Published in final edited form as:
Cell Cycle. 2008 July 15; 7(14): 2225–2233.

Differential regulation of centrosome integrity by DNA damage response proteins

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Abstract

MDC1 and BRIT1 have been shown to function as key regulators in response to DNA damage. However, their roles in centrosomal regulation haven't been elucidated. In this study, we demonstrated the novel functions of these two molecules in regulating centrosome duplication and mitosis. We found that MDC1 and BRIT1 were integral components of the centrosome that colocalize with γ-tubulin. Depletion of either protein led to centrosome amplification. However, the mechanisms that allow them to maintain centrosome integrity are different. MDC1-depleted cells exhibited centrosome overduplication, leading to multipolar mitosis, chromosome missegregation, and aneuploidy, whereas BRIT1 depletion led to misaligned spindles and/or lagging chromosomes with defective spindle checkpoint activation that resulted in defective cytokinesis and polyploidy. We further illustrated that both MDC1 and BRIT1 were negative regulators of Aurora A and Plk1, two centrosomal kinases involved in centrosome maturation and spindle assembly. Moreover, the levels of MDC1 and BRIT1 inversely correlated with centrosome amplification, defective mitosis and cancer metastasis in human breast cancer. Together, MDC1 and BRIT1 may function as tumor-suppressor genes, at least in part by orchestrating proper centrosome duplication and mitotic spindle assembly.

Keywords

DNA damage protein; centrosome duplication; spindle assembly; cytokinesis; mitosis

Introduction

Centrosomes, in addition to their established function as the principal microtubule organizing center for ensuring correct chromosome segregation, are involved in numerous cell cycle regulatory events including entry into mitosis, cytokinesis, G_1/S transition and monitoring of DNA damage.[1–8] Abrogations of the regulatory mechanisms governing centrosome

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duplication as well as defective cytokinesis are common reasons for tumor development and progression.[9–12]

Numerous proteins that are frequently mutated in cancers help regulate the duplication and numerical integrity of centrosomes.[13–17] These proteins belong to various functional groups affecting cell cycle regulation, DNA-damage response, and/or DNA repair. However, the direct links between the localization of these proteins with respect to the centrosome and their specific cellular functions have been unclear. We propose that these regulatory proteins may serve as spatiotemporal organizers where checkpoint components come into proximity in a defined manner by two different mechanisms. We use MDC1/NFBD1 and BRIT1/MCPH1 as examples of each type of regulation to control the numeral integrity of centrosomes and mitosis.

The intriguing roles of MDC1 and BRIT1 in safeguarding genome stability through their indispensable function in early response to DNA damage has been thoroughly studied.[18–24] Both MDC1 and BRIT1 amplify the DNA damage response by binding to γ-H2AX at breakage sites and recruit numerous mediator proteins.[24,25] Furthermore, both MDC1 and BRIT1 are required for intra-S and intra-G_2/M checkpoints and they thus influence the progression of mitosis. Beyond the established functions of MDC1 and BRIT1 in DNA damage signaling and checkpoint activation, we describe their additional functions in maintaining centrosome integrity and mitosis.

Our data indicate that MDC1 and BRIT1 colocalize with γ-tubulin throughout the cell cycle. Depletion of MDC1 or BRIT1 leads to centrosome amplification, mitotic spindle malformation, and delayed cytokinesis, which in turn lead to aneuploidy and polyploidy, promoting genomic instability and malignant transformation. It is noteworthy that while our work was in progress, two other groups also observed the presence of BRIT1 in the centrosome, although its functional significance remains unknown.[26,27]

Results

MDC1 and BRIT1 were associated with the centrosome in vivo

Based on indirect immunofluorescent staining, our data revealed that both MDC1 and BRIT1 were components of the centrosome and colocalized with γ-tubulin throughout the cell cycle (Suppl. Fig. 1A and B). Centrosomal localization of both proteins was further verified by using siRNA. As shown in Figure 1A, depletion of MDC1 and BRIT1 abolished their colocalization with γ-tubulin, indicating that MDC1 and BRIT1 are indeed present in the centrosome. To further locate the structural domains involved in targeting MDC1 to the centrosome, a series of pEGFP-MDC1 deletion mutants was transfected into U2OS cells. As showed in the Figure 1B, the pEGFP-ΔFHA MDC1 failed to colocalize with γ-tubulin, suggesting that the N-terminal FHA domain of MDC1 was the centrosome targeting domain. Although domain analysis of human BRIT1 on centrosome binding has not been fully studied, a recent report of chicken MCPH1 (cMCPH1) demonstrated that the N-terminal BRCT (BRCA1 C-terminal) domain of cMCPH1 (cBRIT1) was required for its centrosomal localization.[27]

MDC1 and BRIT1 were required for the maintenance of centrosome integrity

To address the functional significance of centrosomal localization of MDC1 and BRIT1, we sought to examine how depletion of MDC1 or BRIT1 would affect the centrosome. As shown in Figure 2A, depletion of MDC1 using specific siRNA led to aberration of the centrosome number (more than two centrosomes), compared with findings in the mock-treated or the luciferase-siRNA-transfected cells, our results indicated that 20% of MDC1-depleted cells displayed more than two centrosomes per mononucleated cells as compared to 3% seen in controls (Figure 2B). Consistent with our domain analysis, centrosome amplification in

MDC1-depleted cells could be rescued only with wild-type MDC1 but not with ΔFHA MDC1 (Fig. 2C). Our data therefore suggest that MDC1 is an integral component of the centrosome and is essential for maintaining centrosome integrity.

To ascertain whether supernumerary centrosomes in MDC1 knockdown cells resulted from centrosome overduplication during the S phase or from defective cytokinesis, we compared the hydroxyurea-treated MDC1 knockdown cells with the untreated exponentially growing cells for the induction of centrosome amplification. Compared with the control, MDC1-depleted cells efficiently provoked centrosome amplification despite the presence of hydroxyurea treatment, suggesting an association between MDC1 depletion and centrosome overduplication (Fig. 2D). Since the centrosome does not normally overduplicate during the prolonged S-phase arrest in cells,[28,29] our findings suggest that MDC1 deficiency perturbs the mechanism that regulate centrosome reduplication and, in turn, permits continuous centrosome duplication in the absence of DNA replication.

Recent studies[17] suggest that various elements of DNA damage response regulate the centrosome cycle. Thus, we sought to determine the effect of MDC1 knockdown on centrosome duplication after DNA damage. We assessed the centrosome number in the control-treated or MDC1-siRNA-treated cells that were pretreated with 10 Gy of γ-radiation. Immunofluorescence analysis revealed that irradiation further exacerbated the extent of centrosome amplification in the MDC1 knockdown cells (Fig. 2E). These data collectively indicated that MDC1 plays a pivotal role in regulating centrosome duplication with or without DNA damage.

BRIT1-depleted cells also displayed centrosome amplification. However, unlike in MDC1 knockdown cells, the number of centrosomes did not increase after hydroxyurea treatment, suggesting that BRIT1 was not involved in the centrosome duplication process. In fact, we found that BRIT1-depleted cells were multinucleated, suggesting that the increase in centrosome number might have been an indirect consequence of defective cytokinesis (Fig. 2F).

Depletion of MDC1 or BRIT1 resulted in aberrant mitotic spindles and defective cytokinesis

Centrosome abnormalities lead to aberrant spindle formation and inappropriate chromosome segregation in the mitotic stage.[1–5] Since MDC1 appears to be an important regulator for centrosome duplication, it is vital to investigate how MDC1 deficiency perturbs cell mitosis. As shown in Figure 3A, 17% of MDC1-depleted cells exhibited supernumerary centrosomes that displayed multipolar spindle formation along with altered pattern of chromosome alignment at metaphase. The supernumerary centrosome and the multipolar spindle appeared lead to aneuploidy in MDC1 knockdown cells as monitored by time lapse video microscopy (data not shown).

As in MDC1-depleted cells, the knockdown of BRIT1 also triggered mitotic defects. As shown in Figure 3B, BRIT1 depletion led to a misaligned spindle that failed to align properly to the equatorial plain, along with a chromosome congression failure at the metaphase (see Fig. 3B arrows). In a subset of BRIT1-depleted cells, the two poles were indeed unseparated, and the mass of chromosomes was sprayed away from one side of the spindles. These aberrant spindles were very different from the ones found in the control cells, where the spindles were largely focused and bipolar with chromosomes properly congressed at the metaphase plate.

We also observed a significant portion of BRIT1-depleted cells displaying persistent cytokinesis bridges, binucleated cells (often contain midbody remnants) and cell fusion with widely separated nuclei (data not shown). In Figure 3C, we summarized the mitotic defects detected in BRIT1 knockdown cells. The features observed in the BRIT1-depleted cells were

highly suggestive of aberrant (i.e., delayed) cytokinesis in the progression of mitosis. The delayed cyto-kinesis in BRIT1-depleted cells was further verified by time-lapse video microscopy. In contrast to the control cells that accomplished cell division in an hour, the BRIT1-depleted cells took an additional hour to complete the same process (Suppl. Fig. 2).

The BRIT1-depleted cells also elicited less compacted and lagging chromosomes, as shown by arrows in Supplementary Figure 2. This chromosome lagging and misalignment triggered by BRIT1 depletion likely activated the spindle assembly checkpoint proteins BubR1 or Mad2, which are crucial components that monitor the metaphase-anaphase transition.[30] Of interest, despite the checkpoint activation by BubR1 and Mad2, the BRIT1-deficient cells eventually overcame the checkpoint and entered anaphase. As shown in Figure 3D and E, spindle checkpoint protein BubR1 or Mad2 in the controls was clearly detected as a punctate pattern during prophase and promatephase and then diffused quickly once the biopolar spindle formed in the control cells. However, the punctate pattern of BubR1 or Mad2 staining persisted in the BRIT1-depleted cells, which inappropriately entered anaphase despite the persistent accumulation of Mad2 at the metaphase-anaphase transition (Fig. 3E). Taken together, our data suggest that MDC1 depletion results in centrosome amplification followed by multipolar mitosis and chromosome missegregation, whereas BRIT1 depletion leads to misaligned spindles and/or lagging chromosomes with defective spindle checkpoint activation and delayed cytokinesis.

MDC1 and BRIT1 were negative regulators of aurora A and Plk1

The orchestration of mitosis and cytokinesis is driven by numerous kinases, including Aurora A and Plk1.[31–34] Any error in the choreography of these kinases could result in multipolar mitosis, chromosome missegregation, spindle checkpoint ineffectiveness and defective cytokinesis, and ultimately contributes to aneuploidy, genomic instability, and tumorigenesis.[35,36] The fact that depletion of MDC1 or BRIT1 led to mitotic defects prompted us to explore the potential link between MDC1/BRIT1 and Aurora A/Plk1. Of interest, we detected elevated levels of both Aurora A and Plk1 protein in MDC1- and BRIT1-depleted cells, suggesting the potential roles of MDC1 and BRIT1 as negative regulators of these two kinases (Fig. 4A). Since the overexpression of Aurora A and Plk1 has been previously reported[35] to cause centrosome amplification and spindle checkpoint defects, we suspect that the higher levels of these two kinases may at least partially contribute to the defects in mitosis seen in MDC1- and BRIT1-deficient cells.

The upregulation of Aurora A and Plk1 in MDC1- and BRIT1- depleted cells likely resulted from increased protein stability of these two kinases. As shown in Figure 4B, when de novo protein synthesis in cells was inhibited by treatment with cycloheximide, the control but not the knockdown cells showed decreased levels of Aurora A and Plk1, suggesting that MDC1 and BRIT1 negatively regulate the protein stability of Aurora A and Plk1. Furthermore, wild-type but not ΔFHA MDC1 could rescue the effect of MDC1 deficiency on Aurora A and Plk1 expression (Fig. 4C), supporting the notion that centrosomal MDC1 represses the protein levels of Aurora A and Plk1.

MDC1 and BRIT1 levels inversely correlated with centrosome amplification and defective mitosis in human breast cancer specimens

Centrosome amplification in conjunction with mitotic spindle abnormalities correlates with genomic instability and malignancy during tumor progression in several cancer types, including breast cancer.[37–39] We therefore sought to examine whether the status of MDC1 or BRIT1 was linked to centrosome amplification and mitotic defects in breast cancer specimens. By immunohistochemical analysis, we found that, compared with MDC1 levels in adjacent normal breast tissue, MDC1 expression was significantly decreased in 50% of breast

cancer specimens (5 of 10) (Fig. 5A). BRIT1 protein levels in breast cancer specimens compared with the levels in adjacent normal breast tissue were even more significantly decreased, with 70% of specimens (7 of 10) showing reduced expression (Fig. 5B). Also of note, we observed marked centrosome amplification in MDC1-deficient breast cancer specimens: when 100 total cells were analyzed, 17% showed more than two centrosomes, and 15% displayed multipolar mitosis (Fig. 5C).

We also observed a correlation between BRIT1 status and Plk1 expression level. We found that approximately 12% of the cells in breast cancer specimens showed overexpression of Plk1 at midbody without detectable BRIT1 expression (Fig. 5D). Because centrosome amplification and mitotic checkpoint defects promote genomic instability and cancer progression,[3,10,40] we further analyzed the public cancer database to assess the link between abnormal MDC1 expression and cancer progression. Indeed, the MDC1 level (shown in Fig. 5E) inversely correlated with the metastatic potential in breast cancer. This finding is consistent with our previous finding that BRIT1-deficient breast cancer was more metastatic than was breast cancer associated with normal or high BRIT1 expression.[24]

Discussion

Our study demonstrated a distinct function of two DNA damage response proteins, MDC1 and BRIT1, in maintaining centrosome and/or spindle checkpoint pathways. It has been broadly accepted that disruption of the normal checkpoint function is closely associated with the centrosome cycle.[41] The elevated centrosome number in MDC1 treated cells after hydroxyurea treatment suggests that MDC1 is implicated in regulating centrosome duplication directly. In addition, the integration and interplay between cell cycle progression and the centrosome cycle is also reflected in the presence of cell cycle checkpoint proteins at the centrosome. There is growing evidence that a centrosome inactivation checkpoint exists, which utilizes DNA damage induced centrosome fragmentation or amplification to provoke a “mitotic catastrophe” and eliminate damaged cells.[17] Candidate regulators of this centrosomal checkpoint include the checkpoint kinases, Chk1, Chk2 and its upstream regulators ATM and ATR. Given the specified function of MDC1 and BRIT1 in intra-S and G_2/M checkpoints,[21,23] and their localization on centrosome, we suspect that the cell cycle regulatory functions of MDC1 and BRIT1 might be coupled with their regulation of centrosome integrity and cytokinesis. Consequently, the loss of MDC1 and/or BRIT1 results in an accumulation of damaged DNA and prolonged checkpoint activation, allowing centrosomes to amplify through two different mechanisms.

MDC1-depleted cells result in multipolar spindle formation due to centrosome amplification, thus supporting a potential role for MDC1 in the maintenance of numerical centrosome integrity. Our data also provide new insight into the crucial role of BRIT1/MCPH1 in the spatial regulation of mitotic spindle assembly/chromosome segregation, a function shared with other MCPH gene products (MCPH3/CDKRAP2, MCPH5/ASPM and MCPH6/CENJ).[42,43] Unlike MDC1, which ensures a true centrosome duplication process, BRIT1 seems to be required for proper chromosome alignment and for keeping the spindle checkpoint intact before cytokinesis. BRIT1 regulates the expression of two key checkpoint regulators, BRCA1 and Chk1;[23] it is therefore reasonable to envisage that a BRIT1-defective phenotype may in part correlate with the deficit of BRCA1/Chk1 expression, given that both BRCA1 and Chk1 have been reported to localize to the centrosome and to inhibit the formation of supernumerary centrosomes.[44,45] We will certainly seek to further dissect this complex regulatory network in our future studies. Nevertheless, regardless of the underlying mechanism that leads to centrosome amplification, the depletion of either MDC1 or BRIT1 disrupts mitotic progression and destabilizes the chromosomes in the subsequent cell cycles.

We find it interesting that the depletion of MDC1 or BRIT1 alone led to overexpression of Aurora A and Plk1, suggesting that checkpoint proteins such as MDC1 and BRIT1, perhaps through concerted spatial and temporal interactions at the centrosome, may regulate the protein stability of Aurora A and Plk1 and thereby accelerate the communication between cell cycle and checkpoint signaling networks. Elevated Aurora A and Plk1 in MDC1 and BRIT1 depleted cells lead the cells to override the activated spindle assembly checkpoint, permitting the cells to inappropriately enter anaphase despite the presence of the abnormality. In fact, the abnormal activity of Aurora A and Plk1 has been shown to override the activated spindle checkpoint assembly enabling inappropriate anaphase onset despite spindle checkpoint activation subsequently affect progression through mitosis.[32–34] Our findings, therefore, raise an intriguing possibility that MDC1 and BRIT1 play critical roles not only in the suppression of centrosome amplification and misaligned spindles but also in the regulation of the onset of anaphase by negatively regulating Aurora A and Plk1 to override the activated spindle assembly checkpoint machinery, thus allowing proper mitotic exit.

Interestingly, although both MDC1 and BRIT1 regulate Aurora A and Plk1, knockdown of these two genes leads to different centrosomal defects. We suspect that these may have been due to the mechanisms independent of Aurora A and Plk1. For example, MDC1 but not BRIT1 is known to regulate p53 phosphorylation and stabilization. Since p53 has been shown to regulate centrosome duplication,[46,47] the differential role of p53 in MDC1 knockdown and BRIT1 knockdown cells may have led to different phenotypic defects. More detailed study will certainly be needed in the future to dissect the implication of different specific targets.

Multipolar mitotic spindles, as a consequence of centrosome amplification and binucleated cells, are considered early events in tumor development and subsequent expansion of genomic instability.[2,48–50] In agreement with these findings, we found that the dysfunction of MDC1 and BRIT1 resulted in supernumerary centrosomes followed by aneuploidy and defective cytokinesis. These functional correlations were clearly demonstrated in our knockdown cell systems and in the cancer specimens examined, raising the possibility that defects in these two genes may contribute to global chromosome changes and cancer development. Indeed, studies from Jiri Bartek's and our groups have identified MDC1 and BRIT1 as putative tumor suppressor genes,[24,51] further strengthening their crucial role in safeguarding the genome integrity.

Additionally, in support of the clinical correlation between centrosome/cytokinesis aberrations and cancer prognosis,[2,50] our results demonstrated significant correlations between reduced expression of BRIT1,[24] or MDC1 and the metastatic potential of breast cancer. Thus, the expression of MDC1 and/or BRIT1 is imperative to limit not only the initiation but also the progression of cancer. A proposed model for the function of MDC1 and BRIT1 in maintaining genome surveillance is depicted in Figure 6. Further detailed mechanistic studies are needed to test this important model.

Materials and Methods

Cell lines

U2OS cells were purchased from the American Type Culture Collection (Manassas, VA) and were maintained in McCoy's 5A. HeLa cells were maintained in Dulbecco's modified Eagle's medium. MCF7 cells were maintained in RPMI medium. All three media were supplemented with 10% fetal bovine serum with glutamine, penicillin and streptomycin.

siRNA

The siRNA duplexes were 19 base pairs with a two-base deoxythymidine overhang (Dharmacon Research, Chicago, IL). The sequences of MDC1 siRNA#1 and siRNA#2 oligonucleotides are GUCUCCCAGAAGACAGUGAdTdT and ACAGUUGUCCCCACAGCCCdTdT respectively. The sequences of BRIT1 siRNA#1 and #2 oligonucleotides are AGGAAGUUGGAAGGAUCCAdTdT and CUCUCUGUGUGAAGCACCUdTdT, respectively. The control luciferase siRNA has the following sequence: UAAGGCUAUGAAGAGAUACdTdT. Cells were transfected with siRNA duplexes using Oligofectamine reagent (Invitrogen, Carlsbad, California), following the manufacturer's instructions.

Antibodies

Anti-γ-tubulin clone GTU-88, anti-α-tubulin clone B-5-1-2, anti-MDC1 and anti-Flag M2 affinity gel were purchased from Sigma (St. Louis, MO), and anti-human MDC1 was from AbD Serotec (Oxford, UK). BRIT1 antibody was generated by using a GST-BRIT1 fusion protein synthesized by Proteintech (Chicago, IL). Aurora A and Plk1 were purchased from Cell Signaling Technology (Beverly, MA) and Abcam (Cambridge, UK), respectively, and BubR1 and Mad2 antibody were from Bethyl Laboratories (Montgomery, TX). Secondary antibodies Alexa-488 and Alexa-594 were purchased from Molecular Probes (Invitrogen).

Immunofluorescence microscopy analysis

Cells grown to an exponential phase on cover slips were fixed with methanol/acetone (1:1) for 7 min at −20°C. Similar results were obtained by an alternative fixation protocol that included incubation in 4% paraformaldehyde (10 min at room temperature) followed by 0.2% Triton X-100 (Sigma) for 5 min. Incubation with primary antibodies (anti-mouse γ-tubulin, anti-mouse α-tubulin, anti-human MDC1 and anti-human BRIT1) was carried out for 1 h at room temperature. After three washes with Tris-buffered saline, incubation with secondary antibodies Alexa-488 and Alexa-594 was carried out for 1 h at room temperature, followed by 4'6-diamidino-2-phenylindole-2HCl (DAPI) staining of DNA. Fluorescence images were captured by using a Nicon Eclipse 2000-E and Zeiss Axiovert-200M fluorescence microscope (Germany).

Live cell time-lapse imaging and analysis

Digital time-lapse video microscopy was performed to study mitotic progression after 48 h of MDC1 or BRIT1 knockdown in the stably expressing H2BRFP U2OS cell line. Images were acquired every 3 min for 3 h using a 40X Zeiss Axiovert-200M microscope.

Protein stability assay

After 48 h of luciferase, MDC1 siRNA, and BRIT1 siRNA transfection, the cells were treated with cycloheximide to a final concentration of 10 μg/ml to inhibit protein synthesis. Cells were than harvested at the indicated times after the addition of the inhibitor, and Aurora A and Plk1 levels were analyzed by Western blotting.

Immunofluorescence on frozen breast cancer specimen

Cryosections (12 μm thick) of adjacent normal and breast tissues obtained from Tata Memorial Hospital, India, mounted on coated slides were fixed with methanol for 7 min at −20°C and blocked in phosphate-buffered saline (PBS) containing 5% normal goat serum, 1% glycerol, 0.1% bovine serum albumin (BSA), and 0.1% fish skin gelatin. Incubation with primary antibodies (anti-mouse γ-tubulin anti-human MDC1) was carried out for 1 h at room temperature. After three washes with PBS, incubation with secondary antibodies Alexa-488

and Alexa-594 was carried out for 1 h at room temperature followed by DAPI staining of DNA. Fluorescence images were captured by using a Zeiss Axiovert-200M fluorescence microscope.

Immunohistochemical analysis

Paraffin-embedded sections of adjacent normal and breast cancer specimens stained for centrosome analysis were used to determine the physiologic expression pattern of MDC1 in vivo as follows. After slides were deparaffinized with xylene and rehydrated through ethanol series, microwave antigen retrieval was carried out by placing the slides in a 0.1 M sodium citrate buffer (pH 6.0) and then microwaving them at 98°C for 8 min. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 12 min. Slides were washed three times with PBS and incubated for 1 h in protein blocking buffer (5% normal horse serum plus 1% normal goat serum in PBS). A Vectastain ABC avidin biotin-peroxidase enzyme complex kit (Vector Laboratories, Burlingame, CA) and MDC1 antibody (1:100 dilutions) were used for immunohistochemical staining. Cells were visualized by adding 0.05% diaminobenzidine and counterstained with Gill's No. 3 hematoxylin. The image was captured by using a 40X Zeiss Axioimager Z1 microscope. The studies were performed according to protocols approved by the Tata Memorial Hospital, and all subjects provided written informed consent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by NCI grant R01 CA-112291 and American Cancer Society grant RSG-06-192-01-MGO to S.Y. Lin and an intramural grant from ACTREC, TMC, India to R. Rai. We thank Dr. Junjie Chen (Department of Therapeutic Radiology, Yale University School of Medicine) for valuable advice. We would also like to thank Dr. Harm Kampinga (University of Groningen, The Netherlands) for providing us with the RFP-H2B and GFP γ-tubulin constructs, and Dr. Phang-Lang Chen (University of California, Irvine) for pEGFP MDC1 wild-type and mutant constructs. We also thank Tanuja Dighe for her technical support with the time-lapse experiments.

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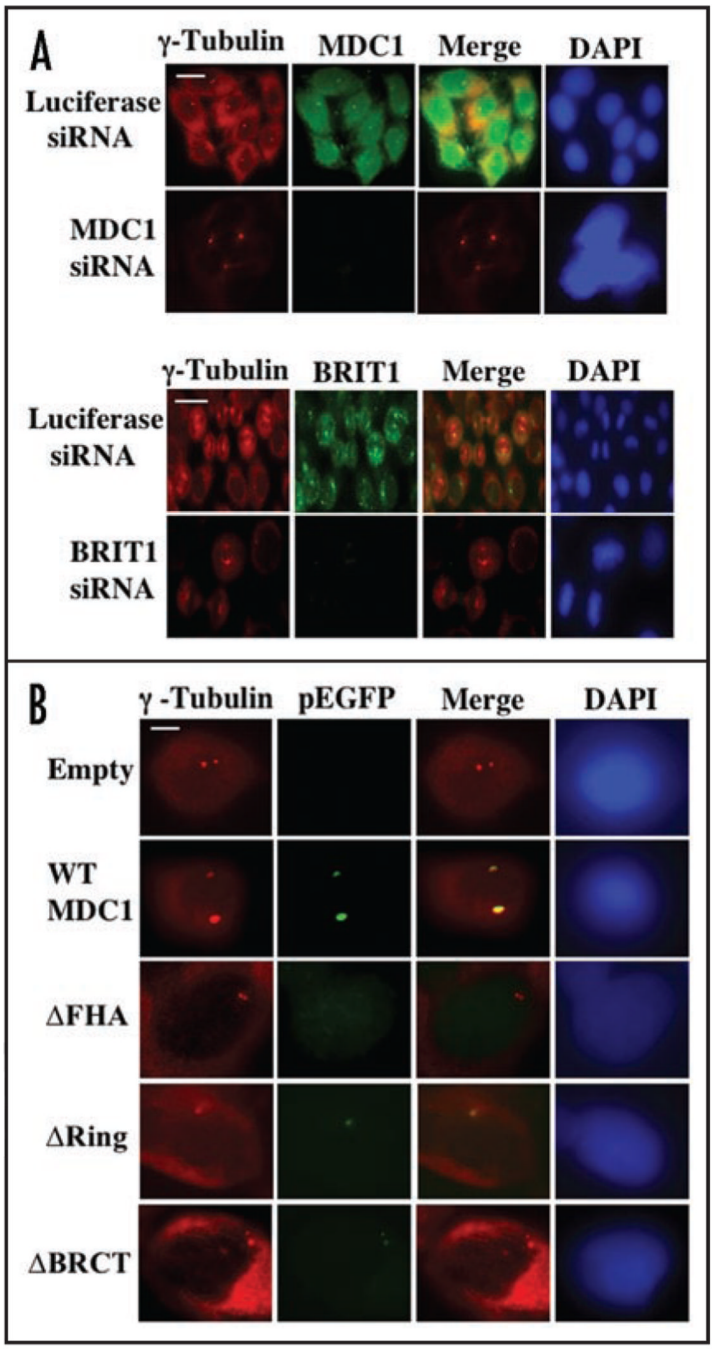

Figure 1. MDC1 and BRIT1 localized on centrosome

(A) U2OS cells grown on cover slips were mock transfected, transfected with luciferase siRNA, MDC1 siRNA, and BRIT1 siRNA. At 48 h after transfection, cells were fixed with methanol/acetone (1:1) and costained with antibodies against γ-tubulin and MDC1 or γ-tubulin and BRIT1 followed by Alexa Fluor dye-conjugated secondary antibodies. Nuclei were visualized with 4'6-diamidino-2-phenylindole-2HCl (DAPI) staining. Scale bar, 20 μm. (B) Wild-type pEGFP-C2-MDC1 and its various deletion mutants were transfected into the U2OS cell line. At 48 h after transfection, the cells were fixed on cover slips with methanol/acetone (1:1) at −20°C and stained with mouse anti-γ-tubulin followed by Alexa Fluor dye-conjugated secondary antibodies. Nuclei were visualized with DAPI staining. Scale bar, 20 μm.

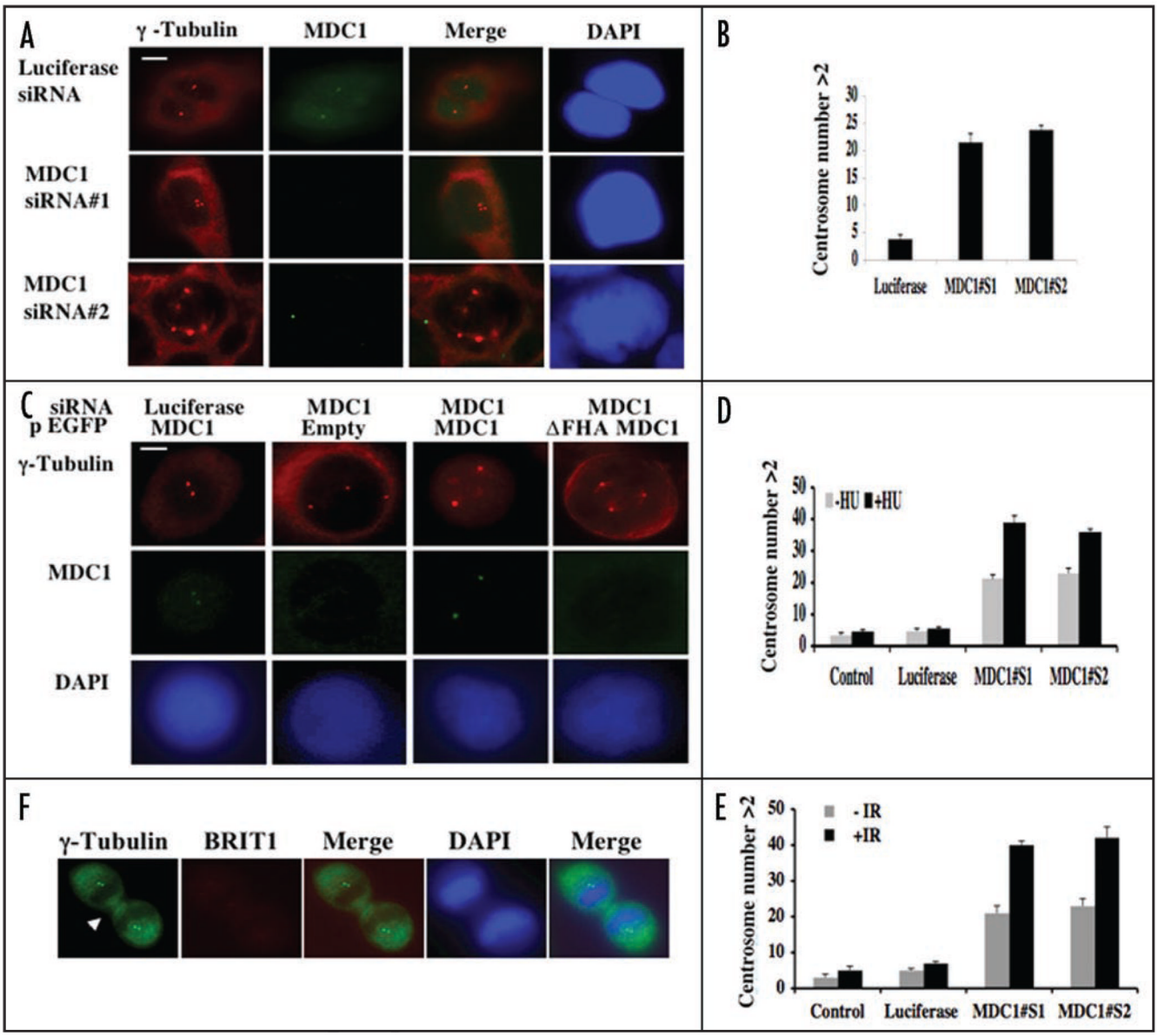

Figure 2. Deficiency of MDC1 or BRIT1 led to centrosome amplification

(A) U2OS cells grown on cover slips were mock-transfected or transfected with luciferase or MDC1 siRNA. At 48 h after transfection, cells were fixed with methanol/acetone (1:1) and costained with antibodies against γ-tubulin and MDC1 followed by Alexa Fluor dye-conjugated secondary antibodies. Nuclei were visualized with DAPI staining. Scale bar, 20 μm. (B) Bar diagram showing that 20% of MDC1-depleted cells contained more than two centrosomes, compared with 3% of luciferase siRNA-treated cells. (C) U2OS cells were treated as described above except that wild-type pEGFP-C2-MDC1 and mutant pEGFP-C2-ΔFHA MDC1 were cotransfected with siRNA to rescue the MDC1-deficient phenotype. Scale bar, 20 μm (D) U2OS cells were grown on cover slips and mock-transfected or transfected with luciferase or MDC1 siRNAs. At 48 h after transfection, cells were treated with 2 mM hydroxyurea for an additional 48 h. Cells were then fixed and costained with antibodies against γ-tubulin and MDC1 followed by Alexa Fluor dye-conjugated secondary antibodies. The percentage of cells containing more than two centrosomes with and without hydroxyurea

treatment was determined. (E) U2OS cells grown as described above except that cells were treated with 10 Gy of γ-radiation instead of hydroxyurea. The percentage of cells containing more than two centrosomes after radiation treatment was determined. (F) U2OS cells grown on cover slips were mock-transfected or transfected with luciferase or BRIT1 siRNA. At 48 h after transfection, cells were fixed with methanol/acetone (1:1) and were costained with antibodies against γ-tubulin and BRIT1 followed by Alexa Fluor dye-conjugated secondary antibodies. Nuclei were visualized with DAPI staining. Arrow indicates centrosome amplification with defective cytokinesis.

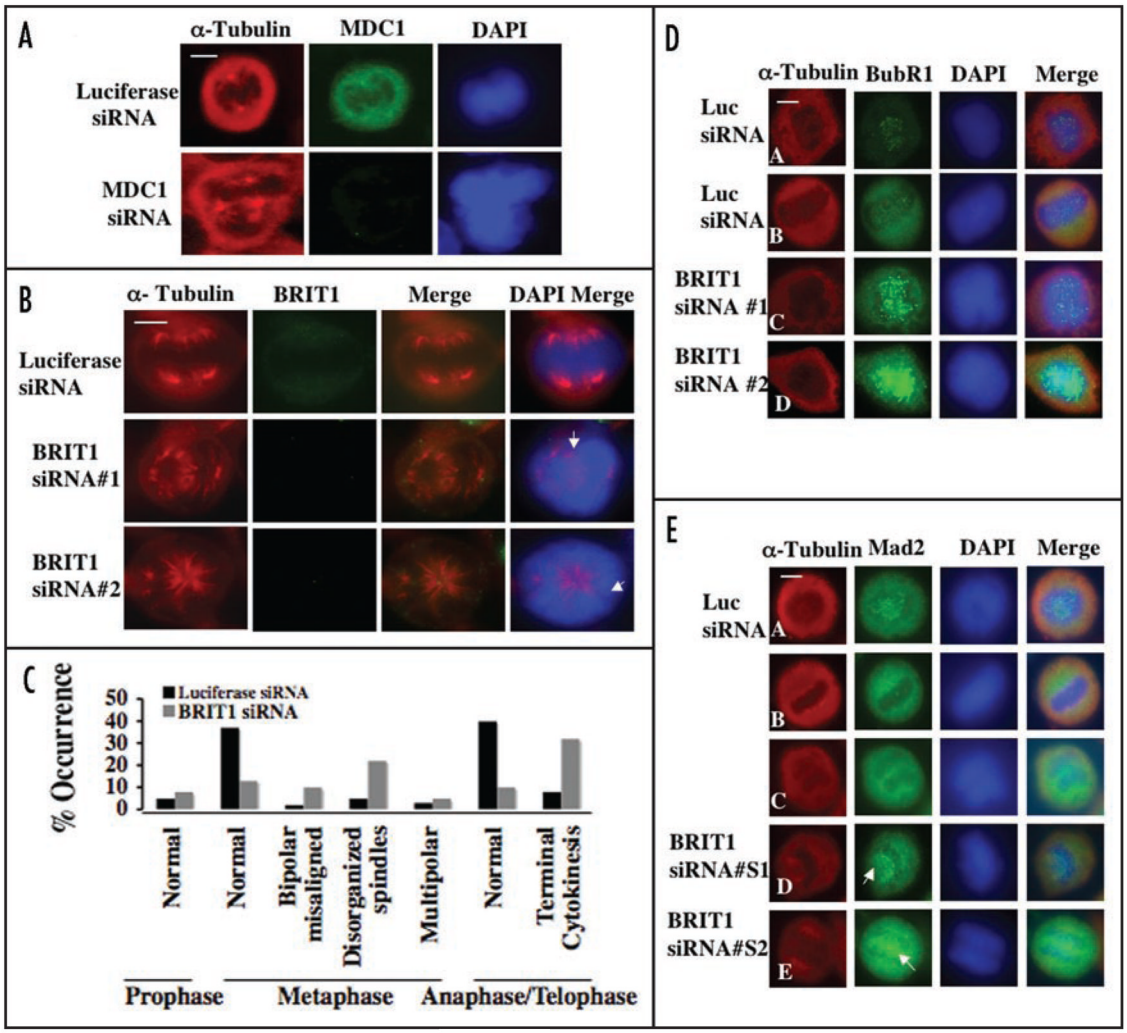

Figure 3. Depletion of MDC1 or BRIT1 leads to aberrant mitotic spindles and defective cytokinesis

(A) U2OS cells grown on cover slips were mock-transfected or transfected with luciferase or MDC1 siRNAs. At 48 h after transfection, cells were fixed and costained with antibodies against α-tubulin and anti MDC1. Cells were washed and subsequently stained with Alexa Fluor dye-conjugated secondary antibodies. Nuclei were visualized with DAPI staining. Scale bar, 20 μm. Mitotic cells with abnormal spindles were captured. (B) Mock-transfected or BRIT1 siRNA-transfected U2OS cells were fixed and costained with antibodies against α-tubulin and BRIT1. Mitotic cells with abnormal spindles were captured. Scale bar, 20 μm. Arrows indicates misaligned spindle that failed to align properly to the equatorial plain, along with a chromosome congression failure at the metaphase. (C) Quantitative analysis of normal and defective mitotic cells observed in luciferase- or BRIT1 siRNA-treated cells. (D) α-tubulin and BubR1 (A, Prometaphase; B, Metaphase; C, Prometaphase; D, Metaphase) or (E) α-tubulin and Mad2 (A, Prometaphase; B, Metaphase; C, Anaphase; D, Metaphase; E, Anaphase) to analyze spindle checkpoint activation. Scale bar, 20 μm. Arrow indicates the persisten accumulation of Mad2 at the metaphase-anaphase transition.

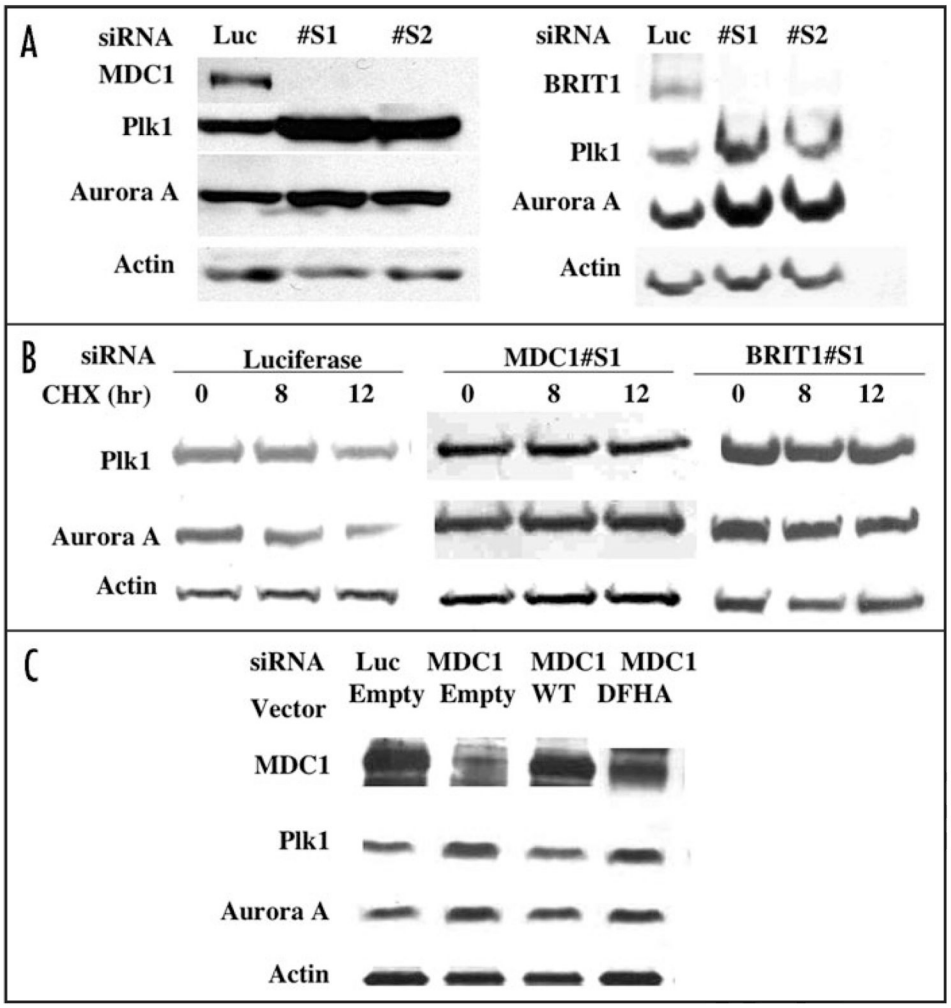

Figure 4. MDC1 and BRIT1 were negative regulators of Aurora A and Plk1

(A) U2OS cells were transfected with luciferase siRNA or MDC1 siRNA (left) or BRIT1 siRNA (right). At 48 h after transfection, cells were harvested and the lysates were subjected to Western blotting using antibodies against (left) MDC1, Plk1 and Aurora A or (right) BRIT1, Plk1 and Aurora A. Actin is for the loading control. (B) U2OS cells were transfected with the siRNA against luciferase, MDC1, or BRIT1. At 48 h after transfection, cells were untreated or treated with cycloheximide for 8 or 12 h. Cells were then harvested, and the lysates were subjected to Western blotting using antibodies against Plk1, Aurora A or actin. (C) U2OS cells were transfected with siRNA against luciferase or MDC1 along with cotransfection of empty expression vector or the vectors expressing wild-type pEGFP-C2-MDC1 or pEGFP-C2-ΔFHA-MDC1 mutant. At 48 h after the transfection, cells were harvested, and the lysates were subjected to Western blotting using antibodies against MDC1, Plk1, Aurora A. Actin is used for the loading control.

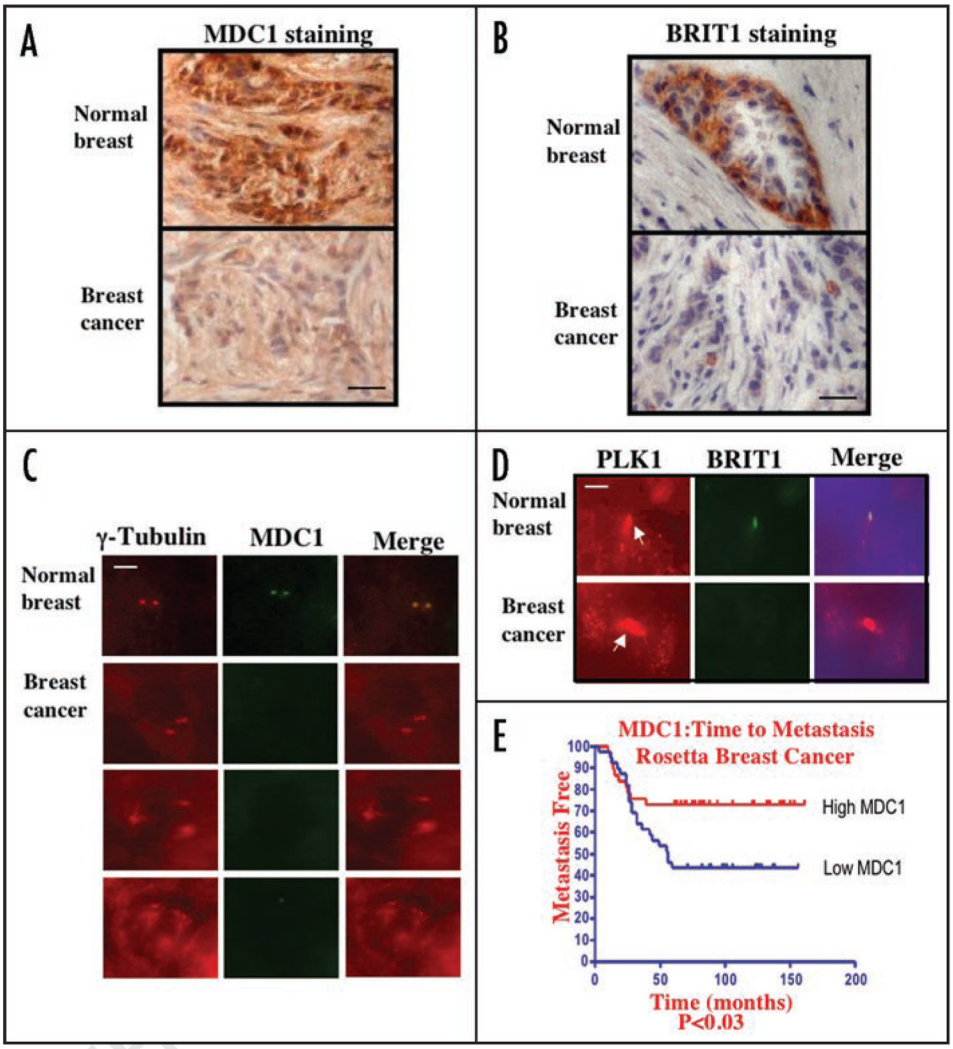

Figure 5. Expression levels of MDC1 and BRIT1 correlated with centrosome amplification and disease progression in breast cancer specimens. Paraffin-embedded adjacent normal and breast cancer specimens were analyzed by immunohistochemical staining

(A) MDC1 and (B) BRIT1 protein expression was correlated with the intensity of brown color in normal and breast cancer specimens. Scale bar, 50 mm. Frozen adjacent normal and breast cancer specimens were immunostained using antibodies against (C) γ-tubulin and MDC1 or (D) Plk1 and BRIT1. Cells were washed and subsequently stained with Alexa Fluor dye-conjugated secondary antibodies. Cells containing more than two centrosomes and mitotic cells with abnormal spindles were visualized. Scale bar, 20 μm. Arrow indicates the overexpression of Plk1 in breast cancer as compared to normal breast specimen. (E) MDC1 mRNA levels from breast cancer were analyzed for correlation with time of freedom from metastasis in a database maintained by Stanford University. Patients whose cancer samples expressed normal to high MDC1 levels were associated with a longer time to develop metastasis and an overall reduction in the incidence of metastasis.

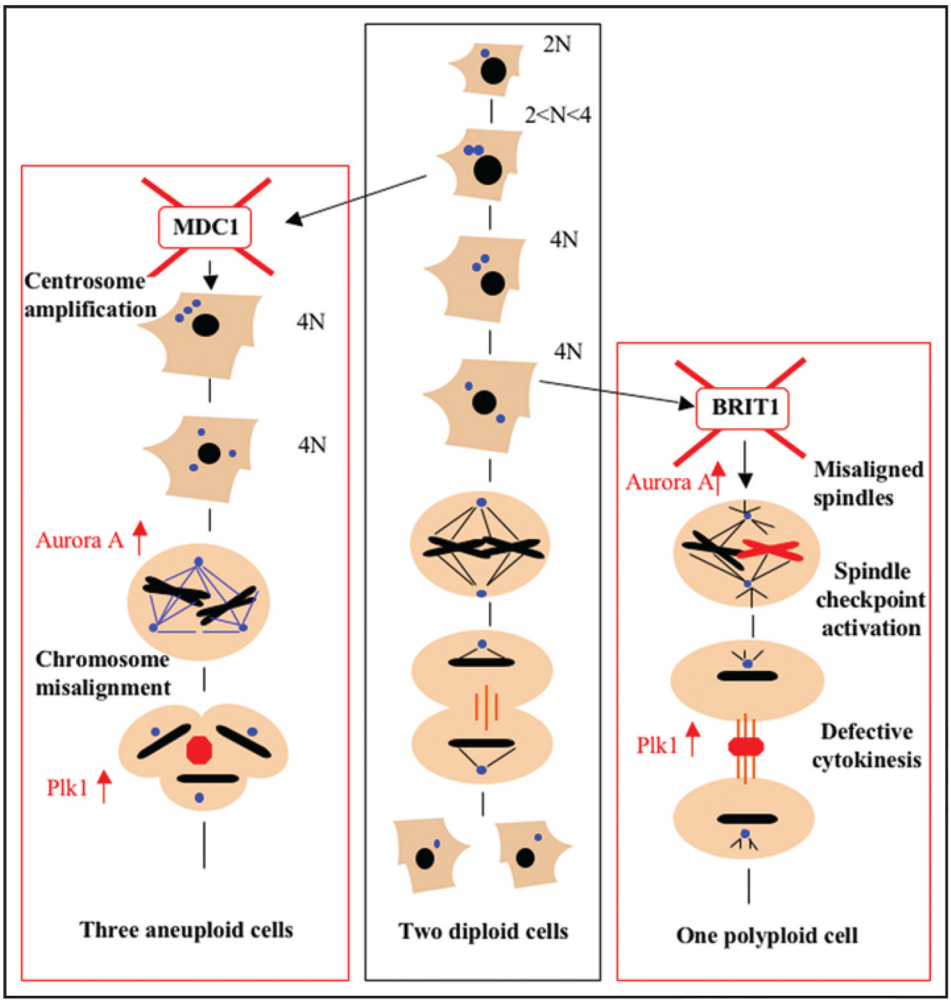

Figure 6.

Model illustrating the role of MDC1 and BRIT1 in maintaining centrosome integrity and cytokinesis. MDC1 and BRIT1 are both required for maintaining numerical centrosome homeostasis, mitotic spindle assembly, and cytokinesis by negatively regulating Aurora A and Plk1. Deficiency of MDC1 led to centrosome amplification followed by multipolar mitosis and chromosome missegregation resulting in aneuploidy. BRIT1 depletion led to misaligned spindles followed by spindle checkpoint activation. Because BRIT1 depletion also led to increased Aurora A and Plk1, the spindle checkpoint was eventually overcome and, in turn, triggered defective cytokinesis, leading to the generation of one polyploid cell.