−/−</sup>/Embryonic stem (ES) cells and Brca1<sup>+/−</sup> ES cells were generous gifts from Dr. M.E. Moynahan (33). Human HeLa S3, U2OS, BJ, MCF7, and mouse NIH3T3 cells were used as control cell lines. A colorectal cancer cell line, LoVo, was used to provide cells that express low levels of NBS1. All cell lines were cultured in DMEM (Sigma Chemical Co.) supplemented with 10% fetal bovine serum (HyClone) at 37°C in a humidified atmosphere containing 10% CO₂.

Fractionation of cells. Centrosomes were isolated as described previously (34). Briefly, the centrosomal fraction was purified from HeLa S3 cells using differential centrifugation. After sucrose density gradient centrifugation, 500 µL aliquots were collected from the top and labeled as fractions 1 to 40.

Plasmid and DNA manipulations. To tag NBS1 cDNA with Myc-His, wt and missense mutants for FHA-2D were subcloned into the BamH I and XhoI sites of the pcDNA3.1/Myc-His(A) vector (Invitrogen) as described previously (22). The entire cDNA insert was confirmed with DNA sequencing. The vectors were transfected into A31-1 cells using Lipofectamine 2000 (Invitrogen).

RNA-mediated interference. The RNA oligonucleotides used in this study were (a) for NBS1, guacguuguuggaaggaaa, gggaaagggaugaagaaaa, and ggacacaaaaccagaguua (THF27A-212, B-Bridge International, Inc.); (b) for ATR, gacggugugcucaugcggc (Qiagen); and (c) for BRCA1, the siGENOME SMART pool reagent (M-003461-00-0005, Dharmacon). These were transfected into cells using Lipofectamine 2000 (Invitrogen). GFP small interfering RNA (siRNA) was used as a negative control in transfections.

Antibodies and Western blots. Cell extracts were prepared using immunoprecipitation assay buffer (50 mmol/L Tris-HCl at pH 7.5, 150 mmol/L NaCl, 0.1% SDS, 1.0% TritonX-100, 0.1% sodium deoxycholate, 1 mmol/L EDTA, 1 × proteinase inhibitor, phenylmethylsulfonyl fluoride, aprotinin, leupeptin), or immunoprecipitation buffer (20 mmol/L HEPES-NaOH at pH 7.4, 0.2% NP40, 150 mmol/L NaCl, 25% glycerol, 0.1 mmol/L EDTA). Western blotting was performed using anti-NBS1 (Novus Biologicals, R&D Systems), anti-MRE11 (Novus Biologicals), anti-BRCA1 (Santa Cruz Biotechnology, Oncogene), anti-ATR (Santa Cruz Biotechnology), anti-γ-tubulin (Sigma Chemical Co.), FK2 anti-monoubiquitin and anti-polyubiquitin (Biomol International), and anti-α-tubulin (Sigma Chemical Co.). Anti-NBS1 (Novus Biologicals) was used for immunoprecipitation of NBS1 protein in the peak fractions obtained from sucrose gradient centrifugation.

Immunostaining. Cells on coverslips were washed with PBS, fixed in cold methanol (−20°C) for 10 min, and incubated with a detergent solution at 4°C for 5 min. The cells were incubated with a blocking solution for 30 min and exposed to primary antibodies for NBS1 (Novus Biologicals), MRE11 (Novus Biologicals), BRCA1 (Santa Cruz Biotechnology), γ-tubulin (Sigma Chemical Co.), and secondary antibodies Alexa 488-conjugated anti-rabbit IgG (Molecular Probes) and Alexa 546-conjugated anti-rabbit IgG (Molecular Probes). Fluorescence from Alexa 488 or Alexa 546 was detected using a microscope equipped with appropriate filters.

Figure 1. NBS1 is localized in the centrosomes. A, NIH3T3, HeLa S3, MCF7, and BJ cells were immunostained with anti-γ-tubulin (red) and NBS1 antibody (green) during interphase. B, NIH3T3 cells were immunostained at M phase. Scale bars, 2.5 µm. C, extracts from HeLa S3 cells were fractionated by centrifugation in a sucrose gradient and immunoblotted using the indicated antibodies. α-Tubulin, primarily present in the cytoplasm, was used as a negative control in centrosome fractions. D, material from centrosome fraction 26 in HeLa S3 cells obtained from sucrose gradient centrifugation was immunoprecipitated with anti-NBS1, and IgG was used as a control. This material was then immunoblotted using anti-NBS1, anti-MRE11, anti-BRCA1, anti-SMC1, or anti-γ-tubulin antibodies.
visualized with a confocal laser-scanning microscope (Olympus). At least 200 cells in each sample were examined for centrosome number and nuclear morphology.

**Cell cycle analysis.** For serum starvation, NIH3T3 cells were grown in DMEM with 0.25% fetal bovine serum, 25 μmol/L of Roscovitine (Calbiochem), 20 μmol/L of Purvalanol (Calbiochem), 50 μmol/L of Olomoucine (Promega) for 48 h. Cells were collected, fixed in 70% ethanol, and stained with propidium iodide. Flow cytometric analysis was performed on a FACSCalibur system (BD Biosciences).

**Results**

**Centrosome localization and the phenotype of NBS1-deficient cells.** NBS1 and γ-tubulin were immunostained in mouse NIH3T3 cells, human HeLa S3 cells, MCF7 cells, and BJ cells by using antibodies for both proteins. All cells showed one or two clear discrete foci for NBS1, and these colocalized with γ-tubulin, a major component of the centrosome (Fig. 1A). This colocalization of NBS1 and γ-tubulin was also observed during mitosis (Fig. 1B). To confirm these immunostaining results, cell lysates from HeLa S3 cells and NIH3T3 cells were fractionated with sucrose gradient centrifugation, and each fraction was analyzed with Western blots. Analysis revealed that NBS1 and MRE11, components of the NBS1 complex, coelute with γ-tubulin in fractions 24 to 27 in HeLa cells (Fig. 1C) and NIH3T3 cells (data not shown). This is consistent with the finding that NBS1 immunoprecipitates with γ-tubulin and MRE11. When the peak sucrose gradient centrifugation fraction containing NBS1 was analyzed with immunoprecipitation, NBS1 was coinmunoprecipitated with γ-tubulin, MRE11, and BRCA1, indicating a possible physical interaction of the NBS1 complex and γ-tubulin in the centrosomes (Fig. 1D).

To obtain additional observations concerning the involvement of NBS1 in the maintenance of centrosomes, the NBS1 protein in NIH3T3 and U2OS cells was knocked down using siRNA (Fig. 2A, right) and the number of centrosomes was scored using immunostaining for γ-tubulin (Fig. 2A, left and middle). Figure 2A (left) shows that interference with NBS1 expression led to a significant increase in the number of induced supernumerary centrosomes. Cells having three or more centrosomes are defined as having a supernumerary number of centrosomes, and the frequency of cells containing supernumerary centrosomes was three to four times higher in U2OS knockdown cells than in untreated control cells. NIH3T3 cells showed a lesser increase in this frequency, but this is possibly due to a high background level of supernumerary centrosomes (35). Furthermore, NBS1-deficient cells were derived from mouse knockout cells and examined for centrosome instability (Fig. 2B). Nbs1 knockout mouse cells were also found to display supernumerary centrosomes, but centrosome numbers were restored to normal levels after the introduction of wt-NBS1 cDNA (full cDNA; Fig. 2C).
were generated in the FHA domain (FHA-2D), which is essential for ATR activation after DNA damage (Supplementary Fig. S1; ref. 36). Interestingly, the restoration of proper centrosome numbers was not observed after the introduction of the NBS1 mutants containing mutations in the FHA domain into these cells (Fig. 2C), although they expressed the NBS1 mutant protein at the centrosomes (Fig. 2B). These results suggest that NBS1 is localized at the centrosomes and plays a role in centrosome maintenance, possibly through ATR activation.

**Association of NBS1 with centrosome overduplication and impaired centriole separation.** The frequency of supernumerary centrosomes increased in NIH3T3 cells treated with hydroxyurea and aphidicolin or which were exposed to ionizing radiation (Fig. 3A). This is consistent with results reported by other groups (7, 37). These genotoxic stress-eliciting instabilities could be due to the continued duplication of centrosomes, whereas DNA replication is halted by checkpoint mechanisms. Therefore, a reduced number of centrosomes was expected to be present in NBS1-deficient cells because these cells can continue DNA replication in the presence of DNA damage (23–25). However, the inactivation of NBS1 enhanced the frequency of supernumerary centrosomes, indicating that the centrosome phenotype of NBS1-deficient cells is not explained by a failure to halt DNA replication. To elucidate the mechanism involved, NIH3T3 cells were treated with cyclin-dependent kinase 2 (CDK2)/cyclin A/E inhibitors, such as roscovitine, purvalanol A, and olomoucine. These treatments abolished supernumerary centrosomes in NBS1-deficient cells, although these inhibitors increased the percentage of the cell population in S-phase (Fig. 3A and Supplementary Fig. S2). Similarly, the frequency of supernumerary centrosomes significantly decreased when NBS1-deficient cells were arrested in G1 phase by serum starvation (Fig. 3A), suggesting the underlying mechanism was something other than the alteration of the cell cycle. Because inactivity of CDK2/cyclin A/E inhibits centriole separation (38–40), the NBS1-mediated centrosome defect could be due primarily to the impaired maintenance of centrioles (Fig. 3A and Supplementary Fig. S2). However, the abnormal centrosomes observed in NBS1-deficient cells did not result only from the effects of centriole separation. Immunostaining analysis with using antibodies for centrin revealed that supernumerary centrosomes in NBS1-deficient cells are due to both excessive centrosome duplication and impaired centriole separation because, in contrast to one paired centriole-positive focus seen in wt cells, five unpaired centrin-positive foci (a total greater than the expected normal four centrioles per cell seen after a normal duplication) are apparent in NBS1-deficient cells (Fig. 3B). These centrosomal hypertrophies after the inactivation of NBS1 are similar to those seen in BRCA-deficient cells, which indicates that the presence of supernumerary centrosomes is accompanied by abnormal centrosome separation and centrosome overreplication (14).

**Regulation of centrosomes by NBS1 through a pathway shared with BRCA1.** The phenotypic similarity of centrosomes seen in NBS1-deficient and BRCA1-deficient cells (11) suggested it would be worthwhile to examine any association between NBS1 and BRCA1 in centrosomes. Immunostaining experiments showed that BRCA1 localizes to the centrosome and colocalizes with MRE11 in HeLa S3 cells (Fig. 4A). Moreover, communoprecipitation experiments showed a physical interaction of BRCA1 with NBS1 and MRE11 (Fig. 1D). NBS1 and MRE11 in the nucleus are components of the BRCA1-associated genome surveillance complex (BASC). However, their physical interaction in the centrosome may not be the same with their interaction in the nucleus, because SMC1, another component of the BASC, was not detected in NBS1 immunoprecipitated material (Fig. 1D).

In a manner similar to NBS1 deficiency, a deficiency in BRCA1 induces supernumerary centrosomes. Brca1 knockout mouse cells displayed a high frequency of centrosomes, as shown in Fig. 4B. However, the frequency remained unchanged when NBS1 in Brca1−/− cells was suppressed by siRNA, although it is increased in Brca1−/−/− cells (Fig. 4B). Similar results were obtained when BRCA1 and NBS1 in U2OS cells were concurrently down-regulated by siRNA (Fig. 4C). These results indicate that BRCA1 and NBS1 function in a shared pathway for proper centrosome maintenance.

The BRCA1 protein seems to be involved in centrosome maintenance through the ubiquitination of γ-tubulin in *in vitro* experiments (12). The ubiquitination of γ-tubulin was investigated...
in cells using immunostaining with the FK2 monoubiquitin and polyubiquitin antibodies. Analysis revealed that FK2 foci are formed and colocalize with γ-tubulin, but they are not detected in the absence of either BRCA1 or NBS1 (Fig. 5A). Because polyubiquitination of γ-tubulin was not detected, FK2 foci seem to recognize the NBS1-dependent monoubiquitin form of γ-tubulin (Supplementary Fig. S3A). This was confirmed by Western blot analysis, in which the ubiquitination of γ-tubulin was significantly reduced in Nbs1 knockout cells and the level was similar to that observed in BRCA1-deficient cells, although expression of NBS1 restored the normal level of ubiquitination (Fig. 5B). This is consistent with the idea that depletion of NBS1 and/or BRCA1 in U2OS cells, as shown in Fig. 4C, were accompanied by a reduction in the ubiquitination of γ-tubulin and a concurrent amplification of centrosomes, indicating a strong association of supernumerary centrosomes with BRCA1 activity, as measured by the ubiquitination of γ-tubulin (Supplementary Fig. S3B). Furthermore, the reduced ubiquitination of γ-tubulin was observed in three cell lines from NBS patients, as well as in the LoVo cancer cell line, which expresses low levels of NBS1 and no NBS1 mutation (ref. 41; Fig. 5C).

Deficient ubiquitination of γ-tubulin in ATR-depleted cells. NBS1 domain analysis suggested that ATR could be involved in centrosome maintenance, because an FHA-2D mutant lacking the region for ATR activation (36) failed to restore the defect in the centrosome phenotype of NBS1-deficient cells (Fig. 2C). Subsequently, the ubiquitination of γ-tubulin was examined in ATR-deficient cells. As expected, ATR down-regulation reduced the FK2 ubiquitination signals in centrosomes (Fig. 6A and B), although it did not affect BRCA1 localization (Fig. 6D). Consistent with this, Western blot analysis revealed that the ubiquitination of γ-tubulin was significantly reduced in ATR-depleted cells (Fig. 6C). Moreover, the NBS1 mutant (FHA-2D) also had an impaired FK2 signal in a manner similar to that seen in ATR-down-regulated cells (Fig. 6A).

Together, these results suggest that centrosome phenotypes in ATR-deficient cells, similar to that of NBS1-deficient cells, are mediated by BRCA1 activity at the centrosomes, as measured by the ubiquitination of γ-tubulin.

Discussion

Centrosome duplication is highly reminiscent of DNA replication, because the centrosome must be duplicated only once in each cell cycle. Although DNA replication is strictly regulated to prevent the rereplication of chromosomes through a control mechanism involving licensing or tagging, NBS1 down-regulation enhanced the rereplication of chromosomal DNA in the presence of SV40 large T antigen or by overexpression of the licensing factor Cdt1. NBS1 is required for the suppression of rereplication through a mechanism in which NBS1 activates ATR and, thereby, phosphorylates downstream proteins, such as RPA (36). In a situation similar to that seen in DNA rereplication, the present results showed that a deficiency in NBS1 causes the creation of supernumerary centrosomes. Moreover, the NBS1 mutation experiments reported here showed that the domain in NBS1, which is essential for ATR activation, is crucial for proper centrosome operation. This is consistent with a recent report, which indicated that cells from patients with mutations in PCNT-Seckel syndrome are deficient in ATR signaling and contain supernumerary centrosomes (31). If it acts in a manner similar to nuclear ATR signal activation in a response to DNA damage (42), NBS1 could potentially function as an upstream protein, which can regulate ATR during centrosome maintenance.

The use of immunostaining and Western blot analysis with the FK2 antibody to examine the BRCA1-mediated ubiquitination of γ-tubulin in cells is reported here for the first time. This analysis revealed that NBS1 and the ATR kinase are required for BRCA1-mediated ubiquitination of the centrosome (Figs. 5 and 6). In
combination with the results from NBS1 down-regulation in Brca1 knockout mouse cells (Fig. 4B) and BRCA1-depleted U2OS cells (Fig. 4C), these observations provide support for a proposed model in which NBS1, ATR, and BRCA1 function in a common pathway to maintain a proper centrosome number (Supplementary Fig. S3C). Sartori and colleagues showed that nuclear BRCA1 ubiquitinates its binding partner CtIP in an ATR-dependent or ATM-dependent manner and, as a result, regulates G2 checkpoints (43, 44). Similarly, BRCA1 ubiquitinates γ-tubulin in vitro (12) and in cells (present results), and the activity is dependent on cellular NBS1 and ATR. As a result, the present results showed that NBS1 and ATR are involved in the regulation of BRCA1 activity at the centrosomes, and this can help maintain the correct number of centrosomes.

Mutant NBS1 carriers have a 3-fold to 9-fold increased risk of breast cancer, although this varies with ethnic background (18, 19). Moreover, epidemiologic studies have indicated that a reduced expression of NBS1 is observed in up to 80% of breast cancer cases without any reported mutations (45, 46). Consistent with this, present results showed a reduced ubiquitination of γ-tubulin in three cell lines from patients with NBS and in one human cancer cell line, LoVo, expressing low levels of NBS1 (Fig. 5C). However, ATR is not a cancer susceptibility gene because it is an essential

**Figure 5.** Disappearances of the FK2 signal and a decreased ubiquitination of γ-tubulin in Nbs1 knockout mouse cells and Brca1 knockout mouse cells. A, Nbs1 knockout A31-1 cells, complemented cells with wt-NBS1, Brca1+/− ES cells, and Brca1−/− ES cells at mitosis were stained using anti-γ-tubulin and FK2 anti-ubiquitin antibody. Scale bars, 2.5 μm. B, centrosome fraction 26 from Nbs1 knockout A31-1 cells, complemented cells with wt-NBS1, Brca1+/− ES cells, and Brca1−/− ES cells after sucrose gradient centrifugation were immunoprecipitated with anti-γ-tubulin and control anti-IgG and immunoblotted using anti-γ-tubulin and FK2 anti-ubiquitin antibodies. C, a colorectal cancer cell line (LoVo), three NBS cell lines (EUFA1020, 2016JSV, and 2083HSV), and two control cell lines (HeLa and U2OS) were immunoblotted using anti-γ-tubulin and FK2 anti-ubiquitin antibodies, as described above.
protein for cell survival, and its disruption in adult mice leads to stem cell losses (47). Interestingly, a high risk of breast cancer is associated with the I171V NBS1 gene mutation, which is within a region that interacts with ATR, and this is consistent with the finding that the FHA-2D clone, which is mutated in the interaction domain for ATR (23), displays supernumerary centrosomes. These observations provide support for the idea that NBS1 contributes to the prevention of carcinogenesis through the NBS1/ATR/BRCA1 pathway in the centrosome. However, other pathways for tumor development in NBS1 carriers cannot be excluded. NBS1 is a modulator of ATM kinase, which is a core protein in the DNA damage response pathway and in the maintenance of genomic stability (48). The in vivo assays for the ubiquitination of \( \gamma \)-tubulin using the FK2 antibody described here could provide a useful tool to investigate the roles of centrosomal BRCA1 in the etiology of breast cancer and the role of the potential modulators, NBS1 and ATR, in the maintenance of centrosomes.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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