

ORIGINAL ARTICLE

BRCA1 transports the DNA damage signal for CDDP-induced centrosome amplification through the centrosomal Aurora A

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Abstract

Breast cancer gene 1 (BRCA1) plays roles in DNA repair and centrosome regulation and is involved in DNA damage-induced centrosome amplification (DDICA). Here, the centrosomal localization of BRCA1 and the kinases involved in centrosome duplication were analyzed in each cell cycle phase after treatment with DNA crosslinker cisplatin (CDDP). CDDP treatment increased the centrosomal localization of BRCA1 in early S–G2 phase. BRCA1 contributed to the increased centrosomal localization of Aurora A in S phase and that of phosphorylated Polo-like kinase 1 (PLK1) in late S phase after CDDP treatment, resulting in centriole disengagement and overduplication. The increased centrosomal localization of BRCA1 and Aurora A induced by CDDP treatment involved the nuclear export of BRCA1 and BRCA1 phosphorylation by ataxia telangiectasia mutated (ATM). Patient-derived variants and mutations at phosphorylated residues of BRCA1 suppressed the interaction between BRCA1 and Aurora A, as well as the CDDP-induced increase in the centrosomal localization of BRCA1 and Aurora A. These results suggest that CDDP induces the phosphorylation

Abbreviations: ATM, Ataxia telangiectasia mutated; ATR, ATM and Rad3 related; BRCA1, *Breast cancer gene 1*; CHK2, Checkpoint kinase 2; DDICA, DNA damage-induced centrosome amplification; NES, Nuclear export sequence; PLK1, Polo-like kinase 1.

Megumi Kikuchi and Yuki Yoshino contributed equally to the study.

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of BRCA1 by ATM in the nucleus and its transport to the cytoplasm, thereby promoting the centrosomal localization Aurora A, which phosphorylates PLK1. The function of BRCA1 in the translocation of the DNA damage signal from the nucleus to the centrosome to induce centrosome amplification after CDDP treatment might support its role as a tumor suppressor.

KEYWORDS

Aurora A, BRCA1, CDDP, centrosome, DNA damage

1 | INTRODUCTION

Germline and somatic mutations in *BRCA1* are present in several cancers, especially in breast and ovarian cancers.^{1–3} *BRCA1* functions in a variety of cellular processes, including DNA repair and centrosome regulation.^{4–6}

The DNA damage response is initiated by recognition of DNA damage by the sensor proteins ATM and ATR. These kinases phosphorylate the effector kinases checkpoint kinase 1 (CHK1) and CHK2 to amplify the signal and induce cell cycle arrest and DNA repair. ATM responds primarily to DNA double-stranded breaks, whereas ATR protects the integrity of replicating chromosomes.⁷ *BRCA1* is phosphorylated by ATM, ATR, and CHK2 after DNA damage.^{8,9}

Centrosomes are the major microtubule (MT) nucleation centers in animal cells and are critical for the formation of a bipolar mitotic spindle.¹⁰ Each centrosome consists of a pair of centrioles, the mother and daughter centrioles, surrounded by the pericentriolar material. Centrosome duplication is controlled by centriole duplication during the cell cycle,¹¹ and is initiated by the separation of a pair of centrioles (centriole disengagement) in late mitosis–early G1 phase. The new daughter centriole starts to form a procentriole perpendicular to each mother centriole in early S phase. Each daughter centriole elongates during the S and G2 phases. In late G2 phase, the two centrosomes separate to form two mitotic spindle poles.¹⁰

BRCA1 localizes to the centrosome throughout the cell cycle¹² and functions in centriole duplication.^{12–14} *BRCA1* binds to γ -tubulin, a component of centrosomes¹⁵ and ubiquitinates centrosomal proteins.¹³ Inhibition of *BRCA1* causes centriole reduplication.¹⁴ We identified Opg-like ATPase 1 (OLA1) and the receptor for activated C kinase 1 (RACK1) as *BRCA1*-interacting proteins.^{16,17} OLA1 regulates centriole duplication together with *BRCA1*.¹⁶ RACK1 regulates centriole duplication by modulating centrosomal *BRCA1*.^{17,18}

Centriole duplication is controlled by mitotic kinases.^{10,19} Polo-like kinase 1 (PLK1) plays a critical role in centriole disengagement.^{20,21} Aurora A phosphorylates PLK1 at Thr210, and activated PLK1 regulates centrosome maturation and mitotic entry.²² PLK4 functions in the initiation of centriole duplication.^{10,19}

Defects in the mechanisms regulating centrosome duplication cause centrosome aberrations that lead to chromosome segregation errors. DNA-damaging agents induce centrosome amplification, a process known as DDICA.^{23–26} The activation of PLK1 and subsequent premature centriole disengagement are associated with

centrosome amplification induced by S or G2 phase arrest or DNA damage.^{27–33} In addition, *BRCA1* is involved in the centrosomal localization of PLK1 and DDICA induced by the DNA crosslinker mitomycin C (MMC).²⁵ However, it is unknown how the DNA damage signal is transported to the centrosome, resulting in centrosome amplification.

In this study, we performed quantitative analyses of the centrosomal *BRCA1* and the kinases that regulate centrosome duplication in each cell cycle phase after treatment with the DNA crosslinker cisplatin (CDDP). The results showed that CDDP increased the centrosomal *BRCA1*, Aurora A, and phosphorylated PLK1 (p-PLK1). These processes involved the phosphorylation by ATM and nuclear export of *BRCA1*. These results suggest that *BRCA1* functions in the transportation of the DNA damage signal from the nucleus to the centrosome to induce centrosome amplification in response to CDDP.

2 | MATERIALS AND METHODS

2.1 | Immunocytofluorescence

For immunostaining with anti- γ -tubulin and anti-cyclin B1 antibodies, cells were fixed with methanol for 10 min at -20°C and washed with phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-T). For immunostaining with other antibodies, cells were permeabilized in PBS containing 0.2% Triton X-100, 1 mM EGTA, and 1 mM MgCl_2 for 1 min, fixed in methanol for 15 min at -20°C , and washed with PBS-T. After blocking with PBS containing 0.5% bovine serum albumin (PBS-0.5% BSA) for 1 h, cells were incubated with primary antibodies in 1% BSA/PBS overnight. After washing with PBS-T, cells were incubated with secondary antibodies in PBS-0.5% BSA for 1 h. Cells were washed with PBS-T and mounted in mounting medium with Hoechst 33342 stain.

2.2 | Image processing and measurement of fluorescence intensity

ImageJ software v1.49 (<http://imagej.nih.gov/ij/>) was used for image analysis. For analysis of fluorescence intensity of endogenous *BRCA1*, Aurora A, and t-PLK1 at centrosomes, the maximum intensity was measured in a fixed-size area around each centrosome

detected with GFP-centrin or staining with anti- γ -tubulin antibody. Cells showing colocalization of BRCA1 nuclear foci with the centrosome were not analyzed. For analysis of p-PLK1, FLAG-BRCA1, and phosphorylated BRCA1, the integrated signal intensity (ID) was measured in a fixed-size area around each centrosome and the adjusted fluorescence intensity was calculated by subtracting the ID of the background at the nucleus or cytoplasm (according to the localization of each centrosome) from the ID at the centrosome.

3 | RESULTS

3.1 | CDDP increases the centrosomal localization of BRCA1 during S and G2 phases

The centrosomal localization of BRCA1 changes during the cell cycle phases,^{13,25} and DNA damage alters the proportion of cells in each cell cycle phase.³⁴ Therefore, we analyzed the centrosomal localization of BRCA1 in each cell cycle phase in response to CDDP.

Here, HeLa cells expressing GFP-tagged centrin, a centriole marker (HeLa-GFP-centrin), were immunostained with anti- γ -tubulin and anti-cyclin B1 antibodies to characterize the centrosome during the cell cycle (Figure S1A–F). Cyclin B1 is a marker of late S–M phase.³⁵ The number of GFP-centrin foci were consistent with the expression of cyclin B1 when analyzed in relation to the number and distance between γ -tubulin foci. In interphase, most cells with two GFP-centrin foci were cyclin B1-negative, indicating cells in G1–early S phase, and most cells with three or four GFP-centrin foci were cyclin B1 positive, indicating late S–G2 phase (Figure S1A,B).

To analyze more precisely, cells were stained with anti-proliferating cell nuclear antigen (PCNA), a marker of S phase.³⁶ Therefore, in cells with two GFP-centrin foci, PCNA-negative cells were in G1 phase and PCNA-positive cells were in early S phase. In cells with three or four GFP-centrin foci, PCNA-positive cells were in late S phase and PCNA-negative cells were in G2 phase (Figure 1A).

To analyze the effect of CDDP treatment, HeLa-GFP-centrin cells were treated with CDDP for 24 h and immunostained with anti-PCNA antibody, and cells in each cell cycle phase were counted (Figure 1B). CDDP decreased the number of cells in G1 phase and increased that of cells in S phase. These results were consistent with those by flow cytometry (Figures 1C, S2A).

BRCA1 knockdown decreased CDDP-induced centrosome amplification in HeLa cells, as reported in U2OS cells (Figure S2B,C).²⁵ To analyze the centrosomal localization of BRCA1 during the cell

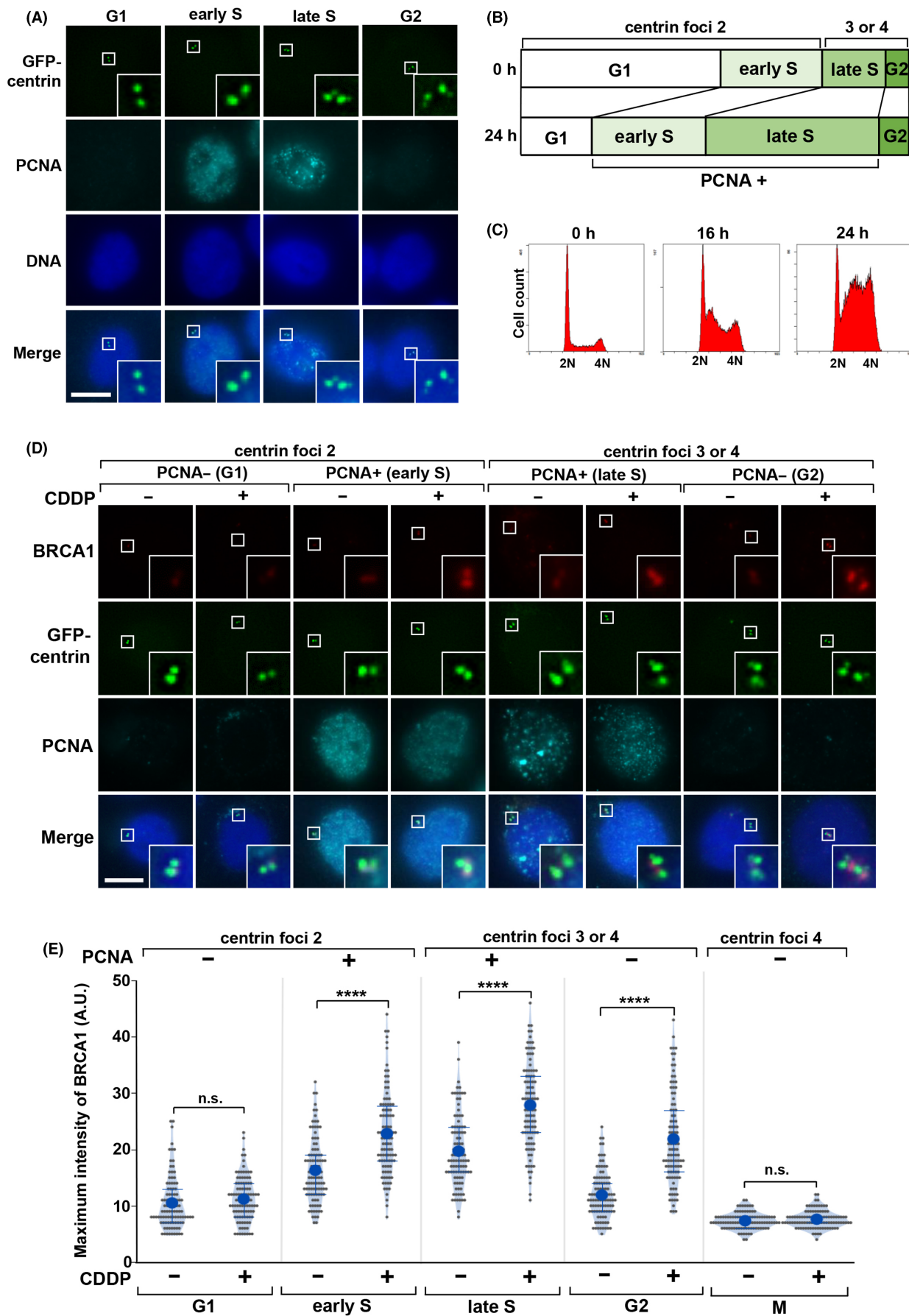
cycle in response to CDDP treatment, HeLa-GFP-centrin cells were treated with CDDP for 24 h and immunostained with monoclonal anti-BRCA1 and polyclonal anti-PCNA antibodies. Cells in the mitotic phase are recognized by chromatin staining using Hoechst 33342. Consistent with previous studies,^{13,25} the centrosomal localization of BRCA1 increased in S phase and decreased in G2 phase in the absence of CDDP (Figure 1D,E). CDDP treatment decreased the amounts of BRCA1 in whole cell lysates, as reported in cells treated with DNA-damaging agents (Figure S2D).^{37,38} However, CDDP treatment increased the centrosomal localization of BRCA1 in early S–G2 phases (Figure 1D,E). BRCA1 localization at the spindle poles was not detected in HeLa-GFP-centrin cells (Figure S2E) and HeLa cells (data not shown). Similar results were obtained using a different polyclonal anti-BRCA1 antibody in HeLa-GFP-centrin cells and normal human mammary epithelium-derived MCF10A cells expressing GFP-centrin (MCF10A-GFP-centrin) (Figure S2F,G). DDICA was induced by CDDP in MCF10A cells similar to HeLa cells (Figure S2H,I).

3.2 | BRCA1 is involved in the CDDP-induced increase in centrosomal localization of Aurora A in S phase

BRCA1 is associated with Aurora A and regulates its activity.^{39,40} We examined the effect of CDDP on the centrosomal localization of Aurora A. HeLa-GFP-centrin cells were treated with CDDP and immunostained with monoclonal anti-Aurora A and polyclonal anti-PCNA antibodies. CDDP treatment increased centrosomal Aurora A in early and late S phases, but not in G1 and M phases (Figures 2A,B, S3A,B). Similar results were obtained using a polyclonal anti-Aurora A antibody in HeLa-GFP-centrin cells (Figure S3C,D) and MCF10A-GFP-centrin cells (data not shown).

BRCA1 knockdown reduced the CDDP-induced increase of centrosomal Aurora A in both early and late S phases in HeLa-GFP-centrin cells and MCF10A-GFP-centrin cells (Figures 2C,D, S3E; data not shown). HeLa-tet-shBRCA1 cells were used to analyze the effects of CDDP and BRCA1 knockdown in PCNA-positive cells considered to be in S phase. CDDP treatment increased the centrosomal localization Aurora A in S phase, and BRCA1 knockdown suppressed this effect (Figure S3F–H). A slight increase of CDDP-treated cells in S and G2/M phases in the BRCA1-knockdown group was observed by flow cytometry (Figure S3I). These results suggest that BRCA1 is involved in the CDDP-induced increase in the centrosomal localization of Aurora A in S phase.

FIGURE 1 CDDP increases the centrosomal localization of BRCA1. (A) HeLa-GFP-centrin cells were immunostained with anti-PCNA antibody and stained with Hoechst 33342. Scale bar, 10 μ m. (B) In total, 600 cells from three independent experiments were counted. (C) HeLa-GFP-centrin cells were treated with 2 mM CDDP for 24 h and analyzed by flow cytometry. At least 10,000 cells were analyzed. (D) HeLa-GFP-centrin cells were treated with CDDP for 24 h, immunostained with monoclonal anti-BRCA1 and polyclonal anti-PCNA antibodies. Scale bar, 10 μ m. (E) The fluorescence intensity data of centrosomal BRCA1 were collected from at least 100 cell images in each cell cycle phase from three independent experiments. Blue points and bars represent the median \pm upper/lower quartiles. **** $p < 0.0001$, n.s., not significant.



3.3 | BRCA1 contributes to the CDDP-induced increase in centrosomal localization of p-PLK1 in late S phase

BRCA1 promotes the centrosomal localization of total PLK1 (t-PLK1) in response to MMC.²⁵ However, CDDP treatment did not increase the centrosomal t-PLK1 in every cell cycle phase (Figure S4A; results of G2 and M phase are not shown).

Because Aurora A phosphorylates PLK1,²² we analyzed the effect of CDDP on the centrosomal p-PLK1 (Figures 3A,B, S4B–D). In a fraction of cells in late S phase, CDDP treatment markedly upregulated centrosomal p-PLK1.

PCNA functions in DNA repair in G2 phase.⁴¹ We presumed that positive PCNA staining would identify cells in G2 phase after CDDP treatment. For the analysis of centrosomal p-PLK1, cells with two GFP-centrin foci were considered to be in G1-early S phase, and cells with three or four GFP-centrin foci were considered to be in late S–G2 phase (Figures 3C, S4E,F). Centrosomal p-PLK1 increased in late S and G2 phases, but not in G1-early S phase, suggesting that CDDP increases centrosomal p-PLK1 in late S phase. In HeLa-tet-shBRCA1 cells, BRCA1 knockdown suppressed the CDDP-induced increase in centrosomal p-PLK1 in S phase (Figure 3D,E), suggesting that BRCA1 is involved in the CDDP-induced increase in the centrosomal localization of p-PLK1.

To confirm the signaling pathways in CDDP-induced centrosome amplification, HeLa-GFP-centrin cells were treated with CDDP for 53 h, followed by an additional 7 h of treatment with CDDP together with inhibitors of Aurora A, PLK1, and PLK4, namely, MLN8054, BI6727, and centrinone B, respectively. Treatment with Aurora A and PLK1 inhibitors decreased the proportion of CDDP-treated cells with centrosome amplification (Figure 3F,G).

3.4 | BRCA1 regulates centriole disengagement induced by CDDP treatment

PLK1 overactivation results in premature centriole disengagement.³⁰ C-Nap1 localizes to the basal part of the mother centriole and disengaged daughter centrioles.⁴² Immunostaining shows that engaged centrioles have one C-Nap1 focus, whereas disengaged centrioles have two foci.⁴³ More than two foci indicate premature centriole disengagement¹⁸ (Figure 4A).

CDDP treatment increased the cells with more than two C-Nap1 foci in a time-dependent manner. BRCA1 knockdown decreased the cells with more than two foci (Figure 4B–E). These results suggest that CDDP promotes premature centriole disengagement, and that BRCA1 contributes to this process.

To determine whether CDDP treatment causes centriole overduplication, HeLa-GFP-centrin cells were treated with CDDP and immunostained with anti- γ -tubulin antibody. Cells with more than five GFP-centrin foci and more than three γ -tubulin foci were counted. CDDP increased the fractions of cells with extra GFP-centrin foci and cells with extra γ -tubulin foci in a time-dependent manner (Figure 4F). Cells were categorized into the following four types: I, cells without extra centrioles or extra centrosomes; II, cells with extra centrioles and without extra centrosomes; III, cells without extra centrioles and with extra centrosomes; and IV, cells with extra centrioles and extra centrosomes. Cells with extra centrosomes without extra centrioles (III) were detected earlier (Figure 4G,H). These results suggest that CDDP treatment causes centriole overduplication by promoting premature centriole disengagement.

3.5 | Nuclear export of BRCA1 contributes to the CDDP-induced increase in the centrosomal localization of BRCA1 and Aurora A and centrosome amplification

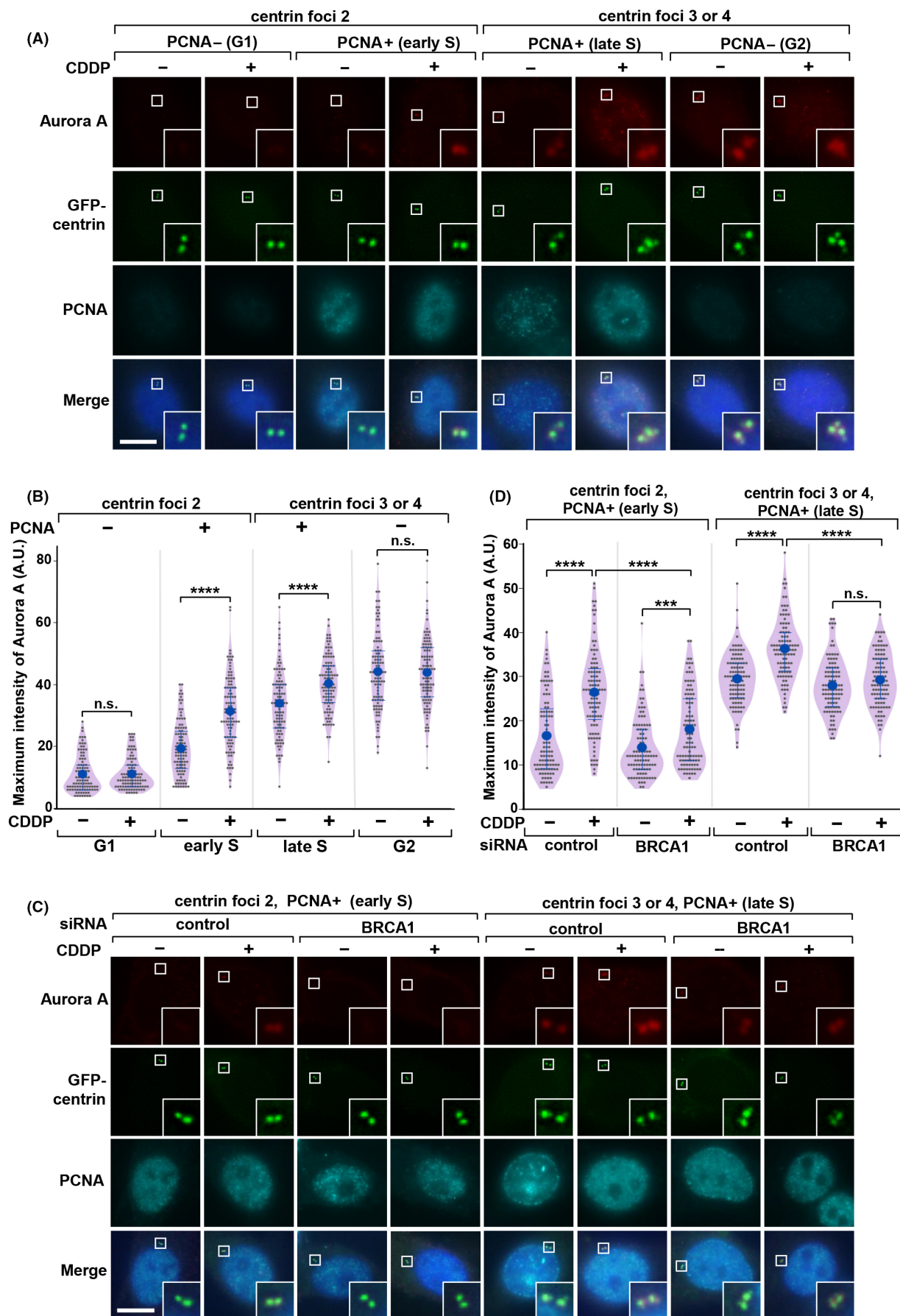
The NES of BRCA1 is important for the centrosomal localization of BRCA1.⁴⁴ To analyze the effect of mutations in the NES, HeLa cells were transfected with FLAG-tagged BRCA1-wild-type (FLAG-BRCA1-WT) or NES mutant (NESm, L86A+I90A)⁴⁴ together with GFP-centrin, which was co-transfected to visualize centrioles and identify transfected cells, and BRCA1 siRNA to knockdown endogenous BRCA1. Mutations in the NES decreased the cytoplasmic localization of FLAG-BRCA1 and the centrosomal localization of BRCA1 and Aurora A after CDDP treatment (Figures 5A–D, S5A–C). No significant changes in the cell cycle were observed by the expression of FLAG-BRCA1-WT or NESm by flow cytometry (Figure S5D).

Next, we analyzed the effect of NES mutations on the CDDP-induced centrosome amplification using HeLa-tet-shBRCA1 cells. BRCA1-WT rescued the reduction of CDDP-induced centrosome amplification by BRCA1 knockdown, but not the NES mutant (Figure 5E–G). These results suggest that nuclear export of BRCA1 is important for the CDDP-induced increase in the centrosomal localization of BRCA1 and Aurora A and centrosome amplification.

3.6 | The kinase activity of ATM contributes to the CDDP-induced increase in the centrosomal localization of BRCA1 and Aurora A

Next, we examined the effect of the kinase inhibitors KU55933, VE821, and PV1019, which are inhibitors of ATM, ATR, and CHK2,

FIGURE 2 BRCA1 is involved in the CDDP-induced centrosomal localization of Aurora A. (A) HeLa-GFP-centrin cells were treated with CDDP for 24 h and immunostained with monoclonal anti-Aurora A and anti-PCNA antibodies. Scale bar, 10 μ m. (B) The fluorescence intensity data of centrosomal Aurora A were collected from at least 100 cell images from three independent experiments. Blue points and bars represent the median \pm upper/lower quartiles. (C) HeLa-GFP-centrin cells were transfected as indicated, treated with CDDP, and immunostained with monoclonal anti-Aurora A and anti-PCNA antibodies. Scale bar, 10 μ m. (D) The fluorescence intensity data were collected from at least 100 cell images from three independent experiments. *** p < 0.001, **** p < 0.0001, n.s., not significant.



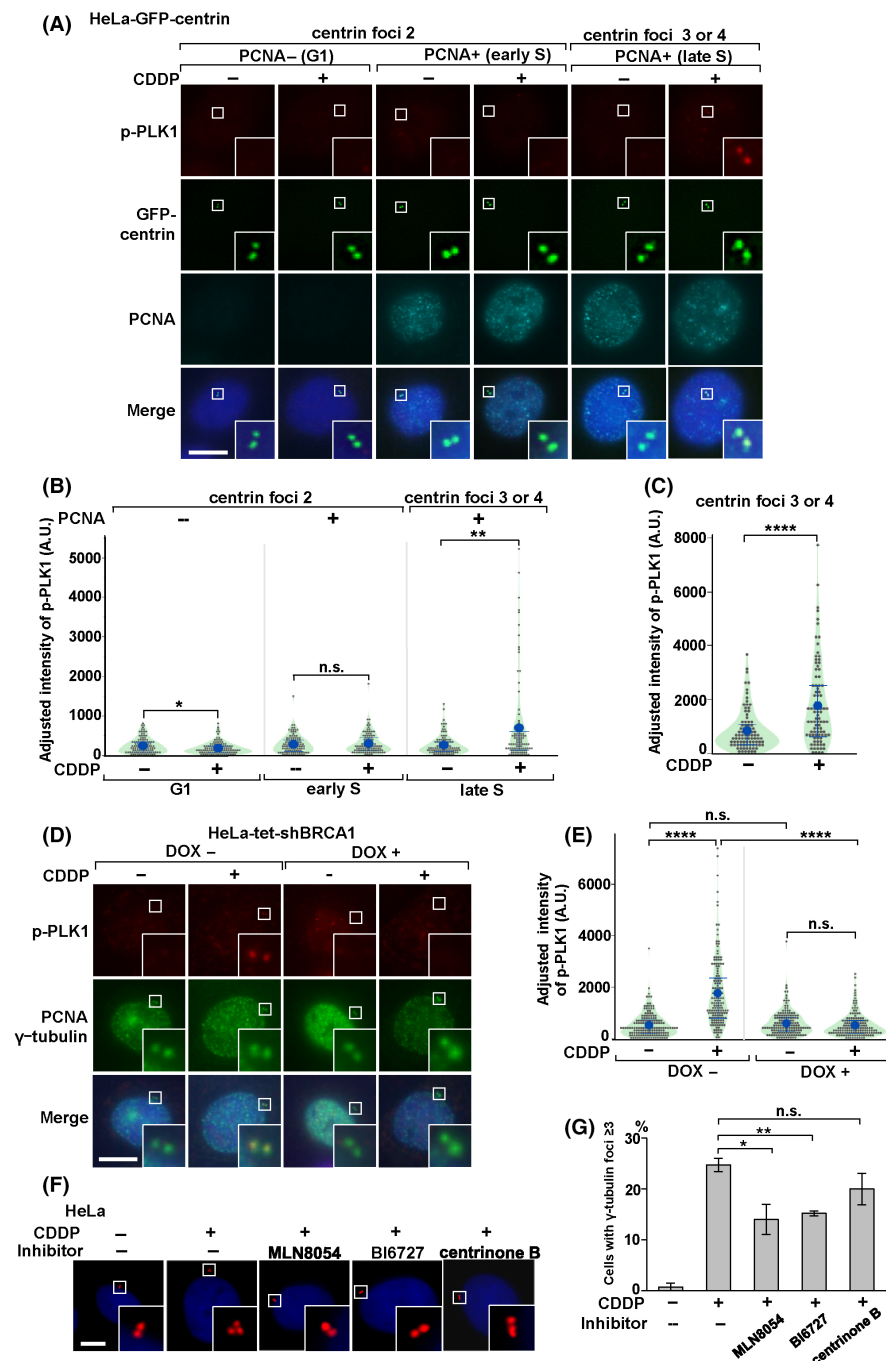
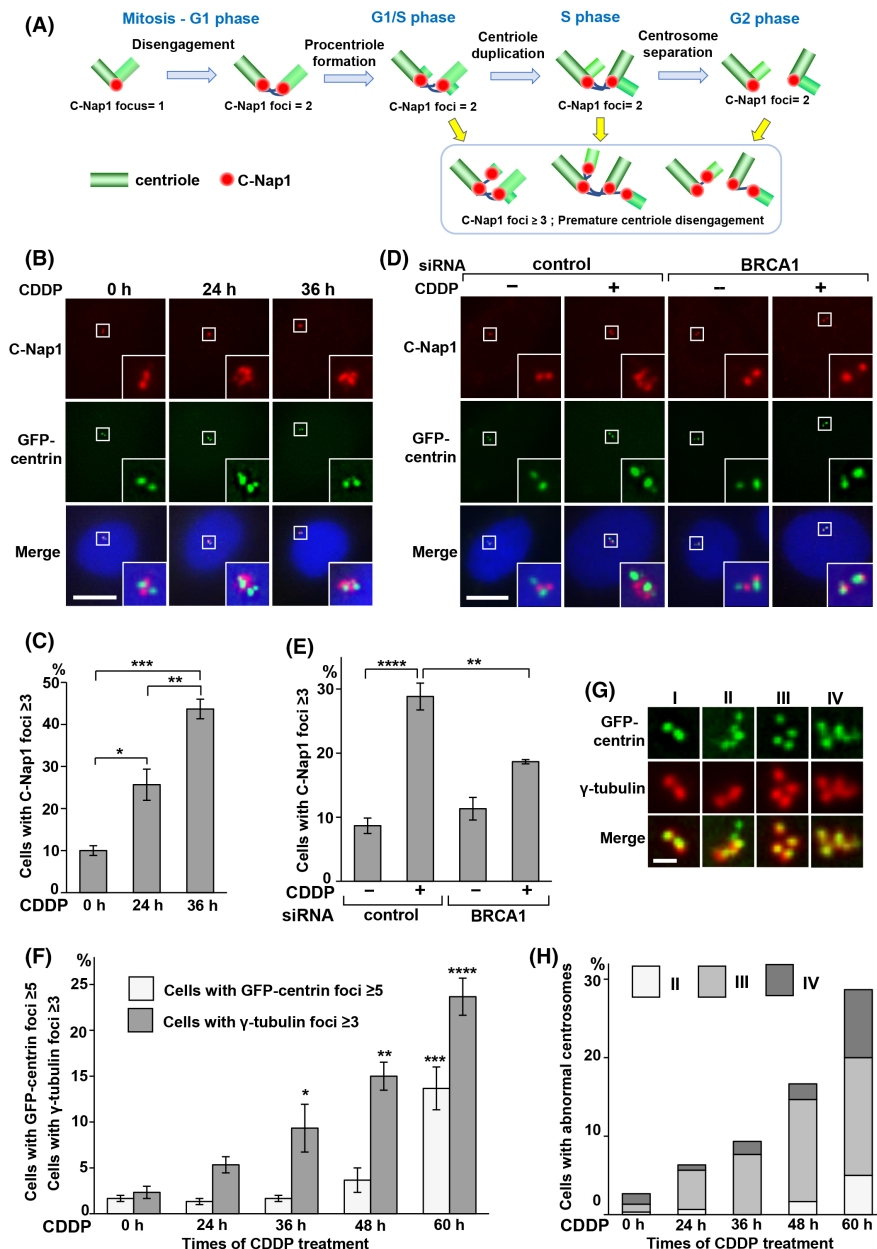


FIGURE 3 BRCA1 is involved in the CDDP-induced centrosomal localization of p-PLK1. (A) HeLa-GFP-centrin cells were treated with CDDP for 24h, and immunostained with anti-p-PLK1 and anti-PCNA antibodies. Scale bar, 10 μ m. (B) The fluorescence intensity data of centrosomal p-PLK1 were collected from at least 100 cell images from three independent experiments. Blue points and bars represent the median \pm upper/lower quartiles. (C) The fluorescence intensity data in cells with three or four centrin foci were collected from at least 90 cell images from three independent experiments. (D) HeLa-tet-shBRCA1 cells were treated with Doxycycline (DOX) for 48h, and then treated with DOX and CDDP for 24h. Cells were immunostained with polyclonal anti-p-PLK1, monoclonal anti-PCNA, and monoclonal anti- γ -tubulin antibodies. Scale bar, 10 μ m. (E) The centrosomal fluorescence intensity in S phase was quantified from at least 150 cell images from three independent experiments. (F) HeLa cells were treated with CDDP for 53h, and then with CDDP and 1 μ M MLN8054, 100nM BI6727, or 500nM centrinone B for 7 h, and immunostained with anti- γ -tubulin antibody. Scale bar, 10 μ m. (G) The percentages of cells with more than three γ -tubulin foci were calculated from at least 100 cells in each sample. The mean of three individual experiments is shown. Error bars indicate \pm SEM. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, n.s., not significant.

respectively. The ATM inhibitor markedly suppressed the CDDP-induced increase in the centrosomal localization of BRCA1 in S and G2 phases, whereas treatment with an ATR inhibitor slightly decreased it only in early S phase; the CHK2 inhibitor reduced it slightly during late S and G2 phases (Figure 6A). Partially consistent with these results, the CDDP-induced increase of centrosomal BRCA1 was suppressed markedly by knockdown of ATM in S and G2 phases and by knockdown of CHK2 in S phase (Figure S6A,B). The CDDP-induced increase of centrosomal Aurora A in S phase was suppressed markedly by ATM and ATR inhibitors, whereas the CHK2 inhibitor reduced it slightly in early S phase (Figure 6B).

S988 of BRCA1 is phosphorylated by CHK2, whereas S1423 and S1524 of BRCA1 are phosphorylated by ATM and ATR.^{8,9} We analyzed the effect of phosphomimetic and nonphosphorylated mutations at these residues. HeLa cells were transfected with FLAG-BRCA1-WT or these variants together with GFP-centrin and BRCA1 siRNA. S1423A and S1524A mutations significantly suppressed the CDDP-induced centrosomal localization of BRCA1 in S and G2 phases, which was significantly restored by the S1524D mutation in S and G2 phase, and moderately restored by the S1423D mutation (Figures 6C, S6C). The CDDP-induced centrosomal localization of Aurora A was decreased by S1423A and S1423D mutations in early and late S phases and by S1524A mutation in late

FIGURE 4 BRCA1 is involved in the CDDP-induced premature centriole disengagement. (A) Schematic illustration of C-Nap1 foci number and centriole disengagement. (B) HeLa-GFP-centrin cells were treated with CDDP and immunostained with anti-C-Nap1 antibody. Scale bar, 10 μ m. (C) The percentages of cells with more than two C-Nap1 foci were calculated from at least 100 cells in each sample. The mean of three individual experiments is shown. Error bars indicate \pm SEM. (D) HeLa-GFP-centrin cells were transfected as indicated, treated with CDDP for 36 h, and immunostained with anti-C-Nap1 antibody. Scale bar, 10 μ m. (E) The percentages of cells with more than two C-Nap1 foci were calculated from at least 100 cells in each sample. The mean of three individual experiments is shown. Significant differences relative to 0 h are indicated. (G) Cells were categorized into four types. Scale bar, 1 μ m. (H) At least 300 cells in each sample from three individual experiments were counted. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.



S phase (Figure 6D). S1524D mutation significantly increased the CDDP-induced centrosomal localization of Aurora A. Mutations at S988 slightly attenuated the CDDP-induced centrosomal localization of BRCA1, but not that of Aurora A. No significant changes in the cell cycle were observed in response to the expression of FLAG-BRCA1-WT or the variants by flow cytometry (data not shown). CDDP treatment increased the centrosomal localization of both S1423- and S1524-phosphorylated BRCA1 in G1-G2 phase (Figure S6D-H).

3.7 | Patient-derived variants of BRCA1 abolish the CDDP-induced increase in the centrosomal localization of BRCA1 and Aurora A

Endogenous BRCA1 co-immunoprecipitated with endogenous Aurora A (Figures 7A, S7A). Exogenous Aurora A interacted with

the full-length, middle portion, and N-terminal region of BRCA1 (Figures 7B, S7B,C). N-terminal region of BRCA1 interacted with Aurora A independently of the RING domain (Figure S7D). Aurora A bound directly to the middle portions of BRCA1, BRCA1-301-660, and BRCA1-594-1080 (Figures 7C, S7E,F).

S1423A and S1524A variants reduced the BRCA1-Aurora A association (Figure 7D). As we possess the vectors for expressing patient-derived BRCA1 variants within amino acids (aa) 1-200 from the Breast Cancer Information Core (BIC) database,⁴⁵ we analyzed the association of 14 variants within aa 70-200 with Aurora A. Three variants, Y105C, N132K, and V191I weakened the BRCA1-Aurora A association (Figure 7E).

The N132K variant, but not the Y105C and V191I variants, suppressed the CDDP-induced increase in the centrosomal localization of BRCA1 (Figure 7F,G). The N132K and V191I variants, but not Y105C, decreased the CDDP-induced increase in the centrosomal localization of Aurora A (Figure 7H).

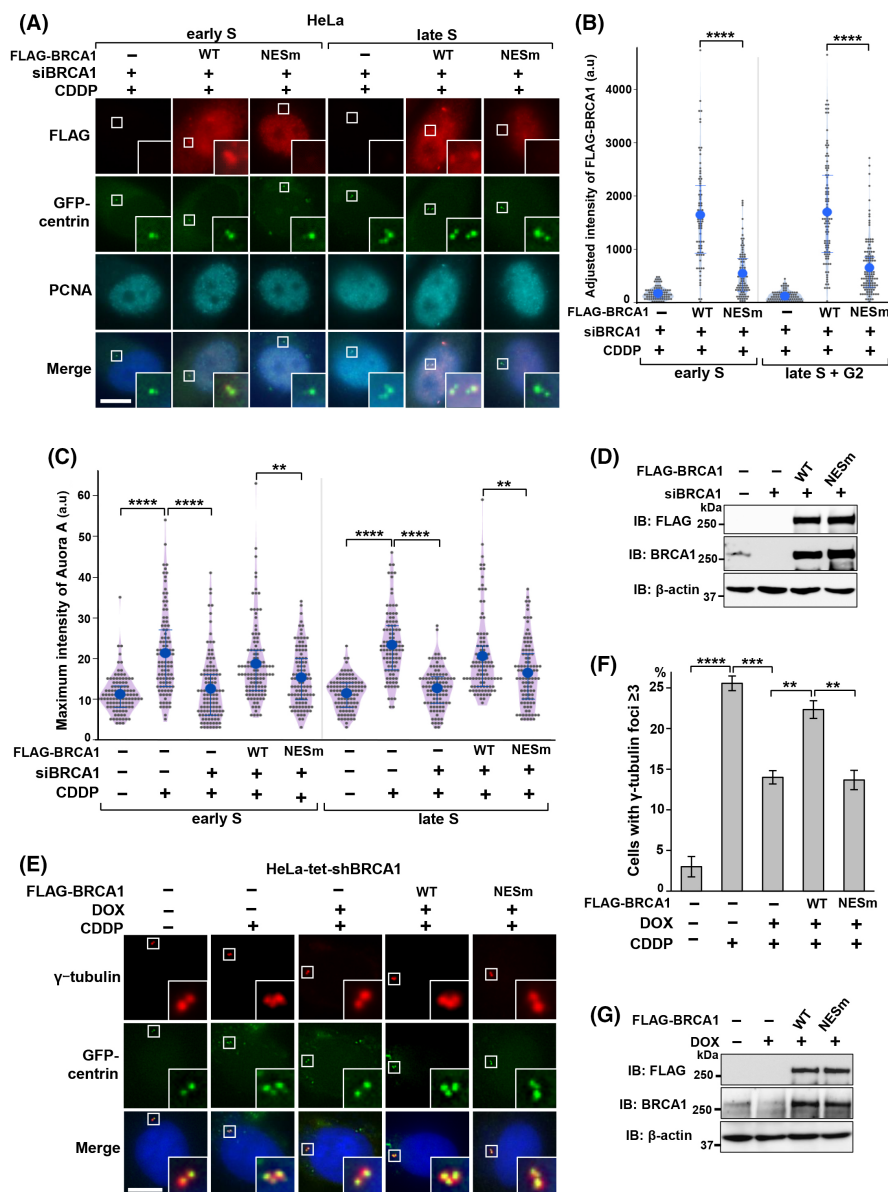


FIGURE 5 Nuclear export of BRCA1 contributes centrosome amplification induced by CDDP treatment. (A) HeLa cells were transfected as indicated, treated with CDDP for 24 h, and immunostained with anti-FLAG (FLA-1) and anti-PCNA antibodies. Scale bar, 10 μm. (B) The fluorescence intensity data were collected from at least 80 cell images from three independent experiments. Blue points and bars represent the median ± upper/lower quartiles. (C) HeLa cells were transfected as indicated, treated with CDDP for 24 h, and immunostained with anti-Aurora A and anti-PCNA antibodies. Centrosomal fluorescence intensity was quantified from at least 100 cell images from three independent experiments. (D) HeLa cells were transfected as indicated. Whole cell lysates were immunoblotted. (E) HeLa-tet-shBRCA1 cells were transfected as indicated, treated with DOX for 8 h, and then treated with DOX and CDDP for 60 h. Cells were immunostained with anti-γ-tubulin antibody. Scale bar, 10 μm. (F) The percentages of cells with more than three γ-tubulin foci were calculated from at least 100 cells in each sample. The mean of three individual experiments is shown. Error bars indicate ± SEM. (G) HeLa-tet-shBRCA1 cells were transfected as indicated. Whole cell lysates were immunoblotted. **p < 0.01, ***p < 0.001, ****p < 0.0001.

4 | DISCUSSION

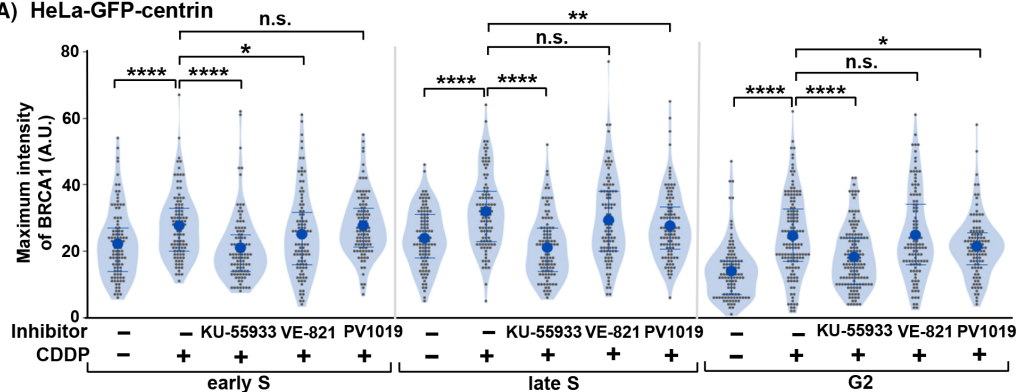
DNA repair factors that localize to the centrosome and are involved in centrosome regulation, including BRCA1, have been identified.⁴⁶ Moreover, deficiencies of centrosomal proteins abrogate the DNA damage response.⁴⁷ These suggest a connection between centrosomal regulation and the DNA damage response. DNA damage causes

centrosome aberrations.²³ However, the underlying mechanism are poorly understood.

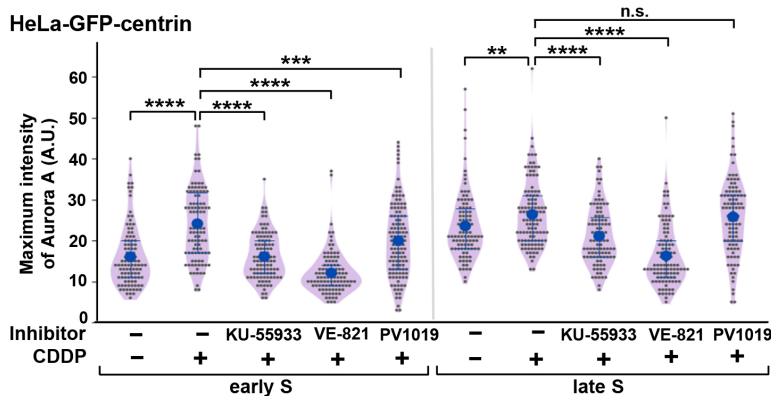
DNA damage activates cell cycle checkpoints and causes cell cycle arrest. We analyzed quantitatively the effect of CDDP treatment on centrosomal BRCA1 and the kinases that regulate centrosome in each cell cycle phase. Analysis of the centrosome during the cell cycle was performed by measuring the number of centrioles and

FIGURE 6 BRCA1 phosphorylation by ATM contributes the CDDP-induced centrosomal localization of BRCA1 and Aurora A. (A) HeLa-GFP-centrin cells were treated with 100 nM KU55933, 5 μM VE821, or 5 μM PV1019 for 1 h and then treated with each inhibitor and CDDP for 24 h. Cells were immunostained with anti-BRCA1 and anti-PCNA antibodies. The centrosomal fluorescence intensity was quantified from at least 100 cell images from three independent experiments. Blue points and bars represent the median ± upper/lower quartiles. (B) The centrosomal fluorescence intensity was quantified from at least 100 cell images from three independent experiments. (C) HeLa cells were transfected as indicated, treated with CDDP for 24 h, and immunostained with anti-FLAG (FLA-1) and anti-PCNA antibodies. The centrosomal fluorescence intensity was quantified from at least 78 cell images from three independent experiments. (D) The centrosomal fluorescence intensity was quantified from at least 100 cell images from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, n.s., not significant.

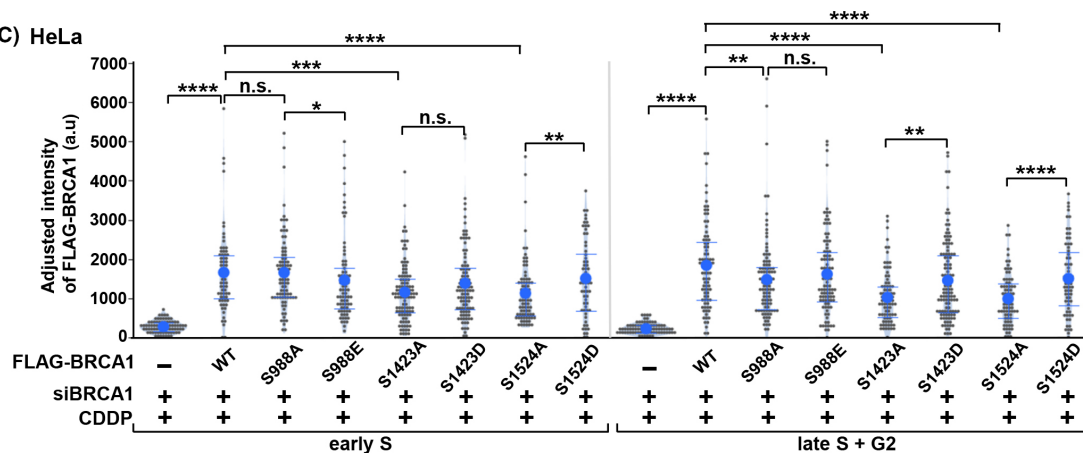
(A) HeLa-GFP-centrin



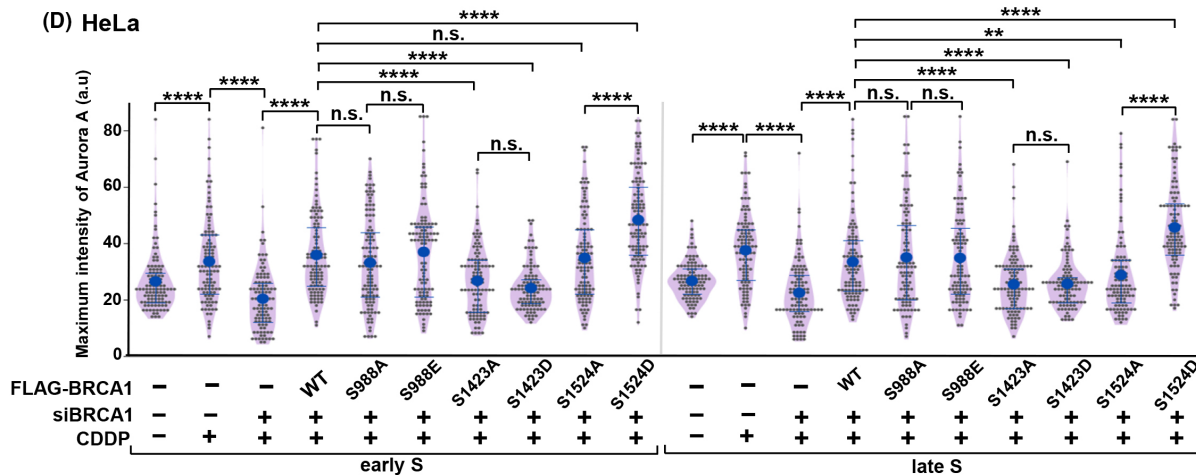
(B) HeLa-GFP-centrin



(C) HeLa



(D) HeLa



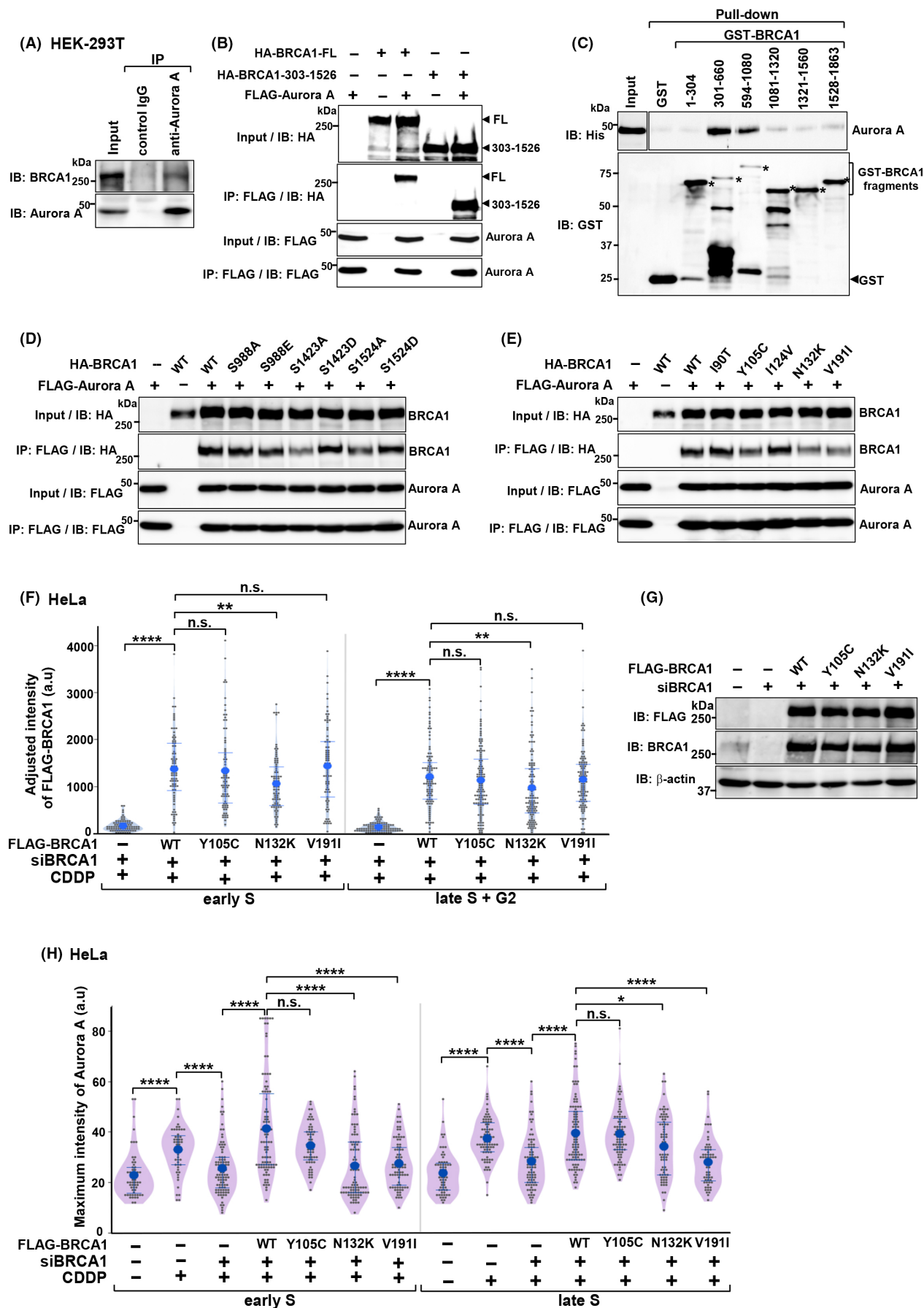


FIGURE 7 BRCA1 binds to Aurora A and promotes the CDDP-induced centrosomal localization of Aurora A. (A) HEK-293T cell lysates were immunoprecipitated (IP) with control IgG or anti-Aurora A antibody and immunoblotted (IB). (B) HEK-293T cells were transfected as indicated. Cell lysates were immunoprecipitated using anti-FLAG antibody. (C) Glutathione-Sepharose beads bound to GST or GST-BRCA1 fragments were incubated with His-Nus Aurora A. The bound proteins were immunoblotted. The GST-BRCA1 bands were marked with asterisks. (D, E) HEK-293T cells were transfected as indicated. Cell lysates were immunoprecipitated using anti-FLAG antibody. (F) HeLa cells were transfected as indicated, treated with CDDP, and immunostained with FLAG (FLA-1) and anti-PCNA antibodies. The fluorescence intensity data were collected from at least 79 cell images from three independent experiments. Blue points and bars represent the median \pm upper/lower quartiles. (G) Whole cell lysates were immunoblotted. (H) The centrosomal fluorescence intensity was quantified from at least 57 cell images from three independent experiments. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, n.s., not significant.

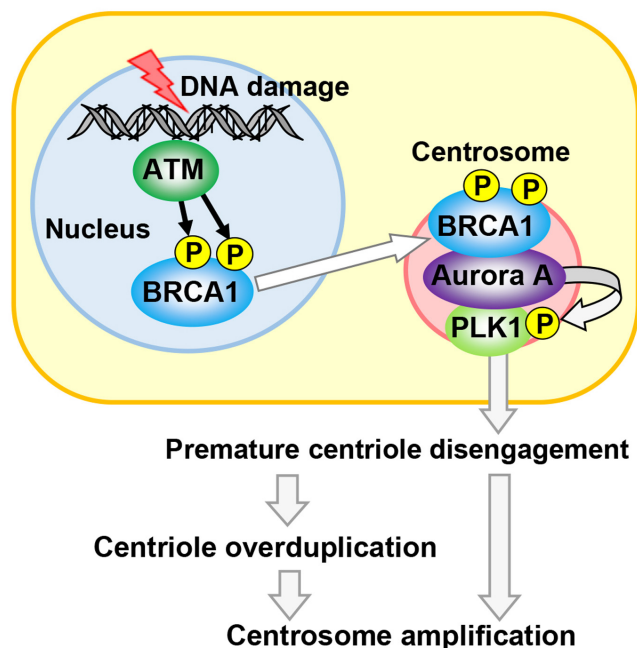


FIGURE 8 Schematic of centrosome amplification induced by CDDP treatment.

by PCNA nuclear staining (Figure 1A,B). MMC increases the centrosomal localization of BRCA1 in G2 phase.²⁵ We showed that CDDP increased the centrosomal localization of BRCA1 in early S–G2 phase (Figures 1D,E, S2F,G).

CDDP increased the centrosomal localization of Aurora A in S phase (Figures 2A,B, S3C,D), and the kinase activities of Aurora A and PLK1 were important for CDDP-induced centrosome amplification (Figure 3F,G). We did not detect increased centrosomal localization of t-PLK1 in response to CDDP treatment (Figure S4A); however, the centrosomal localization of p-PLK1 was significantly increased in late S phase (Figure 3A,B). The increased centrosomal localization of Aurora A in S phase by CDDP treatment was observed earlier than the increase of centrosomal p-PLK1 in late S phase. The results suggest that Aurora A activates PLK1 at the centrosome after CDDP treatment.

In addition, premature centriole disengagement was observed after CDDP treatment (Figure 4B). Centriole disengagement is also induced by cell cycle arrest or DNA damage induced by ionizing radiation and doxorubicin.^{29–31,33} Aurora A inhibitor suppresses G2 arrest-induced centriole disengagement.³¹ CDDP-induced centrosome

amplification seemed to be inhibited by treatment with a PLK4 inhibitor (Figure 3F,G). Centriole overduplication was detected after 60h of CDDP treatment, whereas centriole disengagement appeared earlier than centriole overduplication (Figure 4B,C,G–H). These results suggested that premature disengagement and subsequent centriole overduplication result in CDDP-induced centrosome amplification.

BRCA1 contributed centrosomal localization of Aurora A and p-PLK1 (Figures 2C,D, 3D,E, S3F,G) and premature centriole disengagement induced by CDDP treatment (Figure 4D,E), in addition to CDDP-induced centrosome amplification (Figure S2B). These results suggested that BRCA1 functions upstream of these processes. Conversely, Sankaran and colleagues reported that Aurora A suppresses the ubiquitin ligase activity of BRCA1 that inhibits centrosome microtubule nucleation in M phase.⁴⁸ The increased centrosomal Aurora A in S phase after CDDP treatment might affect the BRCA1 ubiquitin ligase activity and centrosome microtubule nucleation.

Prosser and colleagues used an inhibitor of the CRM/exportin 1 export factor to show that nuclear export of unknown factors is required for centrosome amplification induced by S phase arrest.⁴⁹ Consistent with this, nuclear export of BRCA1 promoted the CDDP-induced centrosomal localization of BRCA1 itself and Aurora A and centrosome amplification (Figures 5, S5A–C).

The phosphorylation of BRCA1 following DNA damage facilitates its function in checkpoint activation.⁵⁰ By contrast, homologous recombination repair requires S988, but not S1423 and S1524.⁵¹ The present results suggest that phosphorylation of BRCA1 S1524 by ATM is important for the BRCA1–Aurora A association and for promoting the centrosomal localization of BRCA1 and Aurora A in response to CDDP treatment, which also involve phosphorylation BRCA1 S1423. S1423D rescued the CDDP-induced centrosomal localization of BRCA1 partially, but not that of Aurora A. Although the S1423D mutant was associated with BRCA1 (Figure 7D), it might not function sufficiently in the CDDP-induced centrosomal localization of Aurora A. In addition, the CDDP-induced increase in the centrosomal localization of Aurora A was suppressed by ATR inhibitor (Figure 6B). ATR may also contribute to the CDDP-induced increase in the centrosomal localization of Aurora A in a manner independent from BRCA1.

Aurora A interacted with the N-terminal region and the middle portion of BRCA1, whereas direct binding of Aurora A occurred at the middle portions of BRCA1 (Figures 7A–C, S7A–F). The middle portion (aa 504–803) is associated with OLA1 via binding to

γ -tubulin,¹⁶ and the aa 802–1002 portion is associated with γ -tubulin.⁵² The N-terminal region corresponds to the binding region of RACK1,¹⁷ which directly binds to Aurora A and functions in the activation of PLK1 by Aurora A.¹⁸ OLA1 and RACK1 might mediate the interaction between Aurora A and BRCA1 and contribute to PLK1 activation after CDDP treatment.

Finally, we identified three patient-derived variants, which reduced the interaction of BRCA1 with Aurora A (Figure 7E). The N132K variant, which moderately decreases homologous recombination activity,⁵³ reduced the CDDP-induced centrosomal localization of BRCA1 and Aurora A, whereas V191I reduced that of only Aurora A (Figure 7F,G). The Y105C variant, which induced a mild reduction of the BRCA1–Aurora A association compared with N132K and V191I, did not affect the CDDP-induced centrosomal localization of BRCA1 and Aurora A. The weak association might be sufficient for the centrosomal localization of BRCA1 and Aurora A. Therefore, the mislocalization of BRCA1 after CDDP treatment and the dissociation of BRCA1 from Aurora A might abolish the CDDP-induced centrosomal localization of Aurora A.

The present data suggest that, in response to nuclear DNA damage, BRCA1 is phosphorylated by ATM in the nucleus, moves to the cytoplasm, localizes to the centrosome, and promotes the centrosomal localization of Aurora A, which phosphorylates PLK1. Activated PLK1 causes premature centriole disengagement and centriole overduplication, leading to DDICA (Figure 8). Therefore, we presume that BRCA1 plays an important role in the translocation of the nuclear DNA damage signal to the centrosome to cause centrosome amplification. Loffler and colleagues reported that DDICA occurs through the excessive formation of centriole satellites.⁵⁴ Involvement of BRCA1 in this process should be investigated.

As centrosome amplification causes mitotic catastrophe,^{55,56} DDICA may be a cell death mechanism for cells with DNA damage that cannot be repaired. Conversely, BRCA1 functions in the regulation of centrosome duplication and microtubule aster formation to maintain genome stability, and its suppression under physiological conditions causes centrosome amplification.^{5,6} BRCA1 variants derived from familial breast cancers causes centrosome amplification,⁵⁷ suggesting that BRCA1 function in centrosomal regulation under physiological conditions is important for tumor suppression. In this study, we identified other BRCA1 variants that abolish the CDDP-induced centrosomal localization of BRCA1 and Aurora A. We propose that the role of BRCA1 in DDICA is crucial for its tumor suppressor function in parallel with its role in the physiological centrosome regulation and the DNA repair pathway. Further investigation of the relationship between centrosomal regulation and the DNA damage response may provide insight into the mechanisms underlying carcinogenesis.

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CONFLICT OF INTEREST

The authors declare no competing interest. Chikashi Ishioka and Yoshio Miki are Associate Editors of *Cancer Science*.

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SUPPORTING INFORMATION

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