Characterization of BRCA1 Protein Targeting, Dynamics, and Function at the Centrosome

A ROLE FOR THE NUCLEAR EXPORT SIGNAL, CRM1, AND AURORA A KINASE

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Background: BRCA1 inhibits centrosome duplication as part of the DNA damage checkpoint.

Results: Binding partners CRM1, BARD1, and Aurora A have distinct roles in targeting BRCA1 to the centrosome and regulating its activity.

Conclusion: BRCA1 nuclear export stimulates its regulation of centrosome duplication.

Significance: CRM1 mediates shuttling of BRCA1 between nucleus and centrosome and may coordinate its DNA damage response.

BRCA1 is a DNA damage response protein and functions in the nucleus to stimulate DNA repair and at the centrosome to inhibit centrosome overduplication in response to DNA damage. The loss or mutation of BRCA1 causes centrosome amplification and abnormal mitotic spindle assembly in breast cancer cells. The BRCA1-BARD1 heterodimer binds and ubiquitinates γ-tubulin to inhibit centrosome amplification and promote microtubule nucleation; however regulation of BRCA1 targeting and function at the centrosome is poorly understood. Here we show that both N and C termini of BRCA1 are required for its centrosomal localization and that BRCA1 moves to the centrosome independently of BARD1 and γ-tubulin. Mutations in the C-terminal phosphoprotein-binding BRCT domain of BRCA1 prevented localization to centrosomes. Photobleaching experiments identified dynamic (60%) and immobilized (40%) pools of ectopic BRCA1 at the centrosome, and these are regulated by the nuclear export receptor CRM1 (chromosome region maintenance 1) and BARD1. CRM1 mediates nuclear export of BRCA1, and mutation of the export sequence blocked BRCA1 regulation of centrosome amplification in irradiated cells. CRM1 binds to undimerized BRCA1 and is displaced by BARD1. Photobleaching assays implicate CRM1 in driving undimerized BRCA1 to the centrosome and revealed that when BRCA1 subsequently binds to BARD1, it is less well retained at centrosomes, suggesting a mechanism to accelerate BRCA1 release after formation of the active heterodimer. Moreover, Aurora A binding and phosphorylation of BRCA1 enhanced its centrosomal retention and regulation of centrosome amplification. Thus, CRM1, BARD1 and Aurora A promote the targeting and function of BRCA1 at centrosomes.

The breast and ovarian cancer susceptibility protein 1 (BRCA1) is a tumor suppressor protein with multiple functions, which is encoded by a gene whose mutational inactivation contributes to hereditary breast and ovarian cancers (1–4). The BRCA1 protein is a key regulator of the cellular DNA damage response and functions in the nucleus as a component of large multiprotein complexes to maintain genomic integrity through homologous recombination and non-homologous end-joining-based DNA repair (5). BRCA1 interacts with many proteins through an N-terminal RING domain and C-terminal tandem BRCT repeats commonly mutated in cancers. The RING domain is a zinc finger motif typical of those found in many ubiquitin ligases and forms part of the BRCA1 sequence that interacts with BARD1 (BRCA1-associated RING domain protein 1) to form a stable BRCA1-BARD1 dimer, with E3 ubiquitin ligase activity (6) that functions in DNA repair (7) and centrosomal duplication (8). Cancer mutations of the BRCT domains, highly folded protein interaction sequences that mediate binding to phosphoserine peptides (9), are known to impair BRCA1 transcription activity (10), DNA repair function (11), and its ability to suppress tumor formation in mice (12).

Centrosomes are non-membranous organelles that nucleate microtubules in interphase and mitosis (13). The centrosome duplicates once during a cell cycle, and the two centrosomes move to opposite poles to form the bipolar mitotic spindle. BRCA1 and BARD1 localize to the centrosome throughout the cell cycle (14, 15) and ensure that centrosome duplication occurs only once during a cell cycle (16), which prevents formation of multipolar spindles, unequal chromosome segregation, and aneuploidy. The loss of BRCA1 by genetic mutation, silencing, or peptide competition results in centrosome amplification in human breast cells, thus contributing to cell transformation

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** This article contains supplemental Figs. S1–S9 and Table S1.

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and ultimately to aneuploidy as detected in breast tumors (16–19). The BRCA1–BARD1 heterodimer ubiquitinates γ-tubulin, a centrosome component of the γ-tubulin ring complex, and this stimulates initial nucleation of microtubules (14). Further studies from the Parvin laboratory (16, 20) show that BRCA1 ubiquitin ligase activity is essential for the negative regulation of centrosome overduplication (generally referred to as amplification in this study) and also for the centrosomal localization of γ-tubulin. BRCA1 regulates the G₂/M cell cycle checkpoint, a mechanism that stalls cell cycle progression after DNA damage to ensure timely repair of the DNA but can inadvertently give time for centrosomes to duplicate more than once in one cell cycle (21). Cell cycle checkpoints are often regulated by kinases, phosphatases, and their substrates. Aurora A kinase is overexpressed in many cancers, including breast cancers (22, 23), and localizes to the centrosome during mitosis (24, 25). Aurora A binds to BRCA1 and moderates its activity at the centrosome by phosphorylation to signal the G₂/M-phase transition (26) and microtubule nucleation inhibition via hindering BRCA1 ubiquitin ligase activity (15).

Our laboratory has studied intracellular transport of BRCA1 since 2000, when we first discovered that BRCA1 contains an N-terminal nuclear export sequence (NES) (27). The BRCA1 NES mediates binding to the major nuclear export receptor, CRM1 (chromosome region maintenance protein 1)/exportin-1, and facilitates the nuclear-cytoplasmic shuttling of BRCA1. The precise role that CRM1 plays in regulation of BRCA1 transport or dynamics in the cell is not well understood. It is also striking to note that despite a decade of research by others into the role of BRCA1 at the centrosome, there is currently no understanding of how BRCA1 moves to the centrosome, what sequences are involved, or which binding partners regulate its recruitment or dynamics. This study was therefore aimed at defining the key sequences that localize BRCA1 to the centrosome and to test the hypothesis that CRM1 contributes to this recruitment process and possibly to the BRCA1 regulation of centrosome amplification.

Previously, we identified N-terminal CRM1-binding NESs in both BRCA1 and BARD1 (27, 28). In addition to its nuclear export function, CRM1 has been shown to bind and regulate (i.e., enhance or reduce) the action of several cellular and viral proteins to maintain normal centrosome duplication (29). Foruges et al. (30) observed that CRM1 localized to the centrosome and that CRM1 inhibition by use of the drug leptomycin B (LMB) or CRM1 sequestration by a hepatitis B viral protein, HBx, resulted in formation of supernumerary centrosomes. Some and that CRM1 inhibition by use of the drug leptomycin B (LMB) or CRM1 sequestration by a hepatitis B viral protein, HBx, resulted in formation of supernumerary centrosomes. Our laboratory has studied intracellular transport of BRCA1 since 2000, when we first discovered that BRCA1 contains an N-terminal nuclear export sequence (NES) (27). The BRCA1 NES mediates binding to the major nuclear export receptor, CRM1 (chromosome region maintenance protein 1)/exportin-1, and facilitates the nuclear-cytoplasmic shuttling of BRCA1. The precise role that CRM1 plays in regulation of BRCA1 transport or dynamics in the cell is not well understood. It is also striking to note that despite a decade of research by others into the role of BRCA1 at the centrosome, there is currently no understanding of how BRCA1 moves to the centrosome, what sequences are involved, or which binding partners regulate its recruitment or dynamics. This study was therefore aimed at defining the key sequences that localize BRCA1 to the centrosome and to test the hypothesis that CRM1 contributes to this recruitment process and possibly to the BRCA1 regulation of centrosome amplification.

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**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfection, and Treatments**—Human breast cancer cell line MCF-7 and human osteosarcoma cancer cell line U2OS were cultured in Dulbecco’s modified Eagle’s medium (DMEM) as described in (38). Human breast cancer cell line HCC1937 (BRCA1 5832InsC mutation) were grown in supplemented RPMI 1640 medium as described (37). At 16 h after seeding, cells were transfected at 50–60% confluence with 2 μg of plasmid DNA (per well in a 6-well plate) using Fugene HD reagent (Promega) according to the manufacturer’s instructions. For siRNA transfections, cells were transfected at 40–50% confluence with 3 μg of siRNA (per well in a 6-well plate) using Lipofectamine 2000 reagent (Invitrogen). At 6 h post-transfection, the transfection mix was removed and replaced with medium containing FBS, as described above. Cells were fixed and processed 24–30 h post-transfection for fluorescence microscopy or Western blotting. For LMB treatment, cells were treated with 5 ng/ml (Sigma) for 12 h before fixation and immunostaining. For nocodazole treatment, cells were treated with 10 μM nocodazole 1 h before fluorescence recovery after photobleaching (FRAP) or fixation and immunostaining.

**Cell Cycle Analysis by Flow Cytometry**—HCC1937 cells were transfected with various yellow fluorescent protein (YFP)-tagged BRCA1 plasmid constructs expressing different forms of BRCA1. 48 h post-transfection, cells were harvested by trypsinization and resuspended 400 μl of PBS, added dropwise to ice-cold 85% ethanol, and incubated for at least 1 h. Prior to flow cytometry, cells were centrifuged at 1500 rpm and resuspended in 600 μl of PBS containing RNase A (1 mg/ml) and propidium iodide (2 mg/ml). Cell cycle/apoptosis profiles were determined using a BD Biosciences FACSCalibur flow cytometer (excitation wavelength 485 nm, emission wavelength 508 nm). The percentage of apoptotic cells was determined by quantifying the sub-G₁ DNA content population using CellQuest software.

**Plasmids**—Many of the plasmids used in this study have been described previously (27, 28, 39–43). Other YFP-labeled BRCA1 constructs were produced by inserting the YFP coding sequence into the NotI site upstream of the BRCA1 coding sequence in FLAG-tagged constructs described previously (27, 39). YFP-BRCA1 297–773 was made using a two-step cloning process. First the NotI/EcoRI site of pF–BRCA1 wt was excised and replaced by a linker made using primers, 5’-G GCC GCA ATG AAT GTA GAA AAG GCT G-3’ and 3’-CGT TAC TTA
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CAT CTT TTC CGA CTT AA-5' to create pF-BRCA1 297–1863. The C-terminal sequence of pF-BRCA1 297–1863 from residue 773 was excised with KpnI and religated. YFP was then inserted as above. Specific details of individual cloning techniques and primer sequences are available on request. RFP-pericentrin C241 was supplied as a generous gift by Dr. Sean Munro (44).

Immunofluorescence Microscopy—Centrosomal protein immunostaining was performed as described previously (38), fixing cells with acetone/methanol and staining cells with the following primary antibodies: rabbit polyclonal BRCA1 Ab-P (1:1000; gift from Prof. Jeffrey Parvin), mouse monoclonal α-tubulin (1:1000; Abcam), mouse monoclonal α-tubulin (1:1000; Sigma), and rabbit polyclonal anti-FLAG (1:2000; Sigma). Bound antibodies were detected with either AlexaFluor 488 (1:500)- or AlexaFluor 594 (1:1000)-conjugated secondary antibodies. Cells were observed and imaged using an Olympus BX-51 fluorescence microscope at ×60 magnification and a Spot RT slider camera 23.1. For each experiment, an average score was obtained from two or three individual experiments, and at least 100 cells were scored for each treatment per experiment. For live imaging, an Olympus FV1000 confocal laser-scanning microscope with a ×60 water objective was used to take z-stack images of co-transfected YFP-CRM1 and RFP-pericentrin C241 at 0.5-μm intervals through the centrosome. The fluorescence intensity of endogenous BRCA1 levels (relative to the centrosome marker, γ-tubulin) in untreated and LMB-treated cells was measured using the Olympus Fluoview version 1.6a software and graphed using Microsoft Excel. For each treatment, at least 200 cells over two experiments were quantified.

Fluorescence Recovery after Photobleaching (FRAP)—FRAP was performed on MCF-7 cells co-expressing similar and moderate levels of YFP-BRCA1 constructs and RFP-pericentrin C241 at 30–48 h post-transfection in a humidified CO2 chamber at 37 °C. RFP-pericentrin C241 was co-transfected to mark the centrosome in live cells. The analysis was performed on an Olympus FV1000 confocal laser-scanning microscope with a ×60 water objective. For YFP-tagged construct FRAP, a cell was scanned with laser power (10–13%), and the region of interest was then photobleached at 100% laser power. For the RFP-tagged pericentrin C241 construct, 20% laser power was used for scanning, and 100% laser power was used for bleaching. Data were analyzed with Olympus Fluoview version 1.6a software. Each FRAP experiment started with two prebleach image scans followed by bleaching 90–100% of the centrosome for 4 s. Images were collected every 4 s for a total of 60 s, and the size of the scan region and digital zoom (×3.2) was kept constant during each experiment. Each type of FRAP analysis was based on 10–15 cells from at least two independent experiments. Average intensities in all regions of interest, including the background signal and whole cell fluorescence bleaching, were calculated using Olympus Fluoview version 1.6a software. In Microsoft Excel (2007), bleaching from imaging and background fluorescence were deducted for each cell, and an average recovery curve was generated. These data were then entered into GraphPad Prism 5 and determined to best fit a two-phase association curve. r² and plateau values were determined and used to compare initial speed of recovery and the recovery percentage at the centrosome. The fraction of protein that contributes to the recovery is called the “mobile” fraction, and the protein that does not is called the “immobile” fraction. Spot bleaching of the cytoplasm was carried out for each construct and showed that fluorescence recovery due to cytoplasmic diffusion was <10% for both BRCA1 and pericentrin proteins (data not shown).

CSK Retention Assay—To compare centrosomal retention of endogenous BRCA1 and γ-tubulin, a detergent extraction assay was used to remove soluble proteins from cells prior to fixation and immunofluorescence staining. Cells were grown on poly-l-lysine (0.1 mg/ml; Sigma)-coated coverslips and then incubated in CSK extraction buffer (10 mM Pipes, pH 6.8, 300 mM sucrose, 5 mM MgCl₂, 100 mM NaCl, 0.5% Triton X-100) for 8 and 40 min at 32 °C or fixed directly. Cells were fixed with cold acetone/methanol for 3 min at room temperature and then blocked in 3% BSA, PBS and probed with previously described primary and secondary antibodies. Cells were scored for centrosomal localization and imaged using an Olympus BX-51 fluorescence microscope at ×60 magnification and a Spot RT slider camera 23.1. For each experiment, an average score was obtained from two or three individual experiments, and at least 100 cells were scored for each treatment.

Centrosome Amplification Assay—To analyze the ability of different forms of BRCA1 to regulate centrosome amplification, cancer cell line HCC1937 (5382InsC BRCA1 mutant) was transfected with various YFP-tagged BRCA1 constructs. At 24 h after transfection, cells were treated with 10 Gy of ionizing radiation (IR) (300 kV, 10 mA) using an X-RAD 320 Biological Irradiator (Precision X-Ray Inc.) and then allowed to recover at 37 °C with 5% CO₂ for 48 h prior to immunostaining and analysis of centrosome number by microscopy.

RESULTS

Cancer Mutations in BRCT Domains Abolish Centrosomal Localization of BRCA1—Because centrosomal localization is integral to BRCA1-dependent regulation of centrosome amplification and tumor suppressor function, we compared the effect of known cancer-associated mutations on the localization of BRCA1 to the centrosome. YFP-tagged forms of BRCA1 were transiently expressed in MCF-7 breast cancer cells and analyzed by fluorescence microscopy for co-localization with γ-tubulin, used to mark the centrosome. Wild-type BRCA1 was detected at the centrosome in most (57%) cells (Fig. 1). The cancer-associated N-terminal RING mutation, C61G, which abolishes ubiquitin ligase activity, did not affect centrosomal targeting. On the other hand, each of the BRCT domain point mutations tested (P1749R, M1775R, Y1853X, 5832InsC) completely abolished centrosomal localization (see Fig. 1). The correct size of all point mutants was confirmed by Western blot (Fig. 1, inset; also see Ref. 40). HCC1937 breast cancer cells harbor an endogenous 5382InsC mutation of BRCA1, and by co-staining with antibodies against BRCA1 (Ab-P) and γ-tubulin, we confirmed the ectopic 5382InsC localization pattern, showing that in HCC1937 cells, less than 5% of endogenous mutant BRCA1 stained at the centrosome (supplemental Fig. S1). Therefore, mutations in the BRCT domain (but not the
The Combination of N Terminus and C-terminal BRCT Domains Targets BRCA1 to Centrosome Independent of BARD1 and γ-Tubulin—BRCA1 localizes to the centrosome throughout the cell cycle; however, the precise targeting sequence(s) remain unknown. A centrosomal localization sequence has been reported in cyclin E (46), cyclin A2 (47), and BRCA2 (36); however, ClustalW protein sequence alignment revealed no similar targeting element in BRCA1. BRCA1 also lacks a PACT domain, required for targeting pericentrin and AKAP450 to centrosomes (44). Consequently, we used an extensive deletion analysis to map the sequences critical for BRCA1 localization to the centrosome. We transfected YFP-tagged BRCA1 peptides into MCF-7 breast cancer cells and analyzed them by fluorescence microscopy for co-localization with γ-tubulin. Cells expressing wild-type YFP-BRCA1 showed centrosomal accumulation characterized by co-localization with γ-tubulin in asynchronous MCF-7 cells (Fig. 1, A and B). The region spanning amino acids 510–622 of BRCA1 has been reported to bind γ-tubulin and seemed a likely candidate for a centrosomal anchor of BRCA1 (48). Two YFP-tagged protein fragments 297–773 and 297–1312 that encompass the γ-tubulin binding region were transiently expressed in MCF-7 cells and analyzed for co-localization with γ-tubulin. These BRCA1 sequences are detectable in both the nucleus and cytoplasm but did not accumulate at the centrosome (Fig. 2). Thus, γ-tubulin binding is

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**FIGURE 1.** Cancer-associated BRCT mutations prevent BRCA1 centrosome localization. Shown is a schematic diagram showing the organization of BRCA1 protein domains (the RING and BRCT domains, the NLS, and the NES) as well as cancer-associated mutations. YFP-tagged BRCA1 cancer-associated mutants were transfected into MCF-7 breast cancer cells and analyzed for co-localization with the centrosome-component γ-tubulin by immunofluorescence microscopy. Representative cell images of YFP-BRCA1 localization are shown in the right-hand panel, in addition to close-up images of the centrosomes, with staining of BRCA1 and γ-tubulin. Cells expressing YFP-tagged BRCA1 were scored for co-localization with the centrosome. Scoring results were obtained from at least three independent experiments, each with at least 100 cells scored (mean ± S.D. (error bars)). Integrity of the BRCA1 point mutants was validated by Western blot (see inset).
essential for BRCA1 centrosomal functions but not for its localization.

Careful scoring of a series of C-terminal deletions revealed that loss of just 112 amino acids from the C terminus (equivalent to BRCA1 peptide with amino acids 1–1751) completely abolished BRCA1 localization at the centrosome (Fig. 2). We therefore tested the BRCT domains alone (residues 1290–1863) and found that they were not sufficient for BRCA1 centrosomal targeting.

Conversely, it is noteworthy that an N-terminal deletion of the BARD1-interacting RING domain (residues 70–1863), which we previously showed abolishes BARD1 interaction (39) (see also supplemental Fig. S2), did not reduce centrosomal targeting, indicating that although BARD1 co-locates with BRCA1, it is not required for its recruitment to the centrosome. This is unexpected, given that BARD1 is the major known binding partner of BRCA1, and the two proteins form an active heterodimer that is important for the role of BRCA1 in preventing centrosome amplification (14). We found that neither the N terminus alone nor the BRCT domains were sufficient to target BRCA1 to the centrosome; however, a fusion peptide comprising both the N terminus (N304; amino acids 1–304) and C terminus (C243; amino acids 1620–1863) localized to the centrosome equally as efficiently, if not slightly better, than wild-type BRCA1 (N304+H11001C243, 69%; Fig. 2B). To underscore the specificity of this unexpected finding, a different fusion construct that combined the N terminus (N304) and amino acids 1078–1312 was also tested but was negative for centrosomal localization. Similar findings were observed in HCC1937 and U2OS cells (data not shown). The integrity and correct size of each construct expressed in cells was verified by Western blot (Fig. 2C) (27, 28, 39, 40, 43). This finding indicates that cooperation of the N terminus and C-terminal BRCT domains is sufficient to mediate effective BRCA1 targeting to the centrosome, in the absence of binding to BARD1 or γ-tubulin.

Minimal Centrosome Targeting Sequence of BRCA1 (N304+C243) Cannot Regulate DNA Damage-induced Centrosome Amplification in HCC1937 Cells—Next we compared the function of specific BRCA1 sequences (Fig. 3A), employing a single cell assay to compare the different sequences for their ability to inhibit centrosome amplification after DNA damage (Fig. 3, B and C). The method utilizes HCC1937 breast cancer cells that express a mutated form of BRCA1 (5282InsC) and mutated p53, resulting in a defective G2/M checkpoint and continuation of the cell cycle after DNA damage, leading to centrosome amplification (50). We observed an increased number of cells (from 20 to 49%) with more than two centrosomes at 48 h after exposure of HCC1937 cells to 10 Gy of IR (Fig. 3), comparable with that previously reported. Centrosome amplification was also observed following overexpression of YFP alone (44%) or YFP-BRCA1 5832InsC mutant (50%), which did not localize to centrosomes. The transient expression of wild-type YFP...
BRCA1 inhibited centrosome amplification by 50% ($p = 0.0001$), as described elsewhere (50). In contrast, the minimal BRCA1 targeting fusion construct N304+C243, which resembles the exon 11 deletion mutant previously shown to disrupt BRCA1 regulation of centrosome number (19), was unable to reduce centrosome amplification (which remained high at 44%). Moreover, the RING mutant YFP-BRCA1 C61G, which can still bind to BARD1 (39) (see also supplemental Fig. S2) but is deficient in ubiquitin ligase activity, was equally as ineffective at inhibiting centrosome amplification in DNA damaged cells (45%). The differences observed were not due to any changes in cell cycle profile, as confirmed by flow cytometry analysis of the cell cycle in transfected HCC1937 cells (supplemental Fig. S3). This is clear evidence that the domains of BRCA1 responsible for centrosome localization are not sufficient for the regulation of centrosome amplification.

**FIGURE 3. Centrosome targeting domains of BRCA1 are insufficient for the regulation of centrosome amplification.** A, diagram of different BRCA1 peptides tested for their ability to regulate centrosome amplification (including the minimal centrosomal targeting sequence) and their main functional defects. B, YFP-tagged BRCA1 proteins were transiently expressed in HCC1937 breast cancer cells, which harbor the endogenous BRCA1 mutation 5382InsC and have a defective DNA damage checkpoint. Cells were treated with 10 Gy of IR and left to recover for 48 h. After fixation with acetone/methanol, cells were immunostained with anti-$\gamma$-tubulin antibody and scored by microscopy for the number of cells displaying centrosome amplification (>2 centrosomes/cell). Representative cell images are shown for each BRCA1 peptide, showing similar expression levels of transiently expressed proteins and close up images of centrosomes. The number of centrosomes in each imaged cell is noted in white. C, IR-induced centrosome amplification was reduced by overexpression of WT BRCA1 but not by various mutated forms of BRCA1, including the minimal targeting sequence (N304+C243). Scoring results were obtained from three independent experiments with at least 100 cells scored (mean ± S.D. (error bars)). Student’s $t$ test was used to determine that only YFP-BRCA1 wild type was statistically significant in regulating centrosome amplification in comparison with YFP control. ***, $p < 0.001$. 
Photobleaching Experiments in Live Cells Identify Dynamic and Immobile Pools of YFP-BRCA1 at Centrosome—To determine whether the central region (including the binding site for γ-tubulin) of BRCA1 contributes to BRCA1 centrosomal dynamics or retention, we employed FRAP analysis to compare mobility of YFP-BRCA1 wild-type and N304+C243 proteins. First, we determined the minimal bleaching time to completely eliminate centrosome fluorescence using fixed MCF-7 cells (4 s, 100% laser intensity). FRAP assays were conducted on live cells co-expressing the fluorescence-tagged C terminus of pericentrilar matrix component pericentrin (RFP-pericentrin-C241) using an Olympus FV1000 confocal microscope (Fig. 4A). The fluorescence pericentrin provided an effective live cell marker of the centrosome. After a high intensity laser spot bleaching of centrosome fluorescence, recovery was measured every 4 s for a period of at least 60 s. YFP-BRCA1 fluorescence (n = 12) recovered at the bleached centrosome with a $t_{1/2}$ of 5.1 s, and YFP-BRCA1 (N304+C243) (n = 10) recovered much faster with a $t_{1/2}$ of 3.9 s. Corresponding FRAP recovery curves are shown for each protein in comparison with RFP-pericentrin C241 (recently described in Ref. 37), indicating the immobile and mobile fractions (left). YFP-BRCA1 (wild type) and RFP-pericentrin were also analyzed by an inverse FRAP (iFRAP) assay, bleaching cellular fluorescence, and then quantifying the rate of loss from the centrosome (right). $t_{1/2}$ (half-time ± S.E. (error bars)) and immobile fraction (percentage ± S.E.) are shown for each protein with an average of 10–15 cells over at least two experiments analyzed for each. Student’s t test was used to show a significant difference in $t_{1/2}$ between wild-type and N304+C243 peptides. **, p < 0.01.

C, in an in vitro assay to measure BRCA1 centrosomal retention, cells were treated with CSK detergent buffer for 0, 8, or 40 min prior to fixation with acetone/methanol to remove soluble protein. Cells were then immunostained with BRCA1 antibody, co-stained for γ-tubulin, and scored by microscopy for the percentage of cells still displaying endogenous BRCA1 at the centrosome.
of 1.4 s (p < 0.01; Fig. 4B). Thus, the centrosomal recovery rate of YFP-BRCA1 (N304+ C243), which cannot bind γ-tubulin, is faster than wild type in the initial stages of centrosomal association (see also supplemental Table S1). All cells analyzed showed ample cytoplasmic BRCA1 and pericentrin C241 available for recovery to the centrosome. The total extent of recovery of the BRCA1 proteins after 25 s was similar and relatively high compared with RFP-pericentrin, and analysis of the 60-s recovery period (data not shown) suggests that ~60% of BRCA1 at the centrosome is mobile and dynamic, in contrast to pericentrin, which is much more stable (~30% mobile; Fig. 4).

On the other hand, we identified a significant immobile pool of BRCA1 (~40% of centrosomal BRCA1) that is relatively stable at the centrosome, independent of γ-tubulin interaction. This was confirmed by a complementary inverse FRAP approach, which involved bleaching of extracentrosomal fluorescence and quantification of the rate of loss from the centrosome (see the graph in the right-hand panel of Fig. 4A). Furthermore, an in vitro assay using mild detergent extraction of soluble proteins revealed centrosome retention of ~50% of the total endogenous centrosomal BRCA1 pool, whereas γ-tubulin was highly resistant and well retained at centrosomes (Fig. 4C).

Interestingly, the BRCA1 kinetics differed from that of BARD1, which is more dynamic with only ~20% immobile at the centrosome (37). Several proteins have been shown to localize to centrosomes via dynein and microtubules (51). To assess the effect of inhibiting CRM1 on endogenous BRCA1, we determined nuclear export of proteins (52). Quantification of fluorescence intensity at hundreds of individual centrosomes showed ample cytoplasmic BRCA1 and pericentrin C241 available for recovery (data not shown) suggests that ~60% of BRCA1 at the centrosome is mobile and dynamic, in contrast to pericentrin, which is much more stable (~30% mobile; Fig. 4).

To determine how nuclear import or export of BRCA1 influenced its localization at centrosomes. In MCF-7 cells, blocking BRCA1 nuclear export by LMB treatment or mutation of the

determine how nuclear import or export of BRCA1 influenced its localization at centrosomes. In MCF-7 cells, blocking BRCA1 nuclear export by LMB treatment or mutation of the
NES decreased staining of YFP-BRCA1 at the centrosome by 30–50% (Fig. 5C), similar to that seen for endogenous BRCA1. This is consistent with a reduced amount of the BRCA1 NES mutant (NESm) protein in the cytoplasm. On the other hand, when the nuclear localization signal (NLS) of BRCA1 is mutated (NLSm), BRCA1 cannot enter the nucleus and is predominantly localized to the cytoplasm and stained strongly at the centrosome in 65% of cells scored (Fig. 5C). Next, we asked what would happen when both the NLS and NES of BRCA1 are mutated (NESm/NLSm), given that this double transport mutant is stuck in the cytoplasm but also cannot bind to CRM1. Surprisingly, the NESm/NLSm form of BRCA1 displayed a 25% decrease in centrosome staining compared with the NLSm form, and this means that disrupting the binding to CRM1 perturbs centrosome localization of BRCA1 even when it is in the cytoplasm. Therefore, we conclude that CRM1 stimulates BRCA1 localization to the centrosome in two ways, first by exporting it from nucleus to cytoplasm and thereby increasing its cytoplasmic concentration and second by enhancing its localization at the centrosome once in the cytoplasm.

**CRM1 Stimulates Dynamic Turnover of BRCA1 at Centrosome**—There is surprisingly little published data on the ability of CRM1, the major nuclear export receptor, to regulate protein kinetics in live cells. Therefore we employed FRAP assays on live MCF-7 cells to quantitatively compare two different forms of YFP-BRCA1, one with an unmasked and highly accessible NES (residues 70–1863) (39) and one with a mutated inactive NES (NESm), measuring fluorescence recovery after 4 s of photobleaching (Fig. 6A). Recovery curves showed that YFP-BRCA1 NESm was slower to recover ($\tau_{1/2} = 6.62$, $n = 16$) than the export-efficient 70–1863 BRCA1 fragment ($\tau_{1/2} = 3.86$, $n = 11$, $p < 0.05$) and also slower than wild-type BRCA1 ($\tau_{1/2} = 5.14$ s). In addition to differences in the recovery rates, the total recovery of fluorescence was slightly reduced for the BRCA1 NES mutant, indicating that it is less mobile than wild-type BRCA1 and considerably less mobile than BRCA1 70–1863 (Fig. 6A). Thus, the NES mutation slows the movement of BRCA1 and causes it to be moderately more retained at centrosomes. The BRCA1 70–1863 sequence does not bind -tubulin (16) and nucleophosmin (7709) enabling assembly of a BRCA1-BARD1 dimer that remained in the absence of BARD1 (Fig. 2). Therefore, we asked whether the dynamics of BRCA1 at centrosomes might differ when BRCA1 was free or bound to BARD1. Normally, this issue would be difficult to test because the co-expression of wild-type BRCA1 and BARD1 results in rapid transport of the heterodimer to the nucleus (28, 39). Therefore, to address this, we employed a combination of NLS-mutated forms of BRCA1 and BARD1, enabling assembly of a BRCA1-BARD1 dimer that remained in the cytoplasm and that localizes to the centrosome (see supplemental Fig. S5) rather than shuttling irreversibly to the nucleus like the wild-type BRCA1-BARD1 complex (39).

To quantify the effect of BARD1 co-expression on the centrosomal dynamics of BRCA1, the nuclear import-deficient BRCA1 (NLSm) was analyzed at the centrosome by FRAP assay with and without co-expression of FLAG-BARD1 NLSm (Fig. 7A). Immunostaining of transfected cells confirmed that the two proteins were co-expressed in 99% of cells (Fig. 7C). First, we noted that after photobleaching, the rate of YFP-BRCA1 NLSm recovery at centrosomes was comparable with that of wild-type BRCA1 ($\tau_{1/2} = 6.95$ s), and it displayed a similar immobile fraction (42%). Analysis of YFP-BRCA1 NLSm showed that it recovered at centrosomes almost twice as fast as BRCA1 ($\tau_{1/2} = 3.69$ s) but with an immobile fraction similar to that of wild-type BARD1 (20.1% immobile; Fig. 7B) (37). Interestingly, in the co-expression experiment when YFP-BRCA1 NLSm recovery was measured in the presence of FLAG-BARD1 NLSm, the BRCA1 mobility and exchange rate increased to a
FIGURE 6. NES mutation disrupts BRCA1 dynamics and function at the centrosome. A, FRAP analysis was performed on live MCF-7 cells co-transfected with RFP-pericentrin C241 and YFP-BRCA1 mutants, 70–1863 and NESm, deficient in binding to BARD1 and CRM1, respectively. The 70–1863 sequence is highly export-active (39). Centrosome fluorescence was photobleached, and fluorescence recovery was then measured by time lapse microscopy. Representative prebleach, first image postbleach, and images for 10.5, 18.5, and 26.5 s after bleach are shown for each protein. The insets show higher magnification views of the target area. Corresponding recovery curves are shown for the above proteins, as well as wild-type BRCA1. The $t_{1/2}$ (half-time ± S.E. [error bars]) and immobile fractions (percentage ± S.E.) are also shown. Statistical significance of changes in immobile fraction and half-time are indicated. *, $p < 0.05$; **, $p$ value $< 0.001$.

B, different BRCA1 mutants defective in binding to BARD1 or in nuclear transport were tested for their ability to regulate centrosomal amplification. YFP-tagged BRCA1 proteins were transfected into HCC1937 breast cancer cells, and cells were then treated with 10 Gy of IR and left to recover for 72 h. Cells were fixed and immunostained with anti-γ-tubulin antibody and analyzed for centrosome amplification (>2 centrosomes/cell). Scoring results are from three independent experiments (mean ± S.D.) and Student's $t$ test was used to determine statistical significance. **, $p$ value $< 0.01$; ***, $p$ value $< 0.001$. 

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similar level as that seen for BARD1. More specifically, when co-expressed with BARD1, the recovery of YFP-BRCA1 NLSm was accelerated ($t_{1/2}/H11005^{3.47}$ s) and recovered to almost 80% of initial fluorescence, which corresponds to a 50% reduction in the immobile retained fraction (from 42 to 22%; Fig. 7B). From these data, we conclude that the co-expression of BARD1, resulting in heterodimer formation, speeds up BRCA1 association with the centrosome and decreases its retention (outlined in Fig. 7D). This might contribute to the biased nuclear localization of the wild-type BRCA1-BARD1 heterodimer normally observed in cells (54).

**C-terminal Binding Partner, Aurora A Kinase, Contributes to BRCA1 Retention and Function at Centrosome**—Thus far, this work has concerned N-terminal partners of BRCA1. In the literature, there are few C-terminal partners that might regulate BRCA1 centrosome dynamics; one of the best candidates is...
Aurora A kinase, which interacts with BRCA1 at the BRCT domain (Fig. 8A) (26). Aurora A is up-regulated in BRCA1mut breast tumors relative to sporadic breast tumors and has a role in the regulation of BRCA1 function at the centrosome (56).

Because the BRCT domains are critical for BRCA1 localization to the centrosome, we used FRAP assays to test the effect of inhibiting Aurora A on the dynamics of YFP-BRCA1 at 72 h after Aurora A knockdown by siRNA (Western blots were per-
formed in parallel and displayed a consistent knockdown of >80%, determined by densitometric analysis (see Fig. 8B). When Aurora A was silenced, YFP-BRCA1 wild-type recovery was moderately slower, with a $t_{1/2}$ value of $\sim 3.9$ s compared with control siRNA ($\sim 4.6$ s). Although this difference was not statistically significant, it could suggest that inhibition of Aurora A binding or of its kinase activity slows BRCA1 recruitment to the centrosome. Moreover, Aurora A siRNA caused a significant decrease in the immobile pool of BRCA1 (28%) relative to control siRNA (37%), demonstrating that Aurora A contributes to BRCA1 retention at the centrosome (Fig. 8B). The effect of Aurora A on BRCA1 regulation of DNA damage-induced centrosome amplification was also assessed in HCC1937 cells. The cells were transfected with either Aurora A or control siRNA for 16 h and then transfected with YFP-BRCA1 or YFP control plasmid. After 24 h, cells were treated with 10 Gy of IR and fixed and immunostained after a further 48 h. As shown in Fig. 6B, YFP-BRCA1 (28%) was able to substantially reduce the number of cells with >2 centrosomes in comparison with YFP alone (55%) in MCF-7 cells treated with control siRNA. However, in the presence of Aurora A knockdown, YFP-BRCA1 was not able to regulate centrosome amplification after DNA damage (Fig. 8D). Moreover, a mutant version of BRCA1 that cannot be phosphorylated by Aurora A, S308N (26), was unable to regulate centrosome amplification. These data suggest a role for Aurora A in BRCA1 retention at the centrosome as well as a role in the BRCA1-dependent regulation of centrosome number.

**DISCUSSION**

BRCA1 gene mutations lead to centrosome amplification and related mitotic abnormalities in human breast cancers (57) and transgenic mice (18, 19). BRCA1 has been known for some time to locate at the centrosome (58, 59) and, through binding and ubiquitinating $\gamma$-tubulin (48), contributes to the regulation of centrosome amplification and microtubule nucleation (14, 16). Despite knowledge of BRCA1 sequences that mediate its ubiquitination activity (amino acids 1–300) (60) and $\gamma$-tubulin binding (amino acids 510–622) (48), surprisingly little is known of the sequences or protein interactions that target BRCA1 to the centrosome. Here we show for the first time that BRCA1 is not targeted to the centrosome via its interaction with $\gamma$-tubulin but instead by the combination of N- and C-terminal end sequences that flank the central $\gamma$-tubulin binding region. The N-terminal nuclear export sequence was important for BRCA1 targeting, turnover, and function at the centrosome, indicating regulation by CRM1. We propose that BRCA1 contributes to the pathway of CRM1-dependent mitotic regulation (model outlined in supplemental Fig. S7).

BRCA1 shuttles between different subcellular compartments, including the nucleus and cytoplasm (54), centrosome (58), and mitochondria (61), to regulate diverse processes. We mapped two separate BRCA1 centrosome-targeting sequences, corresponding to the N terminal (residues 1–304) and C-terminal (residues 1620–1863) BRCT domains, which act cooperatively to localize a GFP marker peptide to the centrosome. Deletion of either sequence abolished BRCA1 localization at the centrosome (Fig. 2). The fusion of N- and C-terminal sequences (named N304+C243) is identical to the core sequence(s) that we previously showed to target BRCA1 to DNA repair foci in cells following exposure to ionizing radiation (40). Although this suggests some overlap in BRCA1 protein complexes at nuclear foci and centrosomes, a critical difference lies in the role of BARD1, which is essential for association of BRCA1 at foci (39) but was dispensable for accumulation at centrosomes (Fig. 2). The binding of BRCA1 to $\gamma$-tubulin and BARD1 was not required for its movement to centrosomes (Fig. 2); however, loss of these binding sequences abolished BRCA1 regulation of DNA damage-induced centrosome amplification (Fig. 3). Therefore, the regulation of centrosome amplification and targeting can be distinguished.

Another intriguing aspect is the striking similarity between the minimal centrosome-targeting fusion peptide (N304+C243) and the naturally occurring BRCA1 splice variant Δexon11, which in mouse embryonic fibroblasts taken from BRCA1Δexon11/Δexon11 transgenic mice was shown to cause a high proportion of centrosome amplification, multipolar spindles, and aneuploidy (19). The BRCA1(N304-C243) protein retained targeting to centrosomes but lacked the ability to regulate their amplification, which is highly consistent with previous findings and predicts that the Δexon11 splice variants will also display centrosomal localization and could potentially compete with wild-type BRCA1 for binding to specific partners, such as CRM1 or BARD1.

We employed fluorescence photobleaching assays and found that ~60% of BRCA1 at the centrosome is highly mobile and in continuous exchange between centrosome and surrounding cytoplasm (Fig. 4). Control experiments confirmed that any differences measured in exchange rates of BRCA1 isoforms were not due to variations in expression levels (see supplemental Figs. S8 and S9). It is intriguing to note that the retained centrosomal pool of BRCA1 was twice the amount recently observed for BARD1 (37). The rapid turnover of the mobile BRCA1 pool is reminiscent of previous reports for the centrosomal regulatory proteins Aurora A, Nek2A, CDC-20, and Dynamic-2 (62–65). This contrasts with the relatively long occupancy times of centrosomal structural proteins.
Regulation of BRCA1 by N-terminal Partners CRM1 and BARD1—Recent studies have shown that the major exportin, CRM1, not only mediates nucleocytoplasmic transport but also regulates centrosome duplication to maintain correct assembly of the mitotic spindle (30). It was proposed that centrosomal localization of CRM1 is mediated by its N terminus and binding partner Ran-GTP (31) and that other CRM1 sequences provide a docking site for NES containing proteins. Prior to this study, CRM1 was implicated in the centrosomal localization of nucleophosmin (NPM1) (34), the BRCA1 homologue BRCA2 (35), and BARD1 (37). Of these, NPM1 and BRCA2 were linked to CRM1 control of centrosome duplication (33, 34). NPM1 is a nucleolar protein that binds to CRM1 and localizes at centrosomes during mitosis via interaction with the Ran-CRM1 complex, leading to inhibition of centrosome reduplication. Its functionality and centrosome association is regulated by phosphorylation. NPM1 also binds the N-terminal amino acids 1–122 of BRCA1 and is monoubiquitinated by the BRCA1-BARD1 ubiquitin ligase (55). Therefore, these previous studies indirectly implicated BRCA1-BARD1 in CRM1 regulation of centrosome duplication.

Because NPM1 and BRCA1 are both nuclear shuttling proteins that modulate centrosome duplication, we tested for potential CRM1 regulation of BRCA1. Inactivation of CRM1 by leptomycin B or mutation of the N-terminal NES reduced staining of BRCA1 at the centrosome (Fig. 5). Interestingly, although CRM1 was apparently essential for targeting of NPM1 (33, 34), we observed that CRM1 contributed to, but was not essential for, BRCA1 recruitment to centrosomes. This is explained by our FRAP experiments in living cells, which showed that CRM1 acted not as a retention factor for BRCA1 but more as a driver to stimulate BRCA1 traffic to the centrosome (Fig. 6A). An export-defective BRCA1 mutant displayed a slower recovery and stronger retention at centrosomes than the export-active form, implicating CRM1 in rapid turnover of BRCA1 at the centrosome. Since we first discovered the NES in BRCA1 (27), it has yet to be assigned functional relevance. We found here that in BRCA1-mutated HCC1937 cells exposed to DNA damage, mutation of the BRCA1 NES completely blocked BRCA1 regulation of centrosome amplification (Fig. 6B), suggesting that interaction with CRM1 is essential for BRCA1 function as a centrosome DNA damage checkpoint protein. We propose that CRM1 regulates BRCA1 at the centrosome by (a) increasing centrosomal localization indirectly through nuclear export, (b) increased targeting and turnover, and (c) stimulating its regulation of centrosome amplification.

We speculate that CRM1 helps target BRCA1 to a functionally relevant compartment of the centrosome, such as the centriole, possibly through CRM1-dependent interaction with an enriched Ran-GTP pool anchored by the large centrosomal scaffolding protein AKAP450 (32). It is important to note that CRM1 can only bind to the undimerized form of BRCA1, because the NES (CRM1-binding site) is a core part of the BARD1-binding domain, and both the BRCA1 and BARD1 NESs become masked upon heterodimer formation (39). When the CRM1-bound BRCA1 is brought into proximity with BARD1, the BARD1 will therefore compete with and displace CRM1 (28) to assemble an enzymatically active BRCA1-BARD1 heterodimer, leading to ubiquitination of several substrates required for centrosome amplification control, including γ-tubulin (16), NPM1 (55), and RHAMM (56). Our findings demonstrate that BRCA1 and BARD1 can locate at the centrosome independently of one another and that both proteins are targeted there by CRM1 prior to assembly of the heterodimer (see supplemental Fig. S6) (37). This new concept of post-targeting assembly could explain how the BRCA1-BARD1 dimer is able to form at the centrosome when normally the dimeric complex is rapidly directed into the nucleus and blocked from exiting back to the cytoplasm (54). Finally, our photobleaching assays showed that heterodimer formation significantly reduced the centrosomal retention of BRCA1, hinting at an unanticipated mechanism to accelerate its release after the dimer forms and ubiquitinates its substrates.

Regulation of BRCA1 by C-terminal Partner Aurora A Kinase—BRCA1 checkpoint functions are regulated by phosphorylation (67). A key regulator of BRCA1 mitotic function, Aurora A kinase, binds to the C-terminal BRCT domain of BRCA1 (26) and was therefore a candidate for regulation of BRCA1 dynamics. Indeed, our photobleaching studies revealed that siRNA-mediated knockdown of Aurora A caused a 25% reduction in the immobile pool of BRCA1, indicating that Aurora A contributes to BRCA1 retention at the centrosome. Aurora A is a serine/threonine kinase overexpressed in breast cancers (22, 23) and accumulates at the centrosome in early G2 phase (24, 25, 68). Aurora A phosphorylates BRCA1 at Ser-308 during early M phase, and this was implicated in regulation of the G2 to M transition (26). This kinase has also been implicated in the regulation of BRCA1 inhibition of microtubule nucleation from the centrosome by decreasing ubiquitin ligase activity of the BRCA1-BARD1 heterodimer (49). A further analysis here showed that not only does Aurora A regulate BRCA1 retention at the centrosome in live cells, but its activity (and specifically the phosphorylation of Ser-308) is crucial for BRCA1 regulation of centrosome amplification after DNA damage.

In conclusion, we propose that CRM1 acts as a chaperone to target BRCA1 to a functionally relevant subcompartment(s) of the centrosome. Based on the evidence presented here, we speculate that the ability of CRM1 to drive BRCA1, BARD1, and several of the BRCA1-BARD1 substrates to the centrosome heightens the chance of their proximity, hence expediting BRCA1-BARD1 dimer formation to catalyze ubiquitination of downstream substrates required to regulate centrosome amplification during the DNA damage response. Future experiments to further characterize the spatial and temporal organization of these protein interactions will yield new insights into the G2/M checkpoint function of BRCA1 and its role at the centrosome.
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