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Cancer Research

BRCA2 and Nucleophosmin Coregulate Centrosome Amplification and Form a Complex with the Rho Effector Kinase ROCK2

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Abstract

BRCA2 germline mutations account for the majority of heredity breast and ovarian cancer. Besides its role in DNA damage repair, BRCA2 also plays an important role in cytokinesis, transcription regulation, and cancer cell proliferation. Recently, we reported that BRCA2 localizes to centrosomes as well as nuclei and the dysfunction of BRCA2 in a centrosome causes abnormalities in cell division. Here, we identified a nucleolar phosphoprotein, nucleophosmin (NPM), as a novel BRCA2-associated protein. We also detected the binding of BRCA2 to ROCK2, an effector of Rho small GTPase. Because it is known that ROCK2 binds to NPM at centrosomes, these 3 proteins may form a complex. NPM-binding region was within amino acids 639–1,000 of BRCA2. Exogenous expression of this BRCA2 region resulted in aberrant centrosome amplification and a high frequency of multinucleated cells. Our results suggested that a complex consisting of BRCA2, NPM, and ROCK2 maintains the numerical integrity of centrosomes and accurate cell division and that dysfunction of this regulation might be involved in the tumorigenesis of breast cancer. *Cancer Res; 71(1); 68–77. ©2010 AACR.*

Introduction

BRCA1 and *BRCA2* germline mutations predispose hereditary breast and ovarian cancer, and mutation of *BRCA2* is involved in approximately 50% of hereditary breast cancers (1, 2). Besides its important role in double-strand break repair by homologous recombination (3, 4), BRCA2 also plays a pivotal role in transcriptional regulation (5), cytokinesis (6), cell proliferation (7), and centrosome duplication (8). However, the exact function of BRCA2 in hereditary breast and ovarian cancer has not been well characterized. Recently, we reported that BRCA2 has a centrosomal localization signal and is a substrate of Crm1. BRCA2 localizes to centrosomes as well as nuclei, and the dysfunction of BRCA2 in centrosomes causes abnormalities in cell division (9, 10). It has been intriguing to identify novel BRCA2-associated proteins at centrosomes to elucidate exact roles of BRCA2 in centrosome regulation and cell division, as well as to reveal the function of BRCA2 in the pathogenesis of hereditary breast cancer.

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The cell cycle is an intricate process, including DNA replication and cell division that lead to the formation of 2 genetically equivalent daughter cells from a bipolar mother cell. In this process, the centrosome duplication occurs once beginning at G_1/S phase and completing within S phase, coinciding with the DNA replication. Disruption of coordination of S-phase events and/or disruption of checkpoints in S and M phases can lead to the genomic instability, and abnormality of centrosome duplication can result in aneuploidy, one of initiation events of carcinogenesis (11, 12).

The centrosome consists of a pair of centrioles and surrounding proteins. Its duplication is tightly controlled and regulated through several mechanisms during cell division. Ran-Crm1 network is involved in this process to ensure the formation of a bipolar spindle (13, 14). It was reported that Plk1 is a parallel activator of centriole disengagement and acts to license centrosome duplication during late G_2 and early M phases (15, 16). Cdk2 and p53 are also required for centrosome duplication. The protein product of p53 localizes to centrosomes and regulates centrosome duplication as a transcription factor (17, 18). Cdk2 is the essential conductor and mediates centrosome amplification by inducing some tumor suppress genes including *p53* and the nucleophosmin (NPM/B23) (19). Especially in p53-null cells, the Cdk2/Cdk4/NPM pathway is a major guardian of centrosome dysfunction and genomic integrity (20).

NPM is a multifunctional nucleolar phosphoprotein that shuttles between the nucleus and cytoplasm during the cell cycle (21, 22). It participates in ribosome assembly, pre-rRNA processing (23, 24), mRNA processing (25, 26), DNA duplication (27), nucleocytoplasmic protein trafficking (28), and molecular chaperoning (29). NPM is also a substrate of

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Crm1 (30) and is associated with unduplicated centrosome as a target of Cdk2/cyclin E. Most of NPM are dissociated from centrosomes after being phosphorylated upon Thr199 by Cdk2/cyclin E during S phase. Some remain at centrosomes and bind to ROCK2, leading to the initiation of centrosome duplication (31, 32). NPM reassociates with centrosomes in mitosis and regulates centrosome duplication (33).

ROCK2 is a Ser/Thr kinase controlled by the small GTPase Rho (34, 35) and regulates the microfilament bundle and focal adhesion site (36), as well as the acetyltransferase activity of p300 (37). ROCK2 is also a Rho kinase and a centrosomal protein that physically interacts with NPM and promotes centrosome duplication. It is superactivated by Thr199-phosphorylated NPM at a centrosome during late G_1 phase and rapidly targets an unknown protein that plays a key role in the initiation of centrosome duplication (32).

In this report, we identified NPM and ROCK2 as binding proteins of BRCA2 by mass spectrometry and further displayed that NPM and ROCK2 interact with BRCA2 in cells. Moreover, physical inhibition of interaction between endogenous BRCA2 and NPM *in vivo* leads to abnormal amplification of centrosome numbers and a high frequency of multinucleated cells in interphase. We propose that NPM, ROCK2, and BRCA2 may form a complex to coregulate centrosome duplication and deregulation of this process may be involved in the tumorigenesis of breast cancer.

Materials and Methods

Cell culture

COS-7 and HeLa S3 cells were purchased from RIKEN BioResource Center and cultured in complete medium [Dulbecco's modified Eagle's medium supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL)] in an atmosphere containing 5% CO_2. These cells were tested and authenticated by the provider. The cumulative culture length of these cells was fewer than 6 months after resuscitation.

Purification of centrosomal proteins and mass spectrometric analysis

Centrosomes were prepared from quiescent HeLa S3 cells as described (38). After centrifugation by a discontinuous gradient consisting of 500 μL of 70%, 300 μL of 50%, and 300 μL of 40% sucrose solutions, fractions (200 μL per fraction) were collected from the bottom (fractions 1–7). The aliquots from each fraction were diluted with 1 mL of PIPES buffer (10 mmol/L of PIPES, pH 7.2) and subjected to centrifugation at 15,000 rpm for 10 minutes. The pellet was subjected to further analysis. To identify the proteins associated with BRCA2 in centrosomes, immunoprecipitates with anti-BRCA2 were resolved by SDS-PAGE and visualized by silver staining. The bands of interest were excised from the gel and subjected to mass spectrometric (LC/MS/MS) analysis as described (39). The acquired collision-induced dissociation spectra were analyzed by Mascot software.

Plasmid construction and transfection

Human NPM (B23.1) full-length cDNA was isolated by RT-PCR from HeLa S3 total RNA using Pyrobest polymerase (Takara Bio) and subcloned into pME18S-HA-A vector in-frame with appropriate N-terminal tags. Plasmids for FLAG-tagged BRCA2 expression were described previously (9). The FLAG-tagged segments of BRCA2 (FLAG-S1 BRCA2 (1–1,000 amino acids), FLAG-S2 BRCA2 (967–2,133), FLAG-S3 BRCA2 (2,093–3,418), FLAG-R1 BRCA2 (1–157), FLAG-R2 BRCA2 (113–685), FLAG-R3 BRCA2 (639–1,508), and FLAG-R3-1 BRCA2 (639–1,000)) were generated by PCR and cloned into pFLAG-CMV-2B or pME18S-FL3-FLAG-B vector. Series of transfection were carried out using TransIT-LT1 reagent (Mirus Bio) according to the manufacturer's instructions.

Antibodies

The antibodies used in this study were anti-ROCK2 polyclonal antibody (Upstate Biotechnology; 07-443), anti-NPM/B23 mouse monoclonal antibody (Chemicon; MAB4500), anti-NPM/B23 mouse monoclonal antibody (Santa Cruz; FC-8791), anti-B23 monoclonal antibody (Sigma; B0556), anti–phospho-NPM (anti–p-NPM; Thr199) rabbit polyclonal antibody (Cell Signaling Technology; 3541), anti-mouse IgM antibody (Sigma; M8644), anti-BRCA2 mouse monoclonal antibody (Oncogene; Ab-1), anti-BRCA2 rabbit polyclonal antibody (Santa Cruz; H299), anti-FLAG monoclonal antibody (Sigma; F3165), anti-γ-tubulin rabbit antibody (Sigma; T3559), anti-γ-tubulin monoclonal antibody (Santa Cruz; C-11), anti-centrin2 goat polyclonal antibody (Santa Cruz; N-17), anti-centrin1/2 rabbit polyclonal antibody (Santa Cruz; H-40), and anti-HA rat monoclonal antibody (Roche; clone 3F10).

Immunoprecipitation and immunoblot analysis

Cells were lysed in 1% NP40 lysis buffer (50 mmol/L of Tris-HCl, pH 7.6, 150 mmol/L of NaCl, 1% Nonidet P-40, 10 mmol/L of NaF, 1 mmol/L of Na_3VO_3, 1 mmol/L of dithiothreitol, 1 mmol/L of phenylmethylsulfonyl fluoride, 10 μg/mL of leupeptin, and 10 μg/mL of pepstatin) and then incubated on ice for 30 minutes. After centrifugation, cell lysates were immunoprecipitated with anti-NPM, anti-ROCK2, or anti-BRCA2 antibodies bound to Protein A (GE Healthcare) or Protein G (Zymed Laboratories) Sepharose, or anti-HA affinity matrix (Roche Diagnostics) or anti-FLAG M2-agarose (Sigma). The immunoprecipitates were washed 3 times with 0.1% NP40 lysis buffer. Cell lysates or immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose filters (Bio-Rad) or polyvinylidene difluoride membranes (Millipore). The membranes were then incubated with anti-NPM, anti-BRCA2, anti-ROCK2, anti-FLAG, and anti-HA antibody, respectively. After extensive washing, the blots were incubated with horseradish peroxidase–conjugated anti-rabbit or anti-mouse or anti-rat immunoglobulin antibodies (Santa Cruz). The antibody–antigen complex was visualized by chemiluminescence (PerkinElmer).

Indirect immunofluorescence microscopy

Proliferating cells cultured in chamber slides were fixed with 3.5% formaldehyde in PBS for 10 minutes on ice and

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permeabilized with 50%, 75%, and 95% cold ethanol on ice for 5 minutes, blocked for 30 minutes, incubated with primary antibody for 1 hour at room temperature, incubated with Alexa Fluor 488– or 594–conjugated secondary antibody (Molecular Probes) for 30 minutes at 37°C, and then preserved in Vectashield (Vector Inc.). DNA was stained with bisbenzimide (Hoechst33258). The samples were examined with an Olympus Power BX51 fluorescence microscope (Olympus).

Results

Identification of NPM and ROCK2 as candidates for BRCA2-associated centrosomal proteins

In an attempt to determine the exact biological significance of BRCA2, we searched candidates for BRCA2-associated centrosomal proteins by mass spectrometry. First, we isolated centrosome fraction from HeLa S3 cells by discontinuous sucrose gradient centrifuge. Immunoprecipitated centrosomal components with anti-BRCA2 antibody were resolved by SDS-PAGE and visualized by silver staining. The protein bands specific to BRCA2 association were subjected to LC/MS/MS as summarized and identified as NPM (37 kDa), ROCK2 (160 kDa), and others (Fig. 1A, lane 3).

Association of NPM with BRCA2 in cells

We tested the interaction between NPM and BRCA2 *in vivo* by coimmunoprecipitation assay. Full-length FLAG-BRCA2 and HA-NPM were coexpressed in mammalian cultured cells. We utilized COS-7 cells in these experiments, as the expression of exogenous FLAG-BRCA2 was the most significant among various cell lines. Cells were collected 30 hours after transfection in 1% NP40 lysis buffer, and the lysates were immunoprecipitated either with anti-FLAG or anti-HA antibody. FLAG-BRCA2 was specifically coprecipitated with anti-HA antibody (Fig. 1B, the third panel, lane 3). Reversely, HA-NPM was specifically coprecipitated with anti-FLAG antibody (Fig. 1B, the sixth panel, lane 3). These results showed that FLAG-BRCA2 is bound to HA-NPM. To examine the interaction between endogenous BRCA2 and NPM *in vivo*, we immunoprecipitated either BRCA2 or NPM from COS-7 cell lysates. Anti-BRCA2 antibody coprecipitated NPM (Fig. 1C, bottom panel, lane 3), and anti-NPM antibody coprecipitated BRCA2 (Fig. 1D, bottom panel, lane 2). These results showed that the physical interaction of these 2 native proteins *in vivo*.

Association of ROCK2 with BRCA2 in cells

Because ROCK2 was detected as a candidate protein binding to BRCA2 in centrosomes, we also tested binding of these 2 proteins *in vivo*. First, COS-7 cells were transfected with an expression vector of full-length FLAG-BRCA2 or an empty vector, respectively. Cells were collected 30 hours after transfection, and the immunocomplex was precipitated with anti-FLAG or anti-ROCK2 antibody. FLAG-BRCA2 was specifically coprecipitated with anti-ROCK2 antibody (Fig. 2A, bottom panel, lane 1), and native ROCK2 was specifically coprecipitated with anti-FLAG antibody (Fig. 2B, bottom panel, lane 1). This indicated that FLAG-BRCA2 interacts with ROCK2. Next, we investigated the binding of endogenous BRCA2 and ROCK2. COS-7 cells were harvested, and the lysates were immunoprecipitated with anti-BRCA2 or anti-ROCK2 antibody (Fig. 2C and D). BRCA2 was specifically coprecipitated with anti-ROCK2 antibody (Fig. 2C, bottom panel, lane 2), and ROCK2 was specifically coprecipitated with anti-BRCA2 antibody (Fig. 2D, bottom panel, lane 2). These results showed that endogenous ROCK2 interacts with endogenous BRCA2.

Analysis of the NPM-binding region of BRCA2

To find out the NPM-interacting region of BRCA2, we further subcloned different segments of BRCA2 and had FLAG-S1 (1–1,000 amino acids), FLAG-S2 (967–2,133), and FLAG-S3 (2,093–3,418). Each of BRCA2 segment was coexpressed with HA-NPM in COS-7 cells. Immunoprecipitation with anti-FLAG or anti-HA antibody was carried out. FLAG-S1 BRCA2 was specifically coprecipitated with anti-HA antibody (Fig. 3B, bottom panel, lane 3), and HA-NPM was specifically coprecipitated with anti-FLAG antibody (Fig. 3C, bottom panel, lane 3). However, FLAG-S2 (967–2,133) or FLAG-S3 BRCA2 (2,093–3,418), and HA-NPM were not coprecipitated each other (Supplementary Fig. S1). These results showed that amino acids 1–1,000 of BRCA2 bind to NPM.

This segment was further separated into 3 smaller fragments: FLAG-R1 (1–157), FLAG-R2 (113–685), and FLAG-R3-1 (639–1,000). To investigate which fragment would bind to NPM, each was expressed in COS-7 cells, and immunoprecipitation with anti-FLAG antibody was carried out. NPM was specifically coprecipitated with FLAG-R3-1 BRCA2 (639–1,000; Fig. 3D, bottom panel, lane 5). Therefore, it was suggested that NPM-binding region of BRCA2 is within the amino acid residues 639–1,000.

Inhibition of the association of endogenous NPM and BRCA2

In an attempt to clarify the significance of NPM–BRCA2 association further, we tried to inhibit the endogenous binding by physical competition. FLAG-R3-1 BRCA2 (639–1,000) was expressed in COS-7 cells, and the lysates were immunoprecipitated with anti-NPM or anti-BRCA2 antibody. Endogenous BRCA2 was not coprecipitated with anti-NPM antibody (Fig. 4A, bottom panel, lane 1), and endogenous NPM was not coimmunoprecipitated with anti-BRCA2 antibody (Fig. 4B, bottom panel, lane 1). These results suggested that exogenous expression of FLAG-R3-1 BRCA2 (639–1,000) could prevent NPM from binding to BRCA2.

It was reported that NPM and ROCK2 have physical interaction (32), and our results discussed earlier showed that ROCK2 binds to BRCA2 (Fig. 2). We examined whether these interactions might be abrogated in the presence of FLAG-R3-1 BRCA2 (639–1,000). In summary, both association between NPM and ROCK2, and that between ROCK2 and BRCA2, were not inhibited by the expression of FLAG-R3-1 BRCA2 (639–1,000; Fig. 4C–F).

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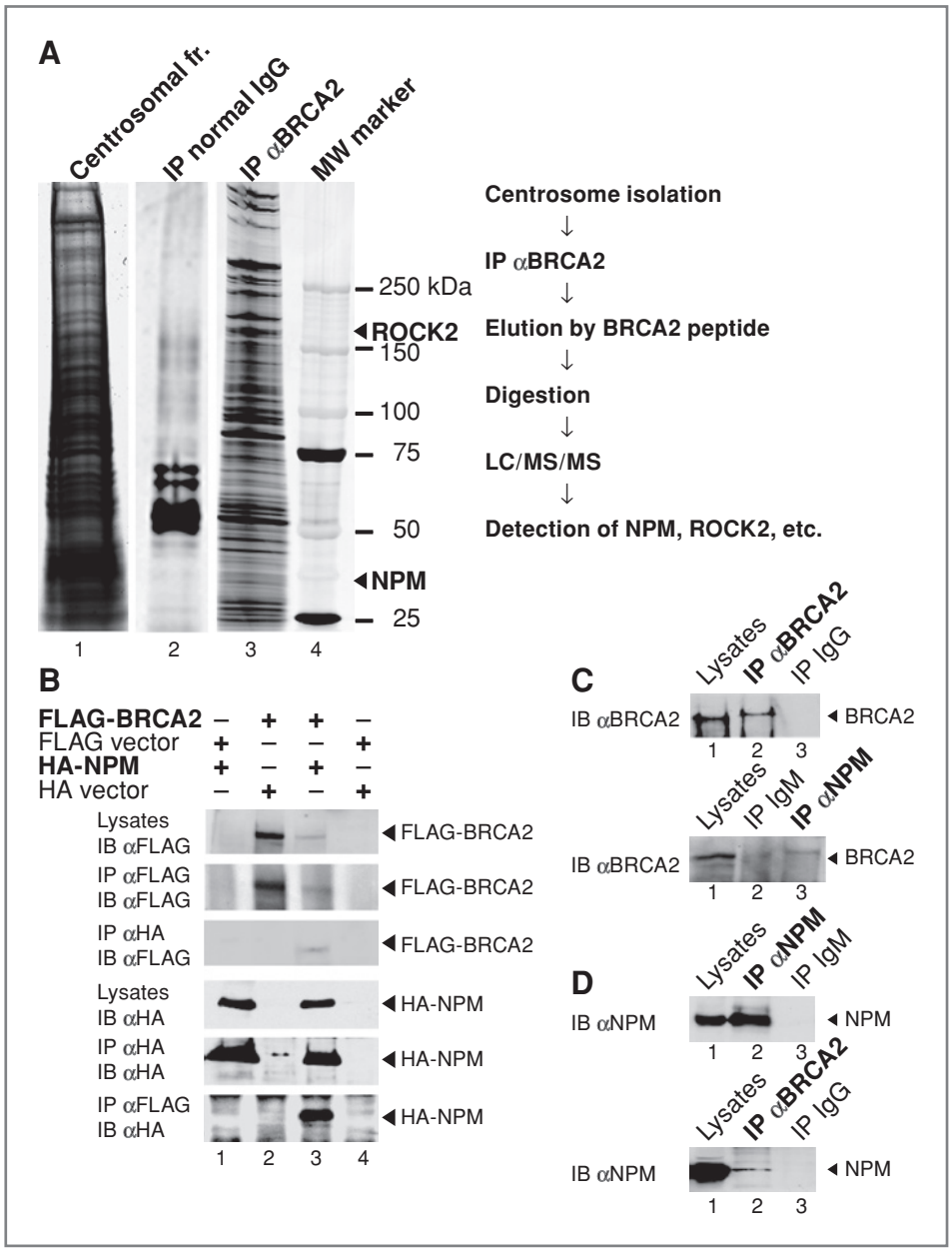

Figure 1. Identification of NPM and ROCK2 as candidates for BRCA2-associated centrosomal proteins and binding of NPM to BRCA2 *in vivo*. A, identification of NPM and ROCK2 as candidates for BRCA2-associated centrosomal proteins by immunoprecipitation and mass spectrometric screening. Centrosomes from HeLa S3 cells were isolated (lane 1), and the centrosomal components were immunoprecipitated with anti-BRCA2 antibody. The immunoprecipitates were subjected to SDS-PAGE and visualized by silver staining (lane 3). The protein bands specific to BRCA2 association were analyzed with LC/MS/MS as summarized. Normal IgG did not precipitated NPM or ROCK2 (lane 2). B, *in vivo* physical interaction between exogenously expressed NPM and BRCA2. COS-7 cells were transiently cotransfected with the combinations of either a FLAG-BRCA2 expression vector or the empty one, and either an HA-NPM expression vector or the empty one. The cell lysates were immunoprecipitated with either anti-HA or anti-FLAG antibody, and the immunoprecipitates were analyzed by immunoblotting with either anti-FLAG (the top 3 panels) or anti-HA antibody (the bottom 3 panels). C and D, binding of endogenous NPM and BRCA2. C, the immunoprecipitates were detected with anti-BRCA2 antibody. Top, lysates from COS-7 cells (lane1) were immunoprecipitated with either anti-BRCA2 antibody (lane 2) or normal mouse IgG (lane 3). Bottom, lysates (lane 1) were immunoprecipitated with either normal mouse IgM (lane 2) or anti-NPM antibody (lane 3). D, The immunoprecipitates were detected with anti-NPM antibody. Top, lysates from COS-7 cells (lane 1) were immunoprecipitated with either anti-NPM antibody (lane 2) or normal mouse IgM (lane 3). Bottom, lysates (lane 1) were immunoprecipitated with either anti-BRCA2 antibody (lane 2) or normal mouse IgG (lane 3). IB, immunoblotting; IP, immunoprecipitation.

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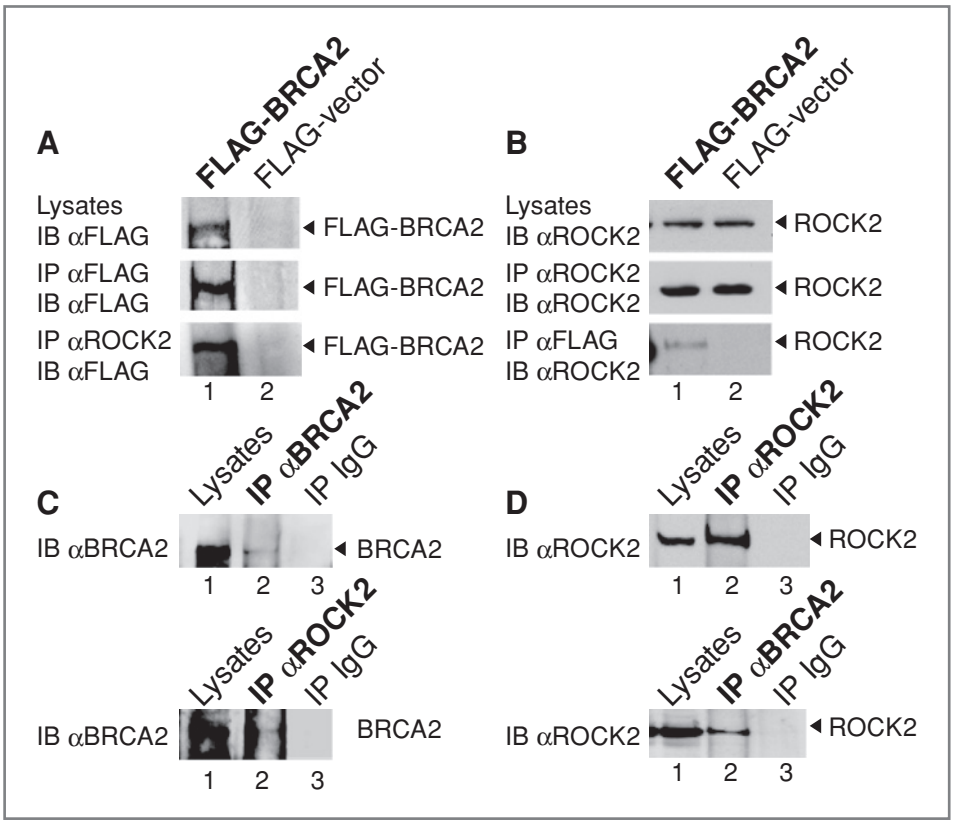

Figure 2. Binding of ROCK2 to BRCA2. A and B, binding of exogenous FLAG-BRCA2 and endogenous ROCK2. COS-7 cells were transiently transfected with either full-length FLAG-BRCA2 expression vector (lane 1) or the empty one (lane 2), respectively. A, The cell lysates (top), the immunoprecipitates with anti-FLAG (middle) and anti-ROCK2 antibodies (bottom) were analyzed by immunoblotting with anti-FLAG antibody. B, the cell lysates (top), the immunoprecipitates with anti-ROCK2 (middle) and anti-FLAG antibodies (bottom) were analyzed by immunoblotting with anti-ROCK2 antibody. C and D, binding of endogenous BRCA2 and ROCK2. C, the immunoprecipitates were detected with anti-BRCA2 antibody. Top, lysates from COS-7 cells (lane 1) were immunoprecipitated with either anti-BRCA2 antibody (lane 2) or the normal mouse IgG (lane 3). Bottom, lysates (lane 1) were immunoprecipitated with either anti-ROCK2 antibody (lane 2) or normal mouse IgG (lane 3). D, the immunoprecipitates were detected with anti-ROCK2 antibody. Top, lysates from COS-7 cells (lane 1) were immunoprecipitated with either anti-ROCK2 antibody (lane 2) or the normal mouse IgG (lane 3). Bottom, lysates (lane 1) were immunoprecipitated with either anti-BRCA2 antibody (lane 2) or normal mouse IgG (lane 3). IB, immunoblotting; IP, immunoprecipitation.

NPM is a substrate of Cdk2/cyclin E and its target is Thr199 (40). We tested whether NPM phosphorylation was changed by inhibiting NPM binding to BRCA2. Expression of FLAG-R3-1 BRCA2 (639–1,000) did not affect NPM phosphorylation on Thr199 (Fig. 4G).

This NPM phosphorylation occurs in early S phase and causes NPM release from the centrosome (31). Costaining studies with NPM and γ-tubulin showed that NPM was absent in centrosomes in all S-phase cells expressing FLAG-R3-1 BRCA2 (639–1,000) as well as in those transfected with an empty vector (Supplementary Fig. S2A). NPM reassociates with centrosomes in M phase (33). It is apparently localized at centrosomes either in the presence or the absence of FLAG-R3-1 BRCA2 (639–1,000; Supplementary Fig. S2B). These results suggested that NPM localization in the cell cycle might not necessarily be affected by the interaction with BRCA2.

The NPM–BRCA2 interaction is required for the numerical integrity of centrosome

To see if the NPM–BRCA2 interaction is required for centrosome amplification, cells were observed by immunofluorescence microscopy when that interaction was inhibited (Fig. 5A). More cells had 3 or more centrosomes in 1 cell when NPM–BRCA2 interaction was abrogated, whereas the control cells had 2 centrosomes at maximum in 1 cell as in the untreated cells (Fig. 5Am–p). We investigated how the amplified centrosomes were generated by the inhibition of NPM–BRCA2 binding. Centrosomes were stained for both γ-tubulin and centrin. A normal centrosome has both γ-tubulin and centrin staining (Fig. 5Am–p). While the all abnormally amplified centrosomes in some cells had both γ-tubulin and centrin staining as a normal centrosome has (Fig. 5Ai–l), some centrosomes in more cells had γ-tubulin staining alone, without centrin (Fig. 5Aa–h, arrows). These results

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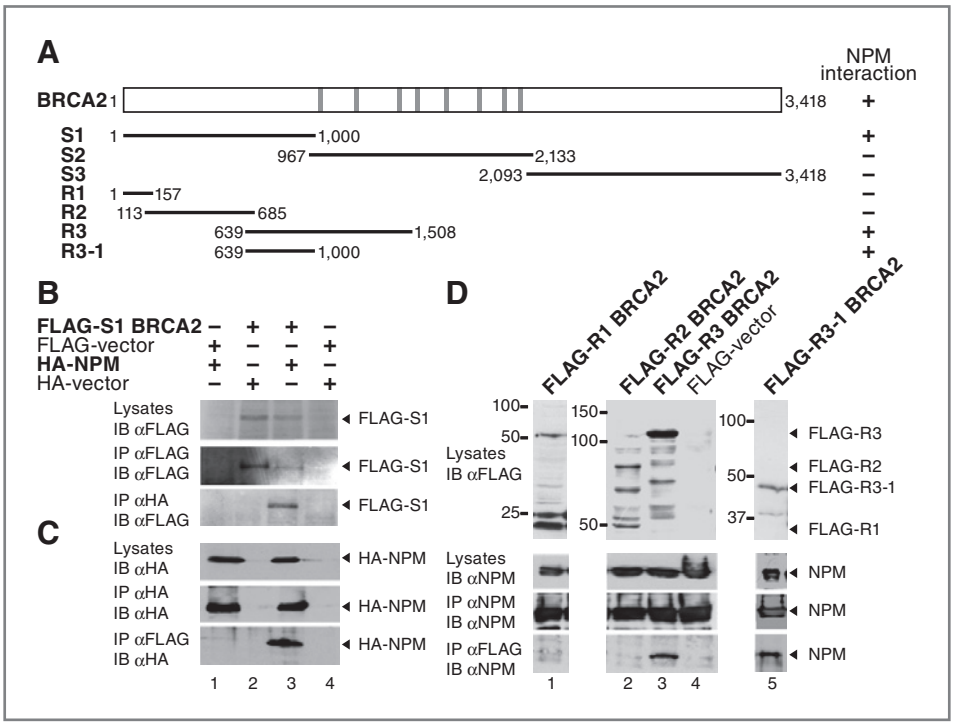

Figure 3. Identification of the binding regions of BRCA2 to NPM. A, diagram of the tested deletion mutants of BRCA2 (1–3,418 amino acids). Abilities to interact with NPM were examined in the following and summarized. The 8 shaded regions stand for BRC repeats, which are responsible for RAD51 binding. S1 BRCA2 (1–1,000) does not contain any BRC repeats. B and C, binding of exogenous FLAG-S1 BRCA2 to HA-NPM. COS-7 cells were cotransfected with the combinations of either a FLAG-S1 BRCA2 expression vector or the empty one, and either a HA-NPM expression vector or the empty one. B, the cell lysates (top) were immunoprecipitated with either anti-FLAG (middle) or anti-HA antibody (bottom). The samples were immunoblotted with anti-FLAG antibody. C, the cell lysates (top) were immunoprecipitated with either anti-HA (middle) or anti-FLAG antibody (bottom). The samples were immunoblotted with anti-HA antibody. D, binding of exogenous deletion mutants of BRCA2 to endogenous NPM. COS-7 cells were transiently transfected with expression vectors for either 4 segments (R1, R2, R3, and R3-1) of FLAG-BRCA2 or the empty one. Immunoprecipitates with anti-FLAG antibody were immunoblotted with anti-NPM antibody (bottom). The arrowheads (top) indicate FLAG-R1 (1–157; lane 1), FLAG-R2 (113–685; lane 2), FLAG-R3 (639–1,508; lane 3), and FLAG-R3-1 (639–1,000; lane 5) BRCA2, respectively. IB, immunoblotting; IP, immunoprecipitation.

suggested that inhibition of NPM–BRCA2 association abrogated the numerical integrity of centrosome and the amplified centrosomes were generated by centrosome fragmentation and to lesser degrees by centrosome overduplication (Fig. 5B).

The NPM–BRCA2 interaction is critical for genomic stability

We further examined the nucleus profiles by immunofluorescence microscopy (Supplementary Fig. S3). A significantly higher fraction of multinucleated cells was observed when NPM–BRCA2 interaction was abrogated (Fig. 5C). These findings suggested that the inhibition of NPM binding to BRCA2 leads to supernumerary centrosomes, which could induce the formation of multipolar spindles and aneuploidy.

Discussion

In an attempt to determine the biological significance of BRCA2 on centrosomal duplication and hereditary breast cancer, we isolated centrosomes and identified NPM and ROCK2 as BRCA2-associated proteins. Among many candidates coimmunoprecipitated with BRCA2, we selected these 2 candidates for further analyses, as we have shown that BRCA2 has a function in centrosomes (9) and NPM and ROCK2 were also already shown to have a function in the regulation of centrosome duplication (32). We showed that endogenous NPM and ROCK2 bind to BRCA2 *in vivo*. While the association of BRCA2 with NPM and ROCK2 has not been previously reported, some functions of NPM and ROCK2 in centrosomes were shown to overlap with those of BRCA2. For example, BRCA2 is a substrate of Crm1 and localizes to centrosomes during G_1 phase to early M phase. Dysfunction of BRCA2 in centrosomes causes abnormal cell division, leading to multicentrosomal and multinucleated cells (9, 10). NPM is also a substrate of Crm1 (30) and is associated with unduplicated centrosome (31). ROCK2 is a Rho effector kinase and a centrosomal protein that physically interacts with NPM and copromotes centrosome duplication (32).

How BRCA2 regulates centrosome duplication together with NPM remained to be elucidated. We attempted to inhibit NPM binding to endogenous BRCA2 to show the functional significance of this binding. Because interference by RNAi

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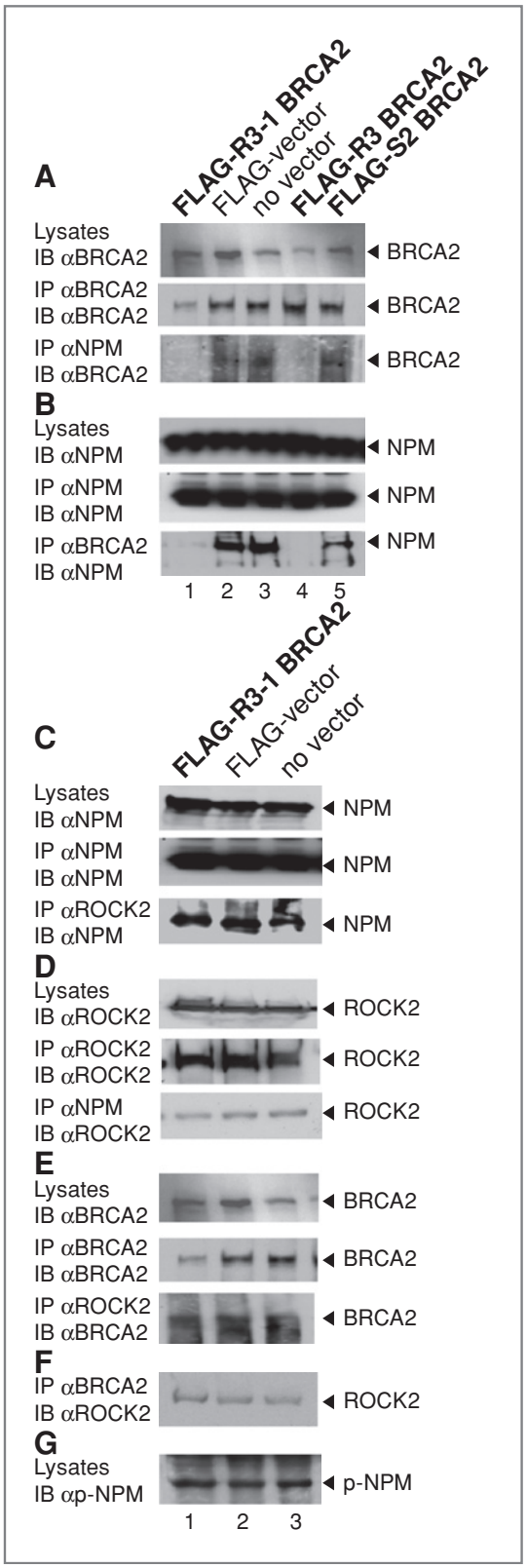

usually leads to the depletion of mRNA expression and suppression of all functions of a target gene in cells, it is difficult to see which ability of the target gene is responsible for the aberrant centrosome amplification. Therefore, we employed physical competition to prevent NPM from binding to endogenous BRCA2 by exogenous expression of a small region of BRCA2 that interacts with NPM. We first determined that NPM-binding site localized to a region between amino acid resides 639–1,000 of BRCA2. Because negative control fragment of 967–2,133 did not bind to NPM (Supplementary Fig. S1A and B) and its expression could not interfere with NPM–BRCA2 binding (Fig. 4A and B, lane 5), actual binding region might be narrowed to 639–966. An exogenous overexpression of FLAG-R3-1 BRCA2 (639–1,000) suppressed NPM–BRCA2 association but did not disturb the interactions between NPM–ROCK2 or BRCA2–ROCK2. This would suggest that the binding sites on NPM to BRCA2 and ROCK2 as well as those on BRCA2 to NPM and ROCK2 might be located in different regions. It also implies that BRCA2, NPM, and ROCK2 might form trimers, though a single-molecule analysis may be required to prove this hypothesis. The numerical integrity of centrosomes was observed by immunofluorescence microscopy. The results showed that physical inhibition of NPM–BRCA2 interaction in cells could lead to an amplification of centrosome numbers and a high frequency of multinucleated cells. This showed that NPM–BRCA2 interaction is required for maintenance of numerical integrity of centrosomes, whose abrogation could result in supernumerary centrosomes. Unrestricted centrosome amplification would induce genomic instability, which could facilitate tumorigenesis. Loss of association between NPM and BRCA2 might play an important role in familial breast carcinogenesis.

Detailed molecular mechanism of NPM and BRCA2 for centrosome regulation remains undefined. Centrosome duplication is also controlled by Ran-Crm1 network and their substrates, such as BRCA1, BRCA2, NPM, p53, and other NES-containing proteins (30). It would be hypothesized that NPM and/or BRCA2 could not associate with Crm1 when NPM–BRCA2 interaction was abrogated.

Figure 4. Inhibition of binding between endogenous NPM and BRCA2 by FLAG-R3-1 BRCA2. A and B, COS-7 cells were transiently transfected with expression vectors for either 3 segments (R3-1, R3, and S2) of FLAG-BRCA2 or the empty one. A, the cell lysates were immunoprecipitated with either anti-BRCA2 or anti-NPM antibody, and the immunoprecipitates were immunoblotted with anti-BRCA2 antibody. B, the cell lysates were immunoprecipitated with either anti-NPM antibody or anti-BRCA2 antibody, and the immunoprecipitates were immunoblotted with anti-NPM antibody. C–G, COS-7 cells were transiently transfected with either FLAG-R3-1 BRCA2 or the empty vector. C, the cell lysates were immunoprecipitated with either anti-NPM antibody or anti-ROCK2 antibody, and the immunoprecipitates were immunoblotted with anti-NPM antibody. D, the cell lysates were immunoprecipitated with either anti-ROCK2 or anti-NPM antibody, and the immunoprecipitates were immunoblotted with anti-ROCK2 antibody. E, the cell lysates were immunoprecipitated with either anti-BRCA2 or anti-ROCK2 antibody, and the immunoprecipitates were immunoblotted with anti-BRCA2 antibody. F, the cell lysates were immunoprecipitated with anti-BRCA2 antibody, and the immunoprecipitates were immunoblotted with anti-ROCK2 antibody. G, the cell lysates were immunoblotted with anti–p-NPM (Thr199) antibody. IB, immunoblotting; IP, immunoprecipitation.

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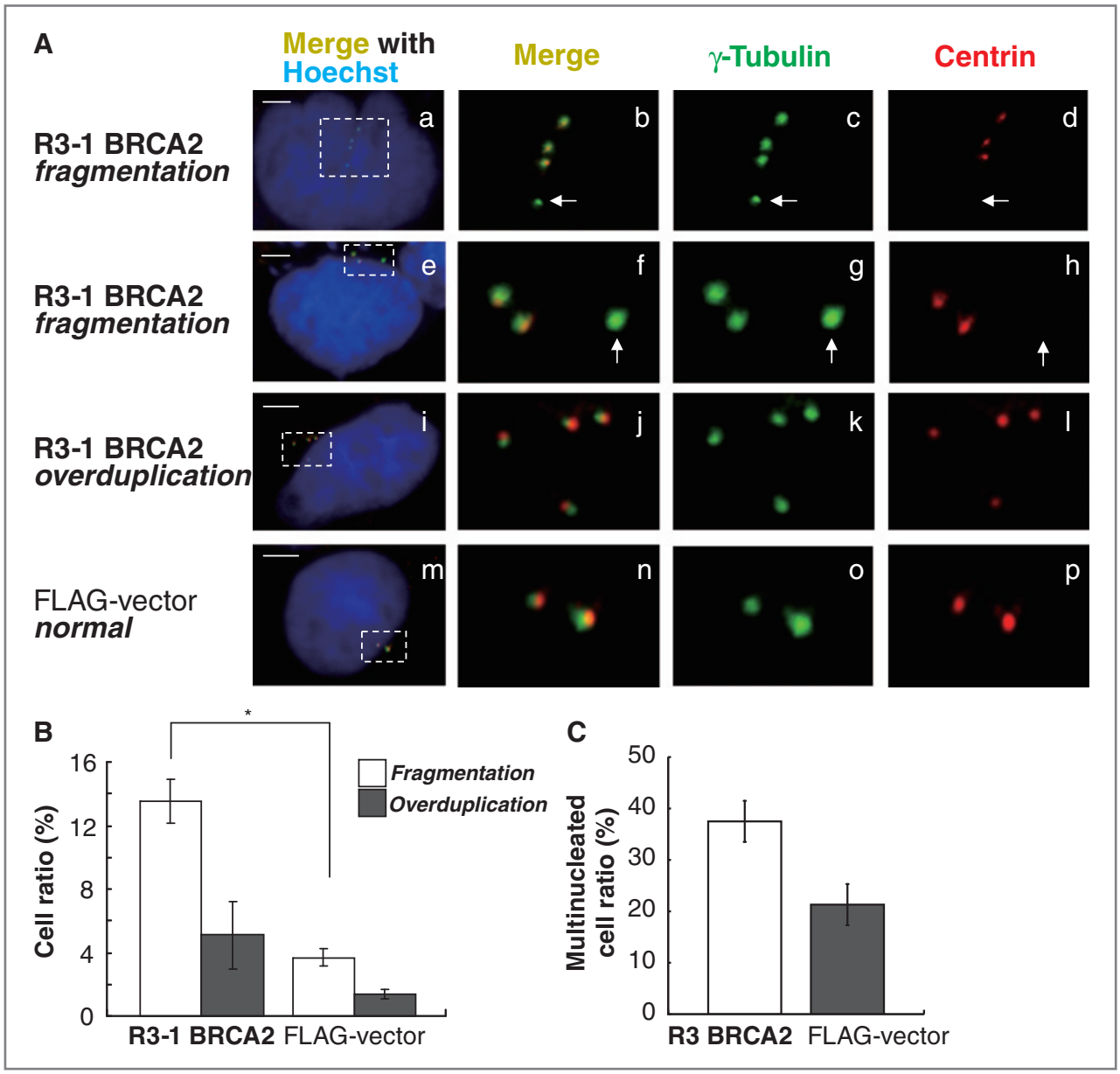

Figure 5. Types of abnormal amplification of centrosomes by inhibiting interaction between endogenous BRCA2 and NPM. A and B, COS-7 cells were transfected with a FLAG-R3-1 BRCA2 expression vector or the empty one. A, the cells were stained for γ-tubulin (green), centrin (red), and DNA (blue). The arrows indicate γ-tubulin staining without centrin. Bar, 5 μm. B, the percentage of the transfected cells that had either centrosome fragmentation or overduplication was plotted. At least 325 cells were quantified. Columns, averages ± standard errors from 3 independent experiments. *, $P = 0.034$ (Mann–Whitney *U* test). C, COS-7 cells were transfected with either a FLAG-R3 BRCA2 expression vector or the empty one. The percentage of multinucleated cells was plotted. At least 321 cells were quantified. Columns, averages ± standard errors from 3 independent experiments.

It was reported that ROCK2 has a high affinity with NPM in centrosomes in late G_1 phase and coregulates the initiation of centrosome duplication (32). NPM is associated with unduplicated centrosome as a target of Cdk2/cyclin E. BRCA2 localizes to the centrosome during G_1 phase to early M phase (12). Taken together with our present data, it would be hypothesized that NPM, ROCK2, and BRCA2 form a complex to maintain integrity of centrosome duplication and genomic stability (Fig. 6).

We showed that NPM-binding region of BRCA2 localized within 639–1,000 amino acids. Other molecular function of this region has not been reported, whereas a lot of missense mutations in this region in the hereditary breast and/or ovarian cancer families were reported (Breast Cancer Information

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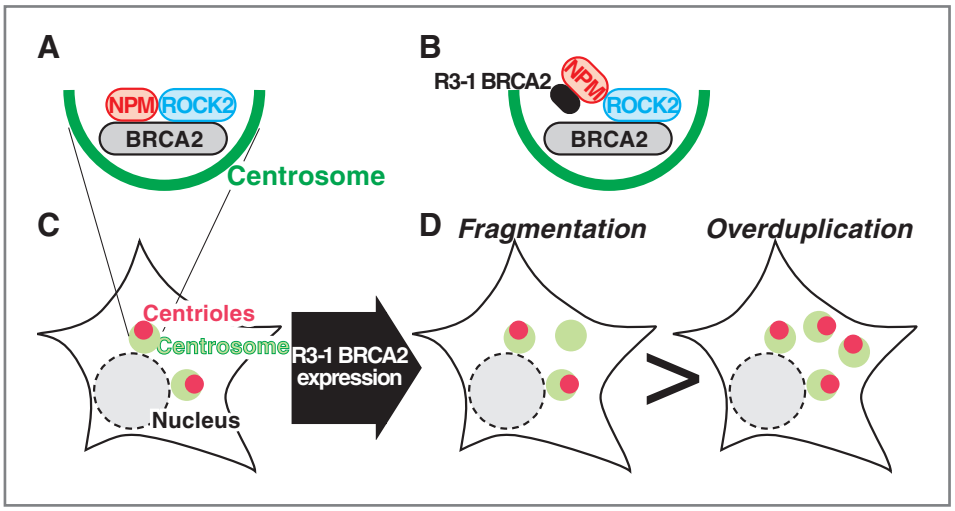

Figure 6. Model of abnormal centrosome amplification by abrogation of NPM–BRCA2 interaction. An exogenous overexpression of FLAG-R3-1 BRCA2 (639–1,000) dissociates endogenous NPM from BRCA2 (B). Under this condition, centrosome duplication is not correctly regulated and centrosome fragmentation and overduplication occur (D). The former happens more.

Core). Interactions between NPM and BRCA2 might be suppressed by these mutations. Our findings would help to elucidate the molecular mechanism of carcinogenesis in these cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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