BRCA2 Phosphorylated by PLK1 Moves to the Midbody to Regulate Cytokinesis Mediated by Nonmuscle Myosin IIC

Miho Takaoka1, Hiroko Saito2, Katsuya Takenaka1, Yoshio Miki1,2, and Akira Nakanishi1

Abstract

Cytokinesis is the critical final step in cell division. BRCA2 disruption during cytokinesis is associated with chromosome instability, but mechanistic information is lacking that could be used to prevent cancer cell division. In this study, we report that BRCA2 phosphorylation by the mitotic polo-like kinase (PLK1) governs the localization of BRCA2 to the Flemming body at the central midbody, permitting an interaction with nonmuscle myosin IIC (NM-IIC). Formation of an NM-IIC ring-like structure at the Flemming body shows that the IIC-ring relies on its ATPase activity stimulated by interaction with BRCA2 and associated proteins. Notably, inhibiting this binding inactivated the ATPase activity, causing disassembly of the IIC-ring, defective formation of the midbody, and interruption of cytokinesis. An analysis of cancer-associated mutations in BRCA2 at the PLK1-binding site suggests that they may contribute to cytokinetic defects by altering BRCA2 localization. Our findings suggest that BRCA2-dependent IIC-ring formation is a critical step in proper formation of the midbody, offering an explanation for how chromosome instability may arise in breast cancer.

Cancer Res; 74(5); 1–11. ©2014 AACR.

Introduction

Germline mutations in the BRCA2 gene have been reported to increase the risk of developing breast and ovarian cancer. The BRCA2 protein has multiple functions, including DNA double-strand break repair (1–3), and the regulation of centrosome amplification and localization (4, 5). BRCA2 also contributes to the regulation of cytokinesis (6, 7). During anaphase, constriction of the actomyosin ring leads to formation of a cleavage furrow (8–10). Continued furrowing results in the formation of a narrow intercellular bridge, which contains the midbody, consisting of a bundled microtubule and a ring-like structure called the Flemming body within its central portion (11). Several studies have implicated various protein factors in the modulation of cytokinesis by BRCA2 (12–14). Disruption of BRCA2 during cytokinesis leads to disorganization of myosin-II at the cleavage furrow and the intercellular bridge (6).

Mitotic polo-like kinase 1 (Plk1) is a key regulator of mitosis from mitotic initiation to cytokinesis. Plk1 contains a serine/threonine kinase domain followed by the carboxy-terminal polo-box domain (PBD), which binds to phosphopeptides within a consensus motif of S-[pS/pT]-[P/X] (15, 16). The PBD regulates cellular function, the interaction with substrates, and the subcellular localization of Plk1 (17). Plk1 is also required for appropriate localization of substrates (18). Previous studies revealed that Plk1 binds to the N-terminal region of BRCA2 and phosphorylates Ser193, and that this phosphorylation is enhanced as mitosis progresses (19, 20). However, the specific Plk1-binding site within this region of BRCA2 has not been identified. The role of BRCA2 phosphorylation in the maintenance of genome stability also remains unclear.

Nonmuscle myosin-II (NM-II) proteins in humans are hexamers, consisting of a pair of heavy chains and two pairs of light chains that hydrolyze MgATP. They are members of a family of actin-binding motor proteins that play essential roles in cellular processes such as cell division and embryonic development. The NM-II family comprises three isoforms: NM-IIA, NM-IIB, and NM-IIC. These contain different nonmuscle myosin heavy chains (NMHC-IIA, NMHC-IIB, and NMHC-IIC) that are encoded by MYH9 and MYH10, respectively (21, 22). The N-terminal region of the NMHC-II protein consists of a globular head containing the actin-binding region, an ATPase domain, and a Src homology 3 (SH3)-like domain (myosin head; refs. 23, 24). NM-IIC is alternatively spliced both in loop-1 and loop-2. Isoform NM-IIC0 contains no inserts in either of the loops. An 8-amino acid extension in the loop-1 region is present in isoforms NM-IIC1 and NM-IIC1C2. Isoform NM-IIC1C2 displays a 33–amino acid extension in the loop-2 region. The presence of 8 amino acid insert in NM-IIC increases the actin-activated ATPase activity (25). The C-terminal deletion isoform of NM-IIC is already present in the Ensembl database (http://www.ensembl.org/index.html; MYH14-007, Protein ID: ENSP00000469573). Jana
and colleagues reported the localization of NM-IIC to the midbody during the process of abscission. In that report, they confirmed that NM-IIC1 is required for cytokinesis (25). Accordingly, we made use of this isoform. In this study, we suggest that BRCA2-dependent IIC-ring formation represents a key step in proper midbody formation, and that a hereditary breast cancer-associated mutation within the Plk1 interaction motif of BRCA2 affects the localization of BRCA2 to the Flemming body, resulting in cytokinesis defects.

Materials and Methods

Detailed descriptions of plasmids, transfections, siRNA treatment, antibodies, immunofluorescence and three-dimensional (3D) reconstitutions, immunoprecipitation, Western blot analysis, binding assays, glycerol density gradient centrifugation, time-lapse microscopy, measurement of ATPase activity, cell-cycle analysis, and statistical analysis are provided in the Supplementary Materials and Methods.

Cell culture

HeLaS3 and COS-7 cells were purchased from the RIKEN GENBANK, and U2OS and A549 were from American Type Culture Collection (ATCC). These cells were cultured in Dulbecco’s Modified Eagle Medium containing 10% fetal calf serum (FCS). MCF7 was purchased from ATCC and cultured in Eagle’s Minimum Essential Medium containing 10% FCS, 0.01 mg/mL bovine insulin, and 1% sodium pyruvate. The identities of HeLaS3, U2OS, A549, and MCF7 cell lines were confirmed that NM-IIC1 is required for cytokinesis (25). We then isolated the midbodies from HeLaS3 cells (Supplementary Fig. S1F) and compared the extent of BRCA2 phosphorylation in midbody lysates with that in whole-cell lysates (Fig. 1B). A greater fraction of the BRCA2 was phosphorylated in the midbody. Using immunofluorescence microscopy, we showed that Plk1 colocalizes with pS193, BRCA2 at the Flemming body (Fig. 1C). Assays of COS-7 cells transfected with BRCA2-FLAG and Plk1-HA demonstrated that BRCA2 and Plk1 coimmunoprecipitated (Fig. 1D). We located a Plk1-binding motif (S-T-P sequence at codons 76–78) in the N-terminal region of BRCA2. A Thr77-phosphorylated peptide corresponding to this region (a.a. 73–82 of BRCA2) was synthesized for in vitro binding analyses. Streptavidin bead-immobilized p-Thr77 peptide precipitated Plk1 from mitotic HeLaS3 cell lysates, whereas unphosphorylated peptide or peptide-free streptavidin beads did not (Fig. 1E). Plk1 was not precipitated from S-phase lysates due to low expression.

This Plk1-binding site is conserved among diverse species (Supplementary Fig. S1G). A hereditary breast cancer-associated missense mutation (T77A) within this site is annotated in the Breast Cancer Information Core (BIC) database (http://research.nhri.nih.gov/bic/). We constructed plasmids encoding a truncated N-terminal region [BRCA2 (R1)-FLAG (WT);1–157 a.a.] containing either the wild-type Plk1-binding site or this mutant, BRCA2 (R1)-FLAG (T77A; Supplementary Fig. S1H). The mutant protein was unable to interact with endogenous Plk1 in HeLaS3 cells (Supplementary Fig. S1I), indicating that this mutation disrupted the interaction with Plk1. Next, we examined whether overexpression of this truncated region could compete with endogenous BRCA2. Coimmunoprecipitation assays were performed in HeLaS3 cells expressing BRCA2 (R1)-FLAG (WT). It was demonstrated that this region inhibited the binding of endogenous BRCA2 to Plk1 (Supplementary Fig. S1J). Also, the endogenous BRCA2 ceased to be detected at the Flemming body following ectopic expression of this region (Supplementary Fig. S1K). These results suggested a dominant-negative effect of HA-BRCA2 (R1) upon endogenous BRCA2. A distinct HA-tagged BRCA2 region [HA-BRCA2 (R2):

Results

Localization of BRCA2 to the Flemming body through phosphorylation by Plk1

To analyze the role of phosphorylation of BRCA2 Ser193 by Plk1, we generated an antibody (93pBRCA2) specific for this phosphoprotein and confirmed the specificity of the 93pBRCA2 antibody by performing a competition assay using pS193- and peptide with protein phosphatase (PP2A; Supplementary Fig. S1A and S1B). This antibody recognized pS193-phosphorylated BRCA2 (pS193-BRCA2) in anti-BRCA2 (Ab-1) immunoprecipitates from midbody lysates (Supplementary Fig. S1C). We observed its localization during the cell cycle by immunofluorescence microscopy. BRCA2 localized to the midbody (particularly at the Flemming body) during cytokinesis of mitotic HeLaS3 cells (Fig. 1A). Similar findings were observed in A549, MCF7, and U2OS cells (Supplementary Fig. S1D). The phosphorylated BRCA2 also localized to the Flemming body (Fig. 1A). In contrast, BRCA1 did not localize to the Flemming body (Fig. 1A and Supplementary Fig. S1E). This result is consistent with that of a previous study by another group (13). We then isolated the midbodies from HeLaS3 cells (Supplementary Fig. S1F) and compared the extent of BRCA2 phosphorylation in midbody lysates with that in whole-cell lysates (Fig. 1B). A greater fraction of the BRCA2 was phosphorylated in the midbody. Using immunofluorescence microscopy, we showed that Plk1 colocalizes with pS193, BRCA2 at the Flemming body (Fig. 1C). Assays of COS-7 cells transfected with BRCA2-FLAG and Plk1-HA demonstrated that BRCA2 and Plk1 coimmunoprecipitated (Fig. 1D). We located a Plk1-binding motif (S-T-P sequence at codons 76–78) in the N-terminal region of BRCA2. A Thr77-phosphorylated peptide corresponding to this region (a.a. 73–82 of BRCA2) was synthesized for in vitro binding analyses. Streptavidin bead-immobilized p-Thr77 peptide precipitated Plk1 from mitotic HeLaS3 cell lysates, whereas unphosphorylated peptide or peptide-free streptavidin beads did not (Fig. 1E). Plk1 was not precipitated from S-phase lysates due to low expression.

This Plk1-binding site is conserved among diverse species (Supplementary Fig. S1G). A hereditary breast cancer-associated missense mutation (T77A) within this site is annotated in the Breast Cancer Information Core (BIC) database (http://research.nhri.nih.gov/bic/). We constructed plasmids encoding a truncated N-terminal region [BRCA2 (R1)-FLAG (WT);1–157 a.a.] containing either the wild-type Plk1-binding site or this mutant, BRCA2 (R1)-FLAG (T77A; Supplementary Fig. S1H). The mutant protein was unable to interact with endogenous Plk1 in HeLaS3 cells (Supplementary Fig. S1I), indicating that this mutation disrupted the interaction with Plk1. Next, we examined whether overexpression of this truncated region could compete with endogenous BRCA2. Coimmunoprecipitation assays were performed in HeLaS3 cells expressing BRCA2 (R1)-FLAG (WT). It was demonstrated that this region inhibited the binding of endogenous BRCA2 to Plk1 (Supplementary Fig. S1J). Also, the endogenous BRCA2 ceased to be detected at the Flemming body following ectopic expression of this region (Supplementary Fig. S1K). These results suggested a dominant-negative effect of HA-BRCA2 (R1) upon endogenous BRCA2. A distinct HA-tagged BRCA2 region [HA-BRCA2 (R2):

Material and Methods

Detailed descriptions of plasmids, transfections, siRNA treatment, antibodies, immunofluorescence and three-dimensional (3D) reconstitutions, immunoprecipitation, Western blot analysis, binding assays, glycerol density gradient centrifugation, time-lapse microscopy, measurement of ATPase activity, cell-cycle analysis, and statistical analysis are provided in the Supplementary Materials and Methods.

Cell culture

HeLaS3 and COS-7 cells were purchased from the RIKEN GENBANK, and U2OS and A549 were from American Type Culture Collection (ATCC). These cells were cultured in Dulbecco’s Modified Eagle Medium containing 10% fetal calf serum (FCS). MCF7 was purchased from ATCC and cultured in Eagle’s Minimum Essential Medium containing 10% FCS, 0.01 mg/mL bovine insulin, and 1% sodium pyruvate. The identities of HeLaS3, U2OS, A549, and MCF7 cell lines were confirmed by short tandem repeat (STR) profiling by BEX CO., Ltd and used within 6 months of testing. COS-7 cells (monkey cell line) were not analyzed by STR profiling because STR profiling is method for authentication of human cell lines.

Midbody isolation

Mitotic cells (A549) were released from colcemid treatment (50 ng/mL) for 17 hours by washing twice with fresh medium. After incubating at 37°C for 60 minutes, the cells were collected. Midbody isolation was performed according to the methods of Mullins and McIntosh (26).

Measurement of ATPase activity

COS-7 cells were transfected with plasmids expressing HA-tagged NMHC-IIC and FLAG-tagged BRCA2. After 24 hours, cells (5 × 10⁶) were lysed in lysis buffer [20 mmol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl, 1 mmol/L EDTA, 1% NP-40, and protease inhibitors]. BRCA2 and NMHC-IIC were immunoprecipitated by anti-FLAG or anti-HA antibodies, respectively. Each sample was eluted by 40 μL elution buffer (50 mmol/L glycine, pH 2.8), which was immediately neutralized with 1 mol/L Tris. BRCA2-FLAG and NMHC-IIC-HA were mixed and incubated at room temperature for 1 minute and then placed into a solution for measurement of ATPase activity. F-actin was added immediately before the measurement. ADP production during ATP hydrolysis was measured as a change in NADH concentration. NADH absorbance was measured every 2 seconds at 340 nm and the rate of NADH consumption was interpreted as the ATPase activity.
112–685 a.a.] did not affect the localization of the endogenous BRCA2 (Supplementary Fig. S1K). To evaluate further the effect of BRCA2 phosphorylation at Ser193 upon its localization, we expressed either wild-type recombinant BRCA2-FLAG or mutants thereof containing substitutions of Ser193 by alanine (S193A) or glutamate (S193E). Although both the wild-type and the S193E mutant localized to the Flemming body, the S193A mutant did not (Supplementary Fig. S1L). This suggested that phosphorylation of BRCA2 at Ser193 by Plk1 is important for its localization to the Flemming body and that a cancer-associated mutation of BRCA2 might prevent phosphorylation leading to its mislocalization.

**Interaction between BRCA2 and NM-IIC**

BRCA2 deficiency causes abnormal accumulation of myosin-II, particularly during cytokinesis (6). However, the physiologic role of colocalization of BRCA2 and myosin-II to the midbody remains unclear. We first examined the subcellular localization of BRCA2 to the Flemming body in HeLaS3 cells. A, cells were fixed and stained for Ser193-phosphorylated BRCA2 (pS193-BRCA2), BRCA2, and BRCA1 throughout the cell cycle. Nuclei were detected by Hoechst 33258 stain (blue). Arrows, the Flemming body. Scale bar, 5 μm. B, total whole-cell and midbody lysates were subjected to Western blot analysis using antibodies against pS193-BRCA2 and BRCA2. IB, immunoblot. C, cells were fixed and stained for pS193-BRCA2 (green) and Plk1 (red) in cytokinesis. White arrows, the Flemming body. Scale bar, 5 μm. D, COS-7 cells were transfected with Plk1-HA and/or BRCA2-FLAG expression plasmids. The expression of transgene-derived proteins in input cell lysates and anti-FLAG immunoprecipitates (IP) analyzed using anti-HA or anti-FLAG antibodies are shown. E, N-terminal biotin-fused pThr77 peptides (QLASpTPIIEK) were linked to streptavidin beads and tested for the ability to precipitate Plk1 from S-phase or mitotic HeLaS3 cell lysates. The phosphorylated Thr77 residue and the Ser76 residue crucial for PBD binding are indicated with asterisks (*). Immunoblot analyses show Plk1 coprecipitated with the peptides.
localization of each NM-II isoform in A549 cells during cytokinesis (Fig. 2A). NM-IIC localized to the Flemming body and formed a ring-like structure. In contrast, NM-IIB was diffusively distributed throughout the midbody, and NM-IIB was localized within an area from the basal portion of the midbody to the cytosol of the dividing cell. Similar results were obtained in human mammary epithelial cells (Supplementary Fig. S2A). Further analysis of 3D images by confocal laser microscopy clearly demonstrated that NM-IIC formed part of a unique ring-like structure within the central portion of the midbody (Supplementary Fig. S2B). This structure, which we term the IIC-ring, was approximately 1.5 μm in diameter and colocalized with the Flemming body. To characterize this BRCA2-NM-IIC colocalization further, we analyzed its sedimentation in a glycerol gradient following isolation from A549 cells (Fig. 2B and Supplementary Fig. S2C). The pS193-BRCA2, NM-IIC, Plk1, and MgcRacGAP (a marker of the midbody) were all detected within fraction 2, whereas NM-IIB was detected in fraction 3, and NM-IIA was present in several fractions (1–8). BRCA1 was not found at the midbody and nonphosphorylated BRCA2 was mainly detected within fraction 4 (Supplementary Fig. S2C). These results suggested that pS193-BRCA2 might interact with NM-IIC and MgcRacGAP in addition to Plk1 at the Flemming body. BRCA2-FLAG and NMHC-IIC-HA were coexpressed in COS-7 cells and cell lysates were subjected to immunoprecipitation with anti-FLAG and anti-HA antibodies. NMHC-IIC-HA and BRCA2-FLAG were both detected in the anti-FLAG or anti-HA immunoprecipitates (Fig. 2C). Endogenous BRCA2 immunoprecipitates from A549 cells were shown to contain NMHC-IIC (Fig. 2D and Supplementary Fig. S2D). This result was validated by two different antibodies that recognized distinct epitopes of NMHC-IIC (Fig. 2D) and by mass spectrometric analysis (Supplementary Fig. S2D). Immunoprecipitations from A549 cell lysates using anti-BRCA2 antibody revealed the presence of several protein components in the BRCA2 complex. Polypeptides with apparent molecular weights of 485, 385, 220, 156, and 122 kDa coimmunoprecipitated with BRCA2 (Supplementary Fig. S2D). As a control, we also performed immunoprecipitation with normal rabbit immunoglobulin G (IgG). These polypeptides were digested in gel by trypsin, and the resulting peptides were sequenced by nanoelectrospray mass spectrometry. Besides NMHC-IIC and BRCA2, polycystin-1 (PKD1), ATM, Rho-associated protein kinase 1 (ROCK1), and PARP1 were coimmunoprecipitated by the anti-BRCA2 antibody (Supplementary Fig. S2D). In contrast, NM-IIB did not bind to BRCA2 in this assay. Endogenous MgcRacGAP did not also bind to BRCA2 or NM-IIC (Supplementary Fig. S2E and S2F) despite its colocalization to the Flemming body.

Abnormal cytokinesis following NMHC-IIC gene silencing

To investigate the role of the IIC-ring during cell division, we observed the formation of the midbody 24 hours following siRNA knockdown of NMHC-IIC (Fig. 3A). Midbody structures in most siRNA-control cells were bundled and formed the Flemming bodies (Fig. 3B, top). In contrast, a majority of the cells treated with the NMHC-IIC siRNA retained midbody fibers, that is, exhibited a nonbundled midbody and lacked the Flemming body (Fig. 3B, bottom). The fraction of abnormal cells (exhibiting a non-Flemming body) was 25.2 ± 3.6% in the control cells and 63.5 ± 3.6% in the knockdown cells (Fig. 3B). At 48 hours, we observed a 1.5- to 3-fold increase in the number of multinuclear cells in response to NMHC-IIC knockdown (IIC-1: 57.2 ± 4.5%; IIC-2: 36.3 ± 1.7%) compared with control cells (scrambled control: 21.0 ± 2.0%; no-transfection: 15.8 ± 4.5%; Supplementary Fig. S3A). The NMHC-IIC knockdown cells were observed using time-lapse differential interference microscopy for 48 hours after siRNA treatment. A subpopulation of the cells had fused back together, resulting in bunucleated cells. Another subpopulation exhibited catastrophic cell death before cytokinesis completion (Supplementary Fig. S3B). This process is shown in greater detail by time-lapse images (Supplementary Fig. S3C).

Inhibition of IIC-ring formation by BRCA2 gene silencing

To see an effect of BRCA2 suppression on the IIC-ring formation, we silenced BRCA2 using siRNA in A549 cells, which express all three isoforms: IIA, IIB, and IIC (Fig. 3C). As observed in the NMHC-IIC knockdown cells, midbody fibers were present in the absence of the Flemming body in the BRCA2 knockdown cells. Consequently, NM-IIC was distributed throughout the nonbundled midbody, and the IIC-ring was not apparent (Fig. 3D and Supplementary Fig. S3D). A greater number of abnormal midbodies were observed in siRNA-BRCA2–treated cells (siRNA-BRCA2-1: 48.3 ± 2.9%; siRNA-BRCA2-2: 43.3 ± 7.6%) than in control cells (siRNA-luciferase: 21.7 ± 2.9%; no-transfection: 10.0 ± 5.0%; \( P = 0.017 \); Fig. 3D, right). Similar results were obtained in U2OS cells treated with siRNA targeting BRCA2 (Supplementary Fig. S3E and S3F). To determine whether BRCA2 is important for the formation of midbody structures and progression of cytokinesis, we analyzed the BRCA2 knockdown HeLa S3 cells undergoing cytokinesis. BRCA2 protein levels were reduced after siRNA application (Fig. 3E), and the length of the midbody during cytokinesis in HeLa S3 cells was greater in BRCA2 siRNA-treated cells than in control cells (siRNA-luciferase or no-transfection; \( P < 0.01 \); Fig. 3F). Next, we examined whether the length of the midbody in siRNA-BRCA2 cells could be restored by expressing BRCA2-FLAG or BRCA2 (S193A)-FLAG (Supplementary Fig. S3G and S3H). Although BRCA2-FLAG localization was observed in the midbody, this was not seen in BRCA2 (S193A)-FLAG cells (Supplementary Fig. S3H, left). When BRCA2-FLAG was expressed, the length of the midbody was similar to that in the control (siRNA-luciferase). In contrast, the length of the midbody did not recover in cells expressing BRCA2 (S193A)-FLAG (\( P = 0.041 \); Supplementary Fig. S3H, right), suggesting that it could not localize to the midbody. We did not observe an abnormal midbody or IIC-ring in cells treated with the BRCA1-siRNA (Supplementary Fig. S4A–S4E). These results suggest that the formation of the midbody and IIC-ring may require the presence of BRCA2, but not BRCA1, at the Flemming body and that the binding of BRCA2 to NM-IIC is necessary for this process.
Figure 2. NM-IIC localizes to the Flemming body and interacts with BRCA2. A, immunofluorescence in A549 cells stained for NMHC-IIA (green), IIB (green), IIC (green), and α-tubulin (red). Cells in cytokinesis were also costained for NMHC-IIC (green or red) and Plk1 (red) or MgcRacGAP (green). Scale bar, 5 μm. B, midbody lysates from A549 cells were separated by 15% to 35% (w/v) glycerol gradient centrifugation and subjected to Western blot analysis using the indicated antibodies. The nuclear marker, TOPO2α, was not detected in any fractions. C, COS-7 cells were transfected with the indicated expression plasmids. The expression of transgene-derived proteins in cell lysates, anti-FLAG immunoprecipitates (IP), and anti-HA immunoprecipitates (IP) revealed by anti-FLAG or anti-HA antibodies are shown. D, A549 cell lysates were immunoprecipitated (IP) with either mouse IgG or anti-BRCA2 antibodies and immunoprecipitates were analyzed using polyclonal antibodies to different epitopes of NMHC-IIC at the N-(NMHC-IIC-N) and C-(NMHC-IIC-C) terminals. Samples were also analyzed by anti-BRCA2 and anti–NMHC-IIB antibodies.
Figure 3. NM-IIIC and BRCA2 are essential for midbody formation in A549 cells. A, lysates from A549 cells treated with NMHC-IIIC siRNA (IIC-1 or IIC-2) or siRNA-scramble (SC), or no-transfection (NT) were subjected to Western blot analysis using anti-NMHC-IIIC, IIA, IIB, and anti-β-actin antibodies. B, NMHC-IIIC siRNA (bottom) and siRNA-scramble (top)-treated cells were observed by phase contrast microscopy. The bar graph shows the mean fractions (± SD) of cells showing abnormal midbodies based on three independent experiments (n = 100). C, lysates from A549 cells treated with two different BRCA2 siRNAs or siRNA-luciferase or no-transfection (NT) were subjected to Western blot analysis with anti-BRCA2 and anti-β-actin antibodies. D, A549 cells treated with BRCA2 siRNA or siRNA-luciferase or siRNA-no-transfection were fixed and stained with anti-α-tubulin (red) and anti-NMHC-IIIC antibodies (green) in cytokinesis. Scale bar, 5 μm. Magnifications of dotted square areas are shown on the right (1–6). The bar graph shows the mean frequencies (± SD) of abnormal midbodies in cells based on three independent experiments (n = 20). E, lysates from HeLa S3 cells treated with three different BRCA2 siRNAs or siRNA-luciferase or siRNA-no-transfection (NT) were subjected to Western blot analysis with anti-BRCA2 and anti-β-actin antibodies. F, HeLa S3 cells treated with BRCA2 siRNA (BRCA2-1) were fixed and stained for α-tubulin (red). White lines, the measured midbody length by Adobe Photoshop CS3 software. Scale bar, 5 μm. The midbody lengths are shown in box plot that signifies the top and bottom quartiles. The medians are represented by bold lines. Nonparametric data were statistically analyzed by the Mann–Whitney U test (n = 81 in each case, P < 0.01).
Figure 4. The binding inhibition between BRCA2 and NM-IIC affects the formation of Flemming body. A, a schematic diagram of NMHC-IIC deletion mutants [IIC (A), (B), (A1), (A2), and (A3)] and the strength of binding to BRCA2, summarizing the data within Supplementary Fig. S5A and S5B. NMHC-IIC fragments were fuzed to an HA tag. B, COS-7 cells transfected with anti-HA and anti-FLAG antibodies. Inputs and IIC precipitates (IP) were visualized by anti-FLAG antibody. Inputs and IIC (A1)-HA or HA and stained with –tubulin antibody 48 hours after IIC (A1)-HA expression. C, A549 cells were transfected with either IIC (A1)-HA or HA and midbodies were collected. Anti-BRCA2 immunoprecipitates (IP) from midbody lysates were analyzed by anti-NMHC-IIC, anti-BRCA2, and anti-HA antibodies. D, A549 cells were transfected with IIC (A1)-HA or HA and stained with anti-NMHC-IIC (green) or BRCA2 (green), and anti-HA (red) antibodies. Scale bar, 5 μm.

Binding of BRCA2 to the N-terminal region of NMHC-IIC

To determine which region of NMHC-IIC interacts with BRCA2, we introduced plasmids encoding HA-tagged NMHC-IIC deletion mutants (Fig. 4A) and BRCA2-FLAG into COS-7 cells, and performed coimmunoprecipitation analyses. BRCA2 bound to the IIC (A)-HA fusion, containing a a.a. 1–1,000 of NMHC-IIC (Supplementary Fig. S5A). We further divided this fragment into three partially overlapping regions and found that only the IIC (A1)-HA fusion bound strongly to BRCA2-FLAG (Supplementary Fig. S5B). When IIC (A1)-HA was coexpressed with BRCA2-FLAG and NMHC-IIC-HA, the interaction between BRCA2-FLAG and NMHC-IIC-HA was abolished (Fig. 4B). The same dominant-negative effect was seen between the endogenous BRCA2 and NM-IIC at the midbody (Fig. 4C). Both the localization of endogenous BRCA2 and IIC-ring formation at the Flemming body were disrupted in A549 cells expressing IIC (A1)-HA (Fig. 4D and Supplementary Fig. S5C).

Abnormalities in cytokinesis induced by expression of NMHC-IIC (A1) or BRCA2 (R1)

We hypothesized that the interaction between BRCA2 and NM-IIC at the midbody might play a role in the regulation of cytokinesis. To explore this possibility, we analyzed A549 cells exposed to anti–α-tubulin antibody 48 hours after IIC (A1)-HA transfection. The midbodies of the IIC (A1)-HA-expressing cells varied in length compared with those of the HA-expressing control cells (Supplementary Fig. S5D; P < 0.01 by Mann-Whitney U test). The IIC (A1)-HA-expressing cells were unable to divide and accumulated at G1 phase (76.09%–48.89% for the cells transfected with the empty vector; Supplementary Fig. S5E). These results indicated that the binding of BRCA2 to NM-IIC could have a function in cytokinesis promotion.

Because the endogenous BRCA2 did not localize to the Flemming body in A549 cells expressing the R1 region of BRCA2 (Supplementary Fig. S1K), we supposed that the abundant expression of the R1 domain would also inhibit IIC-ring formation. As expected, the IIC ring was not observed in A549 cells expressing a high level of HA-BRCA2 (R1), as was the case in cells expressing IIC (A1)-HA (Fig. 4D and Supplementary Fig. S5C and S5F). A greater number of binucleated cells was observed in BRCA2 (R1)-FLAG–expressing cells (18.5 ± 3.4%) than in FLAG-expressing control cells (8.3 ± 2.7%; Supplementary Fig. S5G).

Enhancement of the ATPase activity of NM-IIC by BRCA2

NM-II plays a fundamental role in cell adhesion, migration, and division mediated by inherent actin cross-linking and contractile functions. This activity requires energy, which is provided by the hydrolysis of ATP and the active site of NM-II exhibits ATPase activity. Treatment of A549 cells with blebbistatin, an inhibitor of myosin II ATPase activity, caused decay of the IIC-ring surrounding α-tubulin and imperfections in ring formation (Supplementary Fig. S6A). MgcRacGAP was observed in the decay of the IIC-ring (Supplementary Fig. S6C).
To explore the function of BRCA2 in IIC-ring formation, we analyzed the effect of BRCA2 on the actin-dependent ATPase activity of NM-IIC. The actin-dependent ATPase activity of NM-IIC was measured following incubation of the immunoprecipitated NMHC-IIC-HA in the presence or absence of BRCA2-FLAG. We confirmed that introduction of the plasmid encoding NMHC-IIC into cells leads to binding of the exogenous NMHC-IIC to the 12A and 12B isoforms of endogenous light chain (Supplementary Fig. S6C). The ATPase was activated (1.0 × 10⁻³ unit/minute) when both proteins were present (0.5 μg BRCA2-FLAG and 0.63 μg NMHC-IIC-HA; Fig. 5A, lane 1). ATPase activation following incubation of NMHC-IIC and BRCA2 occurred in a dose-dependent manner (Fig. 5A, lanes 1 and 2). The light chain was phosphorylated following the addition of BRCA2-FLAG to the immunoprecipitated NMHC-IIC (Supplementary Fig. S6D). Furthermore, in the presence of IIC (A1)-HA, which inhibits the binding of BRCA2 to NM-IIC, ATPase activation was inhibited (Fig. 5A, lane 4). The absence of F-actin from the reaction mixture reduced ATPase activity significantly (Fig. 5A, lane 5).

Figure 5. ATPase activity is required for IIC-ring formation. A, the actin-dependent ATPase activity of NM-IIC following incubation of the immunoprecipitated NMHC-IIC-HA with and without BRCA2-FLAG. B, NMHC-IIC-GFP was expressed in COS-7 cells and the reaction mixtures were mounted on a glass slide and observed by fluorescence microscopy. The IIC-rings were not seen following blebbistatin treatment or lacking either ATP or Mg²⁺ in the reaction. Magnified images of NMHC-IIC-GFP are also shown (a–f). C, NMHC-IIC-GFP was expressed in COS-7 cells treated with siRNA-BRCA2 or siRNA-control or no-transfection, and the reaction mixtures were mounted on a glass slide and observed by fluorescence microscopy. The samples were fixed and stained for GFP (green). Scale bar, 1 μm. D, the plasmids indicated in figure were expressed in COS-7 cells and the cell lysates were mounted on a glass slide. The samples were fixed and stained for GFP (green) and BRCA2 (red). Scale bar, 1 μm.
localization of F-actin at the Flemming body was observed by immunofluorescence microscopy (Supplementary Fig. S6E). A mutant NMHC-IIC (AA)-HA in which Lys204 and Thr205 in the ATP-binding site (GESGAGKT; 198–205) were substituted with alanine did not exhibit ATPase activity in response to incubation with BRCA2 (Fig. 5A, lane 9). IIC (A1)-HA also did not exhibit ATPase activity (Fig. 5A, lane 8). A mutant BRCA2 (S193A)-FLAG also supported activation of NM-IIC ATPase activity to a level similar to that observed by the wild-type BRCA2-FLAG (Supplementary Fig. S6F). These results suggested that the ATPase activity is increased by NM-IIC–BRCA2 association. Furthermore, the phosphorylation of BRCA2-Ser193 is not essential for the activation of the ATPase activity of NM-IIC but is required for the localization of BRCA2 to the Flemming body.

To test this further, we attempted an in vitro reconstitution of the IIC-ring using recombinant NMHC-IIC. NMHC-IIC-GFP was expressed in COS-7 cells and examined using the cell lysates. It was observed that NMHC-IIC-GFP formed part of a unique ring-like structure (1.4–2.0 μm) in the presence of both ATP and Mg²⁺ (Fig. 5B, 1). However, NMHC-IIC-GFP failed to organize into a ring-like structure when blebbistatin was added to the reaction (Fig. 5B, 2). The ring-like structure was also not observed in the absence of either ATP or Mg²⁺ (Fig. 5B, 3 and 4).

To see an effect of BRCA2 on IIC-ring formation, we overexpressed recombinant NMHC-IIC-GFP and BRCA2-FLAG in COS-7 cells and investigated localization of BRCA2 in the lysates. We showed that BRCA2 localized to the IIC ring-like structure (Fig. 5D). These results suggest that the ring-like structure is composed of NMHC-IIC-GFP and BRCA2.

The requirement of the ATPase activity of NM-IIC for IIC-ring formation

To demonstrate the significance of the ATPase activity of NM-IIC for IIC-ring formation, we examined whether the IIC-ring could be restored by recombinant NMHC-IIC-HA or NMHC-IIC (AA)-HA expression following endogenous NMHC-IIC knockdown (Supplementary Fig. S6G and S6H). Both were coexpressed with BRCA2-FLAG (Supplementary Fig. S6I). Although cells expressing wild-type NMHC-IIC-HA exhibited IIC-ring formation within the midbody, in cells expressing NMHC-IIC (AA)-HA, the IIC-ring-like structure seemed to degenerate within the midbody area (Supplementary Fig. S6J). These results indicate that the ATPase activity of NM-IIC is required for IIC-ring formation.

Discussion

BRCA2, the product of breast cancer susceptibility gene *BRCA2*, plays important roles in the maintenance of genome stability throughout the cell cycle. Several studies have suggested a role for BRCA2 in regulation of cytokinesis at the late M phase. BRCA2 deficiency impairs completion of cell division. Inhibition of cell separation is accompanied by abnormalities in NM-II organization during the late stages of cytokinesis (6). We identified the subcellular localization of each isoform.
during cytokinesis and found that only the NM-IIC isoform colocalizes and interacts with BRCA2 at the Flemming body (Fig. 2 and Supplementary Fig. S2). The interaction of NM-IIC with BRCA2 at the Flemming body allows the activation of NM-IIC ATPase activity followed by IIC-ring formation (Fig. 6).

Cytokinesis, the final step of cell division in all animal cells, partitions the cytoplasm between two daughter cells. This process depends upon the activity of NM-II, which participates in the formation of the cleavage furrow. Attachment of a contractile ring, consisting of a network of actin filaments (actomyosin) and NM-II, to the cytoplasmic membrane induces the formation of a cleavage furrow. Mammalian cells use not IIC but IIA or IIB during this process (10). NM-IIA exhibited a diffuse distribution throughout the entire midbody as observed by immunofluorescence microscopy (Fig. 2A) and glycerol gradient analyses (Fig. 2B), indicating that it might interact with various proteins. Although NM-IIB localizes to an area from the basal portion of the midbody to the cytosol of dividing cells, glycerol gradient analysis revealed that it is absent from the fraction containing phosphorylated BRCA2. In addition, a coimmunoprecipitation assay did not detect interaction between BRCA2 and NM-IIB (Fig. 2D). In contrast, NM-IIC was detected at the Flemming body and it colocalized with phosphorylated BRCA2 during cytokinesis just before abscission. Depletion of BRCA2 or NM-IIC by siRNA disrupted the IIC-ring and led to abnormal midbody formation followed by failure of abscission and cytokinesis (Fig. 3 and Supplementary Fig. S3). Cells expressing NM-IIC (A1), which exhibited a dominant-negative effect upon the interaction of endogenous BRCA2 and NM-IIC, also failed to progress through cytokinesis (Fig. 4 and Supplementary Fig. S5C–S5E). Taken together, the interaction between BRCA2 and NM-IIC seems essential for abscission or the induction thereof. Some cells, including HeLa S3, have been reported not to express NM-IIC; however, we have performed some experiments to confirm NM-IIC-expression in HeLa S3 cells. These include Western blot data using anti-NMHC-IIC-N antibody and the mass spectrometric analysis of HeLa S3-cell lysates (data not shown). These data indicated the existence of the short type of NM-IIC in HeLa S3 cells.

In this study, the interaction of BRCA2 with NM-IIC was shown to be required for the activation of NM-IIC ATPase activity (Fig. 5A and Supplementary Fig. S6F). Furthermore, the endogenous light chain bound to NMHC-IIC-HA was phosphorylated following the addition of BRCA2-FLAG (Supplementary Fig. S6C and S6D). We speculate that a kinase present in the BRCA2 immunoprecipitate might phosphorylate the light chain, resulting in the activation of ATPase activity. ROCK, which phosphorylates Ser19 of smooth muscle MLC2, was detected in the anti-BRCA2 immunoprecipitates by mass spectrometry. This result raises the possibility that ROCK1 may phosphorylate and activate myosin IIC. This issue should be addressed in future studies.

BRCA2 is a multifunctional protein that functions as a caretaker of genome stability throughout the cell cycle, including during processes such as DNA synthesis, centrosome dynamics, chromosome separation, and cytokinesis. In this study, we demonstrated a role for BRCA2 in the late stage of cytokinesis in collaboration with NM-IIC, which should help clarify the mechanics of cytokinesis and breast oncogenesis. We identified in the BIC database a missense mutation in BRCA2 (T77A, within the PLK1-binding motif), the clinical significance of which had not been established. We showed the possibility that this mutation may abolish the binding between BRCA2 and Plk1, resulting in failure of BRCA2 localization to the Flemming body and cytokinesis incompletion, suggesting that this mutation may underlie the development of breast cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: Y. Miki, A. Nakanishi
Development of methodology: M. Takaoka, Y. Miki, A. Nakanishi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Takaoka, A. Nakanishi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Takaoka, A. Nakanishi
Writing, review, and/or revision of the manuscript: M. Takaoka, K. Takenaka, Y. Miki, A. Nakanishi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Takaoka, H. Saito, Y. Miki, A. Nakanishi
Study supervision: K. Takenaka, c. A. Nakanishi

Acknowledgments
The authors thank Rie Hayashi (Leica Microsystems K.K.) for support in preparing the laser-scanning confocal microscope.

Grant Support
This work was supported by JSPS KAKENHI Grant Numbers 24300328 (Y. Miki), 256-0061 (Y. Miki), 21790311 (K. Takenaka), 23590555 (K. Takenaka), by Ishidsu Shun Memorial Scholarship (M. Takaoka), and by Takeda Science Foundation (K. Takenaka).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 18, 2013; revised December 20, 2013; accepted January 5, 2014; published OnlineFirst January 21, 2014.

References
BRCA2 Regulates Cytokinesis by Myosin-IIC ATPase Activation


www.aacrjournals.org
Cancer Res; 74(5) March 1, 2014 OF11

Downloaded from cancerres.aacrjournals.org on April 12, 2017. © 2014 American Association for Cancer Research.
BRCA2 Phosphorylated by PLK1 Moves to the Midbody to Regulate Cytokinesis Mediated by Nonmuscle Myosin IIC

Miho Takaoka, Hiroko Saito, Katsuya Takenaka, et al.

Cancer Res  Published OnlineFirst January 21, 2014.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-13-0504

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2014/01/20/0008-5472.CAN-13-0504.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.