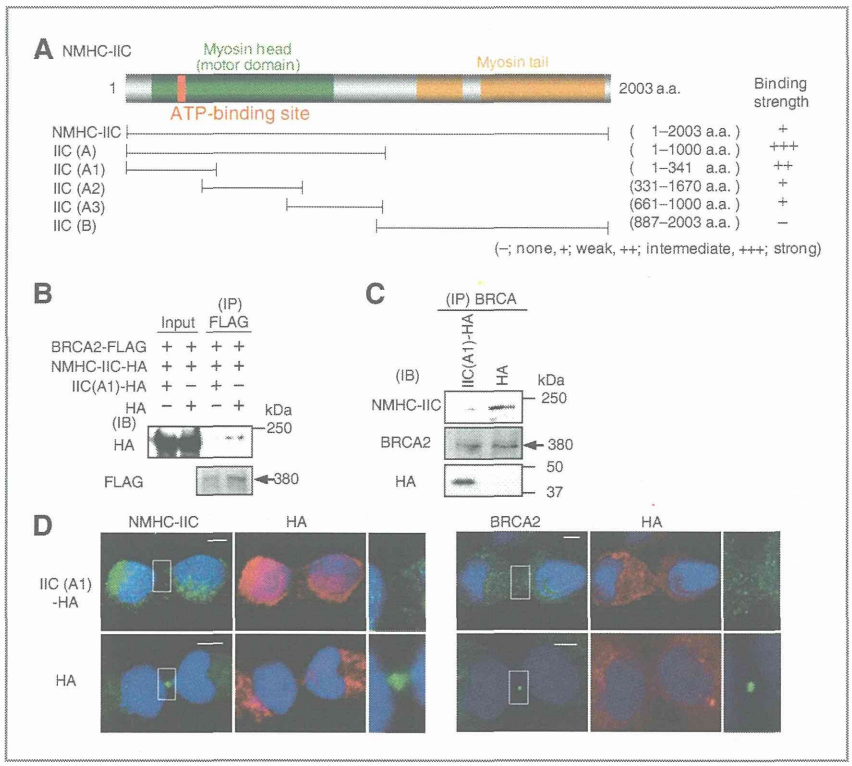

Figure 4. The binding inhibition between BRCA2 and NM-IIC affects the formation of Flemming body. A, a schematic diagram of NMHC-IIC deletion mutants [IIC (A), (B), (A1), (A2), and (A3)] and the strength of binding to BRCA2, summarizing the data within Supplementary Fig. S5A and S5B. NMHC-IIC fragments were fused to an HA tag. B, COS-7 cells transfected with indicated plasmids were lysed and proteins were immunoprecipitated with anti-FLAG antibody. Inputs and precipitates (IP) were visualized by anti-HA and anti-FLAG antibodies. 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. 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 G_1 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.

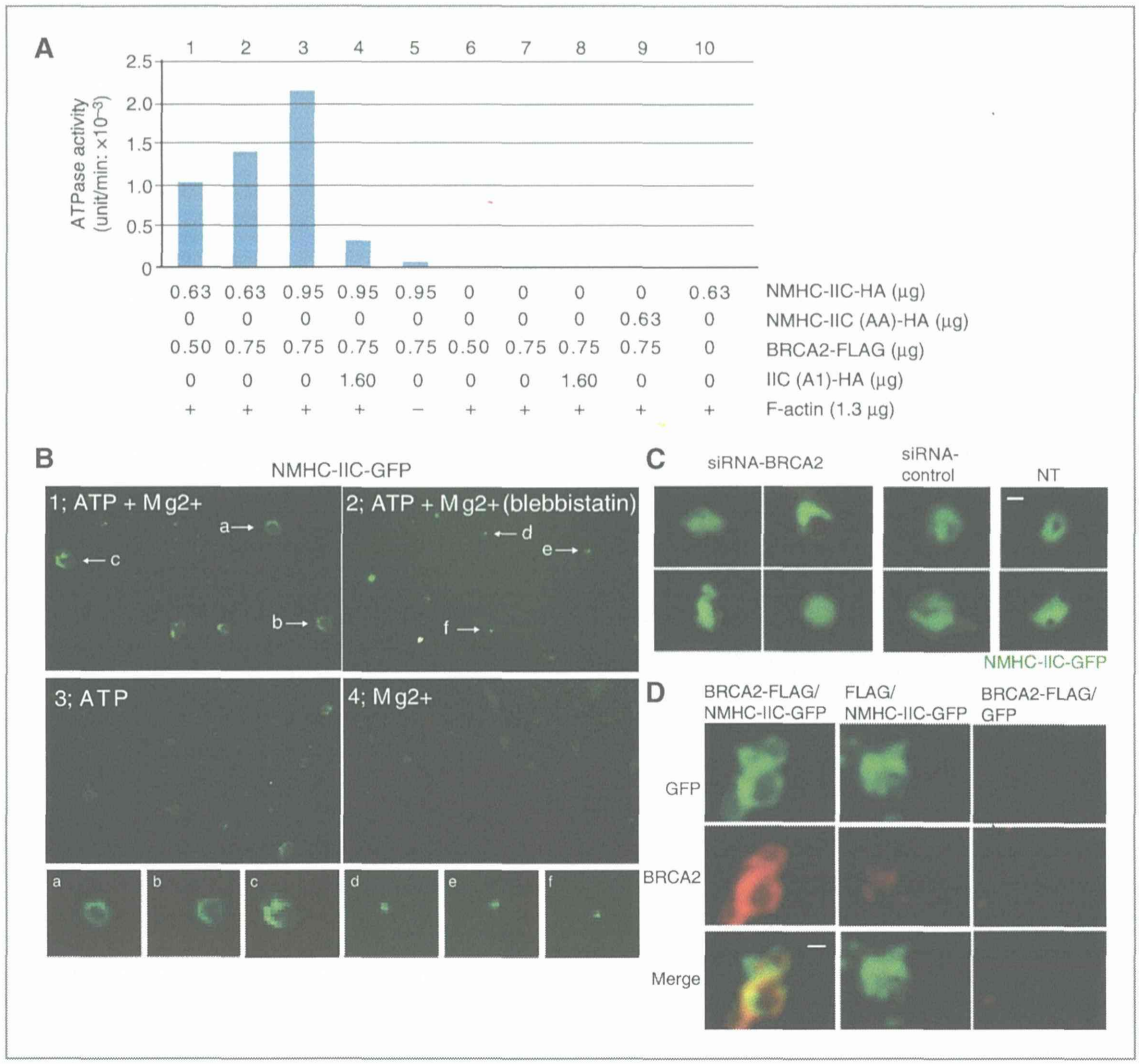

Figure 5. ATPase activity is required for IIC-ring formation. A, the actin-dependent ATPase activity of NM-IIC following incubation of the immunoprecipitated NMHC-IIC-HA with and without BRCA2-FLAG. B, NMHC-IIC-GFP was expressed in COS-7 cells and the reaction mixtures were mounted on a glass slide and 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.

S6B). 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). The

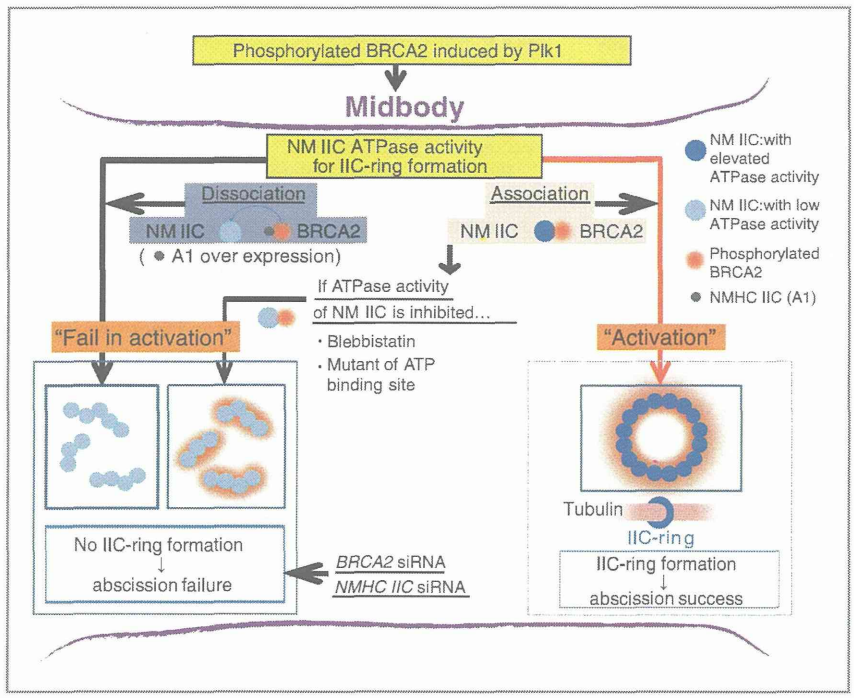

Figure 6. The midbody abscission is modulated by the BRCA2–NM-IIC complex. The phosphorylation of BRCA2 by Plk1 is required for BRCA2 localization to the Flemming body and may support IIC-ring formation through increased NM-IIC-ATPase activity. When BRCA2-NM-IIC binding inhibition induced by IIC (A1) overexpression, the activation of NM-IIC ATPase failed and the IIC-ring was not formed normally as it would be with treatment with blebbistatin (ATPase inhibitor of myosin) to the cells. The IIC-ring is triggered by BRCA2-dependent binding of NM-IIC at the Flemming body, which may allow BRCA2 to modulate the midbody abscission for normal transition of cells through cytokinesis.

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 Mg^{2+} (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 Mg^{2+} (Fig. 5B, 3 and 4). To see an effect of BRCA2 on IIC-ring formation, we overexpressed NMHC-IIC-GFP following siRNA-mediated knockdown of BRCA2 in COS-7 cells. NMHC-IIC-GFP failed to organize into a ring-like structure in siRNA-BRCA2–treated cells (Fig. 5C). Next, we coexpressed 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 coprecipitated with BRCA2-FLAG (Supplementary Fig. S6I). Although cells expressing wild-type NMHC-IIC-HA exhibited IIC-ring 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

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