BRCA1-Interacting Protein OLA1 Requires Interaction with BARD1 to Regulate Centrosome Number

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Abstract

BRCA1 functions as a tumor suppressor in DNA repair and centrosome regulation. Previously, Oba-like ATPase 1 (OLA1) was shown to interact with BARD1, a heterodimer partner of BRCA1. OLA1 binds to BRCA1, BARD1, and γ-tubulin and functions in centrosome regulation. This study determined that overexpression of wild-type OLA1 (OLA1-WT) caused centrosome amplification due to centriole overduplication in mammalian tissue-derived cells. Centrosome amplification induced by overexpression of the cancer-derived OLA1 mutant, which is deficient at regulating centrosome number, occurred in significantly fewer cells than in that induced by overexpression of OLA1-WT. Thus, it was hypothesized that overexpression of OLA1 with normal function efficiently induces centrosome amplification, but not that of OLA1 mutants, which are deficient at regulating centrosome number. We analyzed whether overexpression of OLA1 missense mutants of nine candidate phosphorylation residues, three residues modified with acetylation, and two ATP-binding residues caused centrosome amplification and identified five missense mutants that are deficient in the regulation of centrosome number. Three of them did not bind to BARD1. Two phosphomimetic mutations restored the binding to BARD1 and the efficient centrosome amplification by their overexpression. Knockdown and overexpression of BARD1 also caused centrosome amplification. BARD1 mutant reported in cancer failed to bind to OLA1 and rescue the BARD1 knockdown-induced centrosome amplification and reduced its centrosomal localization. Combined, these data reveal that the OLA1–BARD1 interaction is important for the regulation of centrosome number.

Implications: Regulation of centrosome number by BRCA1/BARD1 together with OLA1 is important for the genome integrity to prevent tumor development. Mol Cancer Res; 16(10); 1499–511. ©2018 AACR.

Introduction

BRCA1 is a breast and ovarian cancer suppressor (1) and forms a heterodimer with BARD1 (2). Both BRCA1 and BARD1 have a RING domain in their amino (N)-terminal region and two BRCT domains in their carboxy (C)-terminal region. The dimerization of BRCA1 and BARD1 is mediated by interaction of their N-terminal regions involving the RING domains and the dimer. BRCT domains are present in many proteins that function in DNA repair and at cell-cycle checkpoints and are responsible for the binding to phosphorylated proteins (4).

The BRCA1/BARD1 heterodimer functions in DNA repair and centrosome regulation (5). Centrosomes are the major microtubule nucleation centers and function in the formation of the mitotic spindle. The single centrosome in G₁ phase duplicates only once in S phase (6). The centrosome contains a pair of centrioles, called the mother and daughter centrioles, and centrosome duplication is initiated by their physical separation (centriole disengagement) in late mitosis-early G₁ phase. In S phase, a daughter centriole forms perpendicular to each mother centriole. Overduplication of centrioles leads to centrosome amplification, which is the best-characterized centrosomal abnormality in cancer and causes aneuploidy and genome instability (7).

BRCA1 and BARD1 localize to the centrosome throughout the cell cycle (8, 9). BRCA1 directly binds to γ-tubulin, a component of centrosomes (10), and the BRCA1/BARD1 heterodimer ubiquitinates γ-tubulin and other centrosomal proteins (11). Fibroblasts of Brca1 knockout mouse embryos show centrosome amplification (12). Suppression of BRCA1 results in centrosome amplification in cells derived from mammary tissue (11). BRCA1 with mutations found in familial breast cancers are deficient in...
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the regulation of centrosome number (13). Centrosome amplification is significantly associated with lack of BRCA1 expression in breast cancer tissue (14).

We identified Obg-like ATPase 1 (OLA1) as a protein that interacts with BARD1 (15). OLA1 is highly conserved from bacteria to humans and is a member of the Obg family and YchF subfamily of P-loop GTPases (16–18), while OLA1 has both ATPase and GTPase activities (19). OLA1 was composed of a central guanine nucleotide-binding domain (G domain), flanked by a coiled-coil domain and a ThrRS-GTP-SpoT (TGS) domain (19). The G domain is the basic functional unit of GTP-binding proteins (G proteins) and contains five characteristic sequences, G1–G5, which are involved in nucleotide binding and hydrolysis (24). The function of the TGS domain is so far unknown. OLA1 is involved in multiple cellular processes including protein translation, the antioxidant response, signal transduction, and cell proliferation (16, 25, 26), and its overexpression is observed in many malignant tumors (22).

OLA1 directly binds to BRCA1, BARD1, and γ-tubulin and localizes to centrosomes and the spindle pole. Knockdown of OLA1 causes centrosome amplification in cell lines derived from mammary tissue. OLA1 with a substitution mutation (E168Q) observed in a breast cancer cell line fails to bind to the amino (N)-terminal region of BRCA1 and does not rescue OLA1 knockdown-induced centrosome amplification. Moreover, a BRCA1 variant, I42V, which is deficient at regulating centrosome number, decreases the binding activity to OLA1. These suggest that OLA1 plays an important role in centrosome regulation together with BRCA1 (15). In general, supernumerary centrosomes arise from centriole overduplication, centrosome fragmentation, and/or centrosome accumulation caused by aborted cell division, for example, through failure of cytokinesis. Inhibition of BRCA1 and knockdown of OLA1 causes centrosome fragmentation and centriole overduplication (15, 27).

In this study, we found that overexpression of wild-type OLA1 (OLA1-WT) causes centrosome amplification. The extent of centrosome amplification induced by overexpression of OLA1–E168Q was significantly lower than that by OLA1-WT. We therefore hypothesized that overexpression of functional OLA1-induced centrosome amplification and analyzed the effect on the centrosome of overexpression of OLA1 mutants. We identified a further five OLA1 mutants, which decreased the number of cells with centrosome amplification. Three of these mutants abolished the direct binding to BARD1. Furthermore, we identified BARD1 mutations that abolished the binding activity to OLA1 and the function in the regulation of centrosome number. Taken together, these suggest that the OLA1–BARD1 interaction is important for the regulation of centrosome number in addition to the OLA1–BRCA1 interaction.

Materials and Methods

Cell lines and transfection

Hs578T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) and 10 μg/mL of insulin. MCF7, PC3, HeLa, U2OS, Saos-2, and HEK-293T cells were grown in DMEM with 10% FBS. HCC1937 cells were grown in RPMI-1640 medium with 10% FBS. T47D cells were grown in RPMI-1640 medium with 10% FBS and 10 μg/mL of insulin. MCF10A cells were grown in DMEM/Ham’s F-12 1:1 with 5% FBS, 10 μg/mL of insulin, 20 ng/mL of epidermal growth factor, and 0.5 μg/mL of hydrocortisone. Hs578T, MCF7, PC3, Saos-2, HEK-293T, HCC1937, T47D, and MCF10A were obtained from ATCC. Cell line identities were verified using the GenomeLab Human STR Primer set (Beckman Coulter). Cells were transfected with vectors using Lipofectamine LTX or with siRNAs using Lipofectamine RNAiMAX (Invitrogen) or TransIT-X2 Dynamic Delivery System (Mirus Bio).

Plasmid construction

To generate the HA-OLA1 expression vector, pCY4B-FLAG-OLA1 was digested and the OLA1 fragment was subcloned into pCY4B-HA (28). pCY4B-HA-OLA1 mutants were generated by site-directed mutagenesis of pCY4B-HA-OLA1. For the pCY4B-FLAG-OLA1 mutants and pGEX-OLA1 mutant, pCY4B-HA-OLA1 mutants were digested and the OLA1 fragments with mutations were subcloned into pCY4B-FLAG and pGEX (GE Healthcare). For pPEGF-centrin, centrin2 fragments were amplified from cDNA from HeLa cells, and the PCR products were subcloned into a pPEGF vector (Clontech). For pCY4B-HA-BARD1-FL-WT, the BARD1 fragments were amplified from p3XFLAG-BARD1, and the PCR products were subcloned into pCY4B-HA. pCY4B-HA-BARD1 mutants were generated by site-directed mutagenesis. For pCY4B-HA-BARD1-546–777-WT and mutants, BARD1 fragments were amplified from pCY4B-HA-BARD1. PCR products were subcloned into pCY4B-HA. For pET-BARD1-546–777-V695L, pCY4B-HA-BARD1-546–777-V695L was digested and the BARD1 fragment was subcloned into pET vector (Novagen). For Myc-BARD1-V695L, the BARD1 fragment was amplified from pCY4B-HA-BARD1-V695L, and PCR products were subcloned into pCMV-Myc (Clontech). pCY4B-FLAG-OLA1, pCY4B-FLAG-OLA1-E168Q, pEFP-OLA1, pCY4B-HA-γ-tubulin, pET-γ-tubulin, pET-BRCA1-1-304, pET-BRCA1-546–777 pGEX-OLA1, pCMV-Myc-BARD1, pcDNA3-HA-BRCA1-WT, pcDNA3-HA-BRCA1-142V, pCY4B-HA-BRCA1-1-304, and p3XFLAG-BARD1 have been described previously (15, 29, 30). All constructs were verified by DNA sequencing.

Immunocytochemistry

Cells were fixed with methanol:acetone (1:1) for 10 minutes at −20°C. After blocking with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (PBS-1% BSA), cells were incubated with anti-γ-tubulin (Sigma), anti-HA (Y-11, Santa Cruz Biotechnology), and/or anti-Cep170 antibodies (Invitrogen) in PBS-1% BSA for 4 hours. After washing with PBS containing 0.05% Tween 20 (PBS-T), cells were incubated with Alexa Fluor 488 and 568-conjugated antibodies (Molecular Probes) in PBS-1% BSA for 30 minutes. Cells were washed with PBS-T and mounted in mounting medium DAPI (Vector Laboratories).

siRNA

siRNAs were synthesized using a Silencer siRNA Construction Kit (Ambion) or synthesized in Japan Bio-service. The siRNA sequence of BARD1-1 was 5′-AAGUGAAUUGUCCGUUAUGUC-3′ (31), that of BARD1-2 was 5′-GAGUAAACGGUACACUGUCGAA-3′ (32), and that of BARD1-3 was 5′-AAGUGCAUGAGUAAUUAUAAU-3′. The siRNA sequence of OLA1-1 was 5′-AAGUGCAAGACACAUAAACAU-3′ and that of OLA1-2 was 5′-AAGGAAAGUACAGAAAAGUG-3′ (15). The Silencer-negative control siRNA template set was used as the control.
Immunoprecipitation and immunoblotting

Cell lysates were prepared in lysis buffer (10 mmol/L Hepes, pH 7.6, 250 mmol/L NaCl, 0.1% Nonidet P-40 (NP-40), 5 mmol/L EDTA) containing a protease inhibitor cocktail (Sigma). Whole cell lysates were prepared in 1 × sample buffer (50 mmol/L Tris–HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 0.67 mmol/L 2-mercaptoethanol, 12% glycerol, 1% Bromphenol blue), sonicated, and incubated at 95°C for 10 minutes. For immunoprecipitation, anti-FLAG (M2, Sigma) or anti-Myc (9E10, Covance) antibodies and Protein G-Sepharose beads (GE Healthcare) were added to each cell lysate. Mixtures were incubated at 4°C for 4 to 16 hours, and the beads were washed with lysis buffer. Samples were subjected to SDS–PAGE and immunoblotted using anti-HA (HA.11, Covance or Y-11, Santa Cruz Biotechnology), anti-β-actin (Sigma), anti-GST, anti-His (GE Healthcare), anti-BARD1 (Santa Cruz Biotechnology, Bethyl), anti-BRCA1 (Ab-1, Calbiochem), anti-Myc, anti-FLAG antibodies, or polyclonal antibodies specific to OLA1 residues 1–171.

GST pull-down assay

His-tagged proteins were expressed in BL21-competent cells. The bacterial cell pellets were lysed with extraction buffer (50 mmol/L phosphate buffer pH 8.0, 0.3 mol/L NaCl, 0.1% NP-40). Purification of His-tagged protein was performed using Ni-NTA-agarose (Qiagen). GST-tagged proteins were expressed in BL21 cells and the bacterial cell pellets were lysed with sonication buffer (20 mmol/L Tris–OAc pH 7.9, 120 mmol/L K2Ac, 10% glycerol, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L DTT). The supernatant was incubated with glutathione-sepharose beads (GE Healthcare) at 4°C for 2 hours. The beads were washed and incubated at 4°C for 2–16 hours with purified His-tagged proteins. The beads were washed and samples were immunoblotted.

Three-dimensional structures and computational docking model

Three-dimensional structures of OLA1 (PDB ID 2OHF) and BARD1 C-terminal domain (PDB ID 2NTE) were downloaded from Protein Data Bank Japan. Missing residues in the OLA1 structure were modeled using MODELLERv9.10 (33). Rigid body docking of OLA1 and BARD1 was performed using ZDOCK3.0.2 (34), generating 2,000 docked poses. Hydrogen atoms were added to these complex structures, and they were reranked using ZRANK (35). The final docking pose was selected by the ZRANK ranking and the interface complementarity.

Results

Overexpression of OLA1 causes centrosome amplification via interaction with BRCA1

OLA1 is overexpressed in many malignancies (22). To analyze whether overexpression of OLA1-WT affects centrosomes in breast cancer cells, Hs578T cells were transfected with either control (HA) or the HA-tagged OLA1-WT (HA-OLA1-WT) plasmid, and immunostained with anti-HA and γ-tubulin antibodies to detect exogenous OLA1 and centrosomes, respectively (Fig. 1A). Cells with amplified centrosomes (more than two) were counted and the percentage was calculated [Fig. 1B]. Of the cells transfected with HA-OLA1-WT, only cells stained with the anti-HA antibody were analyzed (Supplementary Fig. S1A). Overexpression of OLA1-WT caused centrosome amplification [Fig. 1A and B]. Expression of HA-OLA1-WT was confirmed by Western blotting with an anti-OLA1 antibody (Supplementary Fig. S1B).

The effect of overexpression of OLA1 harboring the E168Q mutation (OLA1-E168Q) was also analyzed. Western blot analysis confirmed that the expression level of HA-OLA1-E168Q was similar to that of HA-OLA1-WT [Fig. 1C]. Overexpression of OLA1-E168Q caused centrosome amplification, but the percentage of cells with extra centrosomes was markedly lower than that of the cells transfected with OLA1-WT [Fig. 1B].

Because OLA1-E168Q does not bind to the N-terminal region of BRCA1 (15), we presumed that BRCA1 is involved in the centrosome amplification induced by the overexpression of OLA1. The effect of overexpression of OLA1 on centrosomes was therefore analyzed in the BRCA1-deficient breast cancer cell line HCC1937 (Fig. 1D). Although transfection of HA-OLA1-WT alone increased the number of cells with extra centrosomes, cotransfection with HA-BRCA1-WT markedly enhanced the centrosome amplification, while cotransfection with HA-BRCA1-I42V, which reduces the binding activity to OLA1, did slightly. These suggest that overexpression of OLA1 induces centrosome amplification via an interaction with BRCA1 protein.

Abnormal OLA1 expression causes centrosome amplification in cell lines derived from mammary tissue

Centrosome amplification by BRCA1 inhibition is observed only in cell lines derived from mammary tissue (11). We have reported that OLA1 knockdown results in centrosome amplification in cell lines derived from mammary tissue (15). We analyzed the effect of overexpression of OLA1 on centrosomes in three cell lines derived from mammary tissue and four cell lines derived from other tissue (Fig. 1E; Supplementary Fig. S1C). MCF7 and T47D are breast cancer cell lines and MCF10A is a cell line derived from normal human mammary epithelia. HeLa and PC3 are derived from cervical cancer and prostate cancer, respectively; U2OS and Saos-2 are derived from osteosarcoma. Transfection with HA-OLA1 caused centrosome amplification in cell lines derived from mammary tissue, but not in those derived from other tissues.

We analyzed the effect of OLA1 knockdown on centrosome in cell lines, not derived from mammary tissue (Supplementary Fig. S2A and S2B). In these cell lines, knockdown of OLA1 did not cause centrosome amplification.

Overexpression of OLA1 causes overduplication of centrioles

Cep170 localizes to both mother and daughter centrioles and is used as a marker for both centrioles. Each animal cell normally has two or four centrin-positive spots distributed in pairs. Cep170 is used as a marker for mother centrioles only and, therefore, double-immunofluorescence staining for Cep170 with centrin could discriminate centriole overduplication from centrosome accumulation (36). Cells with extra centrioles due to centriole overduplication have only one or two Cep170-positive centrioles. By contrast, cells with extra centrioles due to abscopal cell divisions accumulate at least two and often more than two Cep170-positive centrioles.

Inhibition of BRCA1 and knockdown of OLA1 increase the number of cells with extra centrioles, which are found frequently not paired and have only one or two Cep170-positive centrioles, suggesting centrosome fragmentation and centriole overduplication (15, 27). Therefore, we analyzed the effect of OLA1 overexpression on centrioles. To visualize centriole and identify the transfected cells, Hs578T cells were transfected with control vector or HA-OLA1 along with GFP-centrin and then immunostained.
with an anti-Cep170 antibody. We found that overexpression of HA-OLA1 increased the number of cells with extra centrioles (more than four; Fig. 2A and B) but only one or two Cep170-positive centrioles (Fig. 2C); these centrioles were mostly unpaired (Fig. 2A). These suggest that overexpression of OLA1 causes centriole overduplication. However, most of the extra daughter centrioles in OLA1-overexpressing cells were close to the mother centrioles, and centrosome fragmentation was unclear (Fig. 2A).

**Overexpression of missense mutants of OLA1 fails to cause centrosome amplification**

OLA1-E168Q mutant does not rescue OLA1 knockdown-induced centrosome amplification (15). This suggests that
OLA1-E168Q is deficient in the regulation of centrosome number. The proportion of cells overexpressing OLA1-E168Q and showing centrosome amplification was significantly lower than that in cells overexpressing OLA1-WT (Fig. 1B); we hypothesized that overexpression of OLA1 with normal function efficiently induces centrosome amplification, but that overexpression of OLA1 mutants, which are deficient at regulating centrosome number, causes centrosome amplification in significantly fewer cells. To identify the residues of OLA1 that are important for centrosome regulation, we analyzed the efficiency of overexpressed OLA1 missense mutants at inducing the centrosome amplification in breast cancer cells.

Nine candidate phosphorylation residues, S36, S45, T124, T320, T325, T336, Y350, S358, and Y375, were chosen by using NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/), and three residues modified with lysine acetylation, K216, K242, and K296, and two ATP-binding residues, F127 and N230, which have been previously identified (19, 37), were substituted alanine or arginine (Fig. 3A; Supplementary Table S1). Expression of the HA-OLA1 mutants was confirmed by Western blot (Fig. 1C, Fig. 3C–E).

We analyzed whether these five mutants could rescue the centrosome amplification induced by knockdown of OLA1. Hs578T cells were transfected with OLA1 siRNA, OLA1-1 against the 3′UTR of OLA1, and the expression vectors, HA-OLA1-WT or mutants, and stained with anti-γ-tubulin and anti-HA antibodies. Cotransfection of OLA1-WT partially, but significantly, rescued the centrosome amplification induced by knockdown of OLA1, which were not observed with these five mutants similar to E168Q mutant (Fig. 3F). These suggest that these five mutants are also deficient in the regulation of centrosome number (Supplementary Table S2).

The structure of OLA1 has been determined (19); S36, T124, F127, K242, and K296 exist in the G domain, and E168 and T325 are located in the coiled-coil domain and TGS domain, respectively (Fig. 3A and G). The F127 side-chain interacts with the adenine base of AMPPCP (an ATP analogue), whereas S36 hydrogen bonds to a phosphate group of AMPPCP.

The OLA1–BARD1 interaction is an important regulator of centrosome number. Because OLA1 binds to the C-terminal region of BARD1 (BARD1-546–777), γ-tubulin, and the N-terminal region of BRCA1 (BRCA1-1–304) (Fig. 4A), we analyzed the binding ability of OLA1 mutants. We prepared purified GST-tagged OLA1 (GST-OLA1) mutants and His-tagged BARD1-546–777 (His-BARD1-546–777), γ-tubulin, and BRCA1-1–304 and performed

**Figure 2.**
Centrosome amplification induced by OLA1 overexpression is due to overduplication of centrioles. A, Hs578T cells were transfected with control vector (HA) or HA-OLA1 expression vector along with GFP-centrin expression vector. Cells were fixed and stained with anti-Cep170 antibody and DAPI. Representative images of the cells are shown. Scale bar, 10 μm. B, The cells were counted, and the percentages of cells with amplified centrioles (more than four) were scored. C, Quantification of the numbers of Cep170-positive centrioles in the cells with more than four centrioles from the experiments shown in A. For B and C, at least 100 cells were counted in each experiment. Histograms show the mean and SEM of three independent experiments. Statistical significance is indicated by asterisks (***, P < 0.001).
Figure 3.
Centrosome amplification induced by the overexpression of OLA1 missense mutants. A, Diagram of the structure and the locations of missense mutations of OLA1. OLA1 is composed of an N-terminal G domain, a coiled-coil domain, and a C-terminal TGS domain. The G domain has characteristic sequence motifs, G1-G5. B, Hs578T cells were transfected with control vector (HA) or vector expressing HA-OLA1-WT or mutants. Cells were coimmunostained with anti-HA and anti-γ-tubulin antibodies, and stained with DAPI. The cells were counted, and the percentages of cells with amplified centrosomes were scored. C-E, Cell lysates were immunoblotted. F, Hs578T cells were transfected with control or OLA1 siRNA, OLA1-1, and vectors for HA-OLA1-WT, S36A, T124A, F127A, E168Q, K242R, or T325A. Cells were stained with anti-γ-tubulin and anti-HA antibodies. The cells with amplified centrosomes were counted, and the percentages of cells were scored. For B and F, at least 100 cells were counted in each experiment. Histograms show the mean and SEM of three independent experiments. Statistical significance is indicated by asterisks (**, P < 0.01) as calculated by the Student t test. G, Mapping of the residues apparently important for the regulation of centrosome number. The G domain, coiled-coil domain, and TGS domain are shown in red, yellow, and green, respectively. Residues that are deficient in the regulation of centrosome number are indicated with space filling. The ATP analogue AMPPCP is shown in sticks.
pulldown assays. GST-tagged OLA1-WT, T124A, E168Q, and K242R bound directly to BARD1, whereas S36A, F127A, and T325A mutants did not (Fig. 4B and C; Supplementary Tables S1 and S2). By contrast, all OLA1 mutants bound to γ-tubulin and to BRCA1-1–304 (Supplementary Fig. S3A–S3B). We previously found that GST-BRCA1-1–304 did not bind to His-OLA1-E168Q (15); in the current study, GST-OLA1-E168Q showed weaker binding to His-BRCA1-1–304 than GST-OLA1-WT. The exchange of tags might affect BRCA1–OLA1 interaction. All mutants bound to γ-tubulin in a manner similar to OLA1-WT.

Next, we analyzed the association in cells of the OLA1 mutants with the C-terminal region of BARD1, γ-tubulin, and the N-terminal region of BRCA1 by immunoprecipitation. HEK-293T cells were transfected with FLAG-OLA1 mutants and HA-BARD1-546–777, HA-γ-tubulin, or HA-BRCA1-1–304. Cell lysates were immunoprecipitated (IP) using an anti-FLAG antibody.

S36, T124, and T325 in OLA1 are the likely phosphorylated residues

As S36, T124, and T325 are candidate residues of phosphorylation, we compared the phosphorylated and nonphosphorylated mutants for their effect on centrosome amplification. We found that the OLA1–BARD1 interaction is important for the regulation of centrosome number. A, Model of the OLA1-containing complex (15). OLA1 binds to the N-terminal region of BRCA1, the C-terminal region of BARD1, and γ-tubulin. The N-terminal region of BRCA1 binds to the N-terminal region of BARD1. The middle portion of BRCA1 is associated with OLA1 via γ-tubulin. The C-terminal region of BRCA1 are likely to be associated with OLA1 via the unknown protein X. Protein X is an unknown protein. "N," the N-terminal region. "C," the C-terminal region. B, Glutathione-sepharose beads bound with GST, GST-OLA1-WT, or mutants were incubated with His-BARD1-546–777. The bound proteins were immunoblotted. C, Model of the conformation changes of the OLA1-containing complex induced by mutations of OLA1. OLA1 with the mutations (M; S36A, F127A, and T325A) do not bind to the C-terminal region of BARD1. D–F, HEK-293T cells were transfected with FLAG-OLA1-WT or mutants and HA-BARD1-546–777, HA-γ-tubulin, or HA-BRCA1-1–304. Cell lysates were immunoprecipitated (IP) using an anti-FLAG antibody.
constructed plasmids to express the phosphomimetic mutants, OLA1-S36D, T124E, and T325E. These mutants caused centrosome amplification to a significantly higher extent than nonphosphorylated mutants (Fig. 5A). Expression of the HA-OLA1 mutants was confirmed by Western blot (Fig. 5B). These results suggest that S36, T124, and T325 are the likely phosphorylated residues (Supplementary Table S2), and that these phosphorylations are involved in the regulation of centrosome number. Because nonphosphorylated S36A and T325A mutants failed to bind to the C-terminal region of BARD1, we prepared purified GST-OLA1-S36D and T325E and analyzed binding to the C-terminal region of BARD1. GST-OLA1-S36D and T325E bound to the C-terminal region of BARD1, GST-OLA1-S36D and T325E bound to BARD1 (Fig. 5C).

S36C has been reported as a mutation observed in cervical cancer (Catalogue of Somatic Mutations in Cancer (COSMIC); http://cancer.sanger.ac.uk/cosmic); we therefore tested its role in centrosome amplification. Overexpression of OLA1-S36C failed to cause efficient centrosome amplification and this mutant did not bind to BARD1 (Fig. 5A and D; Supplementary Table S1).

BARD1 is involved in the regulation of centrosome number
Because the three of six OLA1 mutants that were deficient in the regulation of centrosome number lost the binding activity to BARD1 (Supplementary Tables S1 and S2), we presumed that the OLA1–BARD1 interaction is important for the regulation of centrosome number. We therefore analyzed the effect of knocking down BARD1 on centrosome. We used two different siRNAs: BARD1-1 and BARD1-2. Both siRNAs efficiently reduced the expression of BARD1 (Fig. 6A). Knockdown of BARD1 caused centrosome amplification among T47D and Hs578T cells, in a similar manner to that seen with the knockdown of OLA1 (Fig. 6B and C).

Three mutations, C645R, V695L, and S761N, are reported in the C-terminal region of BARD1 (Fig. 6D). C645R has been identified in breast and ovarian cancers, V695L is reported in breast cancer, and S761N is found in breast and uterine cancers (38, 39). We analyzed the association in cells of HA-BARD1-546–777 bearing each of these mutations with FLAG-OLA1 by immunoprecipitation. HEK-293T cells were transfected with HA-BARD1-546–777-C645R, -V695L or -S761N, and/or FLAG-OLA1. Cell lysates were immunoprecipitated with an anti-FLAG antibody (Fig. 6E). HA-BARD1-full-length with each of the mutations was also analyzed (Fig. 6F). The association of the C-terminal region of BARD1 with the V695L or the S761N mutation with OLA1 reduced markedly. In the case of the full-length BARD1, the presence of each of the three mutations decreased the association with OLA1.

Because the V695L mutation showed the most severe effect on the OLA1–BARD1 association, we prepared purified His-BARD1-546–777 with the V695L mutation and analyzed the binding to OLA1. BARD1-546–777-V695L failed to bind to OLA1 (Fig. 6C). Furthermore, we analyzed whether HA-BARD1-V695L could rescue the centrosome amplification induced by BARD1 knockdown. Hs578T cells were cotransfected with BARD1 siRNA, BARD1-3, against the 3’ UTR of BARD1, and the expression vector, HA-BARD1-WT or -V695L, and stained with anti-γ-tubulin and anti-HA antibodies (Fig. 6G). Staining with the HA antibody showed cells with a normal number of centrosomes, whereas staining with the γ-tubulin antibody showed cells with an increased number of centrosomes. These results suggest that V695L is involved in the regulation of centrosome number.
BARD1 is involved in the regulation of centrosome number via the interaction with OLA1. A, Hs578T cells were transfected with control siRNA, BARD1 siRNA, BARD1-1, or BARD1-2, or OLA1 siRNA, OLA1-2. Whole cell lysates were immunoblotted with anti-BARD1, OLA1, and β-actin antibodies. B, Hs578T and T47D cells were transfected with control, BARD1, or OLA1 siRNAs. Cells were stained with anti–γ-tubulin antibody and DAPI. Representative images of H578T and T47D cells transfected with control or BARD1-1 siRNA are shown. Scale bar, 10 μm. C, The cells with amplified centrosomes were counted, and the percentages of cells were scored. D, Diagram of the location of missense mutations in BARD1-full-length (FL) and BARD1-546–777. RING, RING domain. ANK, Ankyrin repeats. BRCTs, BRCT domains. E, HEK-293T cells were transfected with HA-BARD1-545–777-WT or mutants and FLAG-OLA1. Cell lysates were immunoprecipitated using anti-FLAG antibody. F, HEK-293T cells were transfected with HA-BARD1-FL-WT or mutants and FLAG-OLA1. Cell lysates were immunoprecipitated using anti-FLAG antibody. G, Glutathione-sepharose beads bound with GST or GST-OLA1 were incubated with His-BARD1-546–777-WT or -V695L. The bound proteins were immunoblotted. H, Hs578T cells were transfected with control or BARD1-3 siRNA and vectors for HA-BARD1-WT or V695L. Cells were stained with anti–γ-tubulin and anti-HA antibodies. The cells with amplified centrosomes were counted, and the percentages of cells were scored. For C and H, at least 100 cells were counted in each experiment. Histograms show the mean and SEM of three independent experiments. Statistical significance is indicated by asterisks (**, P < 0.01) as calculated by the Student t test.
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anti-HA antibodies. Cotransfection with BARD1-WT partially, but significantly, rescued the BARD1 knockdown–induced centrosome amplification, but BARD1-V695L did not (Fig. 6H). Knockdown of BARD1 and expression of HA-BARD1-WT and V695L in Hs578T cells were confirmed by Western blot (Supplementary Fig. S4).

The BARD1–OLA1 interaction is important for BARD1 centrosomal localization and centrosome amplification induced by BARD1 overexpression

Next, we analyzed the centrosomal localizations and the effects of overexpression of HA-BARD1-WT and the V695L mutant. HA-BARD1-WT or -V695L was overexpressed in Hs578T and T47D cells. BARD1-WT localized to the centrosome and caused centrosomal amplification in these breast cancer cells (Fig. 7A–C). HA-BARD1 colocalized to only half of centrosomes in amplified centrosomes. The V695L mutation significantly reduced both centrosomal localization of BARD1 and centrosome amplification. In HeLa cells, the V695L mutation slightly decreased the centrosomal localization of BARD1 protein and overexpression of BARD1 did not cause centrosome amplification.

Finally, we confirmed the association in cells of Myc-tagged BARD1-WT (Myc-BARD1-WT) and -V695L with endogenous BRCA1 by immunoprecipitation. HEK-293T cells were transfected with Myc-BARD1-WT or -V695L. Cell lysates were immunoprecipitated with an anti-Myc antibody (Fig. 7D). The association of BARD1-WT and -V695L with endogenous BRCA1 was clearly detected. These suggest that the association of BARD1 with BRCA1 is not involved in the centrosomal localization and the effect of overexpression of BARD1 on centrosome number.

Discussion

Here, we showed that overexpression of OLA1 causes centrosome amplification in cell lines derived from mammary tissue. Suppression of BRCA1 and/or OLA1 causes centrosome amplification in cell lines derived from mammary tissue (11, 15). We found that overexpression of BRCA1 also caused centrosome amplification in breast cancer lines, Hs578T and MCF7 (Supplementary Table S1 and S2). S36A, F127A, and T325A mutants failed to bind to the C-terminal region of BARD1. S36C, a mutation reported in cancer, also failed to bind to BARD1 and cause efficient centrosome amplification by its overexpression. Thus, analyzing centrosome amplification induced by the overexpression of OLA1 variants is useful assay of OLA1. Overexpression of BRCA1-I42V, which is deficient in the regulation of centrosome number (13) and binding to OLA1, also caused centrosome amplification, whereas the percentage of cells with extra centrosomes was significantly lower than that of the cells transfected with BRCA1-WT (Supplementary Fig. S5A). Furthermore, overexpression of BARD1-V695L also failed to cause efficient centrosome amplification. Therefore, centrosome amplification by the overexpression of BRCA1 and BARD1 might also be useful for the evaluation of their function in the regulation of centrosome number.

That overexpression of the phosphomimetic mutants S36D, T124E, and T325E recovered centrosome amplification activity suggests that phosphorylation of S36, T124, and T325 is important for the regulation of centrosome number (Supplementary Tables S1 and S2). S36D and T325E recovered the binding activity to the C-terminal region of BARD1 that contains BRCT domains that bind to phosphorylated proteins (40). This suggests that S36 and T325 of OLA1 are first phosphorylated and then OLA1 binds to BARD1 to regulate the centrosome. In Escherichia coli, the S16 of the OLA1 homolog, YchF, which corresponds to S36 of OLA1, has been identified as a phosphorylation site in a phosphoproteome study (41). Phosphorylation of S16 in YchF affects the ATPase activity and is important for the inhibitory effect of YchF on the oxidative stress response (42).

F127 is important for nucleotide binding site and F127A mutation abolishes ATP binding drastically (19). In the tertiary structure, S36 locates close to F127 and the ATP analogue in the G domain. By contrast, T325 locates to the TGS domain in the C-terminal region, distant from S36 and F127. Consistent with this, S36A and F127A similarly abolished the association with BRCA1, BARD1, and γ-tubulin, whereas T325A diminished the association with BARD1 and γ-tubulin, but not BRCA1. The C-terminal domain of OLA1 (300–396 aa) binds to the C-terminal region of BARD1 (15). The structure of the C-terminal region of BARD1 has also been determined (43, 44). Consistent with a role in direct physical interaction, when the structure of OLA1 is computationally docked to the C-terminal region of BARD1, energetically favorable predicted interactions show that T325 of OLA1 locates close to the binding surface to BARD1 (Fig. 7E).

BARD1 mutations, C645R, V695L, and S761N, decreased the association with OLA1. C645R and V695L destabilize the BRCT domain, and while normal S761 is exposed to the surface, the S761N mutation reduces affinity of BARD1 for its binding partner (43). When the structure of OLA1 is computationally docked to the C-terminal region of BARD1, energetically favorable predicted interactions show that S761 locates at the binding surface to OLA1 (Fig. 7E). The V695L mutation, which did not affect the interaction with BRCA1, abolished the binding to OLA1, the localization to the centrosome, and the regulation of centrosome number. These suggest that interaction with OLA1 via its C-terminal region is important for the localization and function in centrosome of BARD1. Consistent with these findings, the C-terminal region of BARD1 is required for the centrosomal localization of BARD1, but not the RING domain, which is responsible for the interaction with BRCA1 (45). We previously proposed the existence of a
Figure 7.
The BARD1–OLA1 interaction is important for BARD1 centrosomal localization and centrosome amplification induced by BARD1 overexpression. A, Hs578T, T47D, and HeLa cells were transfected with control vector (HA) or HA-BARD1-FL-WT or -V695L expression vectors. Thirty-six hours after transfection, cells were fixed and stained with anti-HA and anti-γ-tubulin antibodies and DAPI. Representative images of Hs578T cells are shown. Scale bar, 10 µm. B, The percentages of cells showing colocalization of HA-BARD1 with γ-tubulin were scored. C, The cells with amplified centrosomes were counted, and the percentages of cells were scored. For B and C at least 100 cells were counted in each experiment. Histograms show the mean and SEM of three independent experiments. Statistical significance is indicated by asterisks (*, P < 0.05; **, P < 0.01) as calculated by the Student t test. D, HEK-293T cells were transfected with Myc-BARD1-FL-WT or -V695L. Cell lysates were immunoprecipitated using anti-Myc antibody. E, Computational docking model of OLA1 and the BARD1. T325 of OLA1 (color coded as in Fig. 3G) and S761 of the C-terminal region of BARD1 (blue) are indicated with space filling. F, Model of the conformation changes of the BRCA1/BARD1/OLA1/γ-tubulin complex induced by OLA1 and BARD1 mutations. OLA1 mutations (M), S36A, S36C, F127A, and T325A abolish the binding to the C-terminal region of BARD1. BARD1 mutation, V695L, abolishes binding to OLA1. These missense substitutions cause similar alterations in the conformation of the protein complex. Dissociation of OLA1 with BARD1 might reduce the association of OLA1 with BRCA1 and γ-tubulin.
BRCA1/BARD1/OLA1/γ-tubulin complex, which is important for the regulation of centrosome number. Both E168Q mutant of OLA1 and I427V variant of BRCA1 impair the BRCA1/OLA1 interaction and are deficient at regulating centrosome number (15). In this study, we demonstrated that the V695L mutation could cause alterations in the conformation of the protein complex similar to those caused by the OLA1 mutations S36A, S36C, F127A, and T325A, which abolish the binding to the C-terminal region of BARD1 (Fig. 7F). The BRCA1/BARD1 ubiquitinates centrosomal proteins, including γ-tubulin; it is thus feasible that OLA1 regulates the BRCA1/BARD1 E3 ubiquitin ligase.

BRCA1/BARD1 functions in homology-directed DNA repair, whereas mutations of V695L and S761N in BARD1 do not affect homology-directed DNA repair (46, 47). In BRCA mutation carriers, centrosome amplification is observed even in normal mammary tissue (48). Therefore, centrosomal regulation could contribute to the function of the BRCA1/BARD1 as a tumor suppressor together with OLA1 in mammary tissue. On the other hand, OLA1 is overexpressed in many other malignancies, including of the colon and lung, but not in breast cancer (22). Both lower expression of OLA1 in breast cancer and higher expression of OLA1 in lung adenocarcinoma are associated with poor prognosis (23, 25). OLA1 might therefore play a cancer type–specific role in carcinogenesis and progression. Further study is needed to clarify the function of the BRCA1/BARD1/OLA1 complex in tumor development.

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

Authors’ Contributions
Conception and design: Y. Yoshino, N. Chiba
Development of methodology: Y. Yoshino

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BRCA1-Interacting Protein OLA1 Requires Interaction with BARD1 to Regulate Centrosome Number

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