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Interference with BRCA2, which localizes to the centrosome during S and early M phase, leads to abnormal nuclear division

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Abstract

BRCA2 is responsible for familial breast and ovarian cancer, and its gene product is linked to DNA repair and transcriptional regulation. The BRCA2 protein exists mainly in the nucleus. Here, we show that BRCA2 has a centrosomal localization signal (CLS), localizes also to centrosomes during S and early M phases, and may regulate duplication and separation of the centrosomes. Green fluorescent protein (GFP) fused to the CLS peptides from BRCA2 (GFP–CLS) localizes to centrosomes and prevents endogenous BRCA2 from localizing to centrosomes. In addition, expression of GFP–CLS in cells leads to the abnormal duplication and positioning of centrosomes, resulting in the generation of multinuclear cells. These results thus implicate BRCA2 in the regulation of the centrosome cycle, and provide new insight into the aneuploid nature of many breast cancers.

Keywords: BRCA2; Centrosome; CLS; Capan-1; Aneuploid

Individuals with germ-line mutations in the breast cancer susceptibility gene *BRCA2* are at risk of developing breast and ovarian cancer [1,2]. Consistent with a critical function for *BRCA2* in tumor suppression, tumors that develop in carriers of heterozygous *BRCA2* mutations are frequently associated with loss of heterozygosity at the *BRCA2* locus. However, mutation of the *BRCA2* gene is rarely found in sporadic breast cancer, and the role of the *BRCA2* gene in the development of sporadic breast cancer is unclear [3]. BRCA2 plays important roles in both transcriptional regulation [4] and DNA damage repair mediated by homologous recombination [5,6]. Furthermore, it has been reported that embryonic fibroblasts with a $BRCA2^{Tr2014/Tr2014}$ mutation have an abnormal number of centrosomes [7]. Recent studies indicate that BRCA2 inactivation in murine embryo fibroblasts and HeLa cells by targeted gene disruption or RNA interference delays and prevents cell division [8].

Centrosome defects are characteristic of many solid tumors and may be responsible for the origin of the mitotic spindle abnormalities [9] and DNA aneuploidy found in cancer [10]. Both G1 arrest and mitotic errors at cytokinesis occur in cells from which centrosomes have been removed by microsurgery [11]. The centrosome is the principal microtubule-organizing center and consists of an amorphous material, called the centrosome matrix, which contains a variety of proteins. Most importantly, it contains the γ-tubulin ring complex, the component largely responsible for nucleating microtubules [12]. The centrioles and associated centrosome matrix are duplicated during interphase, but remain together as a single complex on one side of the nucleus. In early M-phase, this complex separates into two, each of which nucleates its own aster.

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The two asters, which initially lie side by side and close to the nuclear envelope, subsequently move to opposite sides of the nucleus. In this way, the two poles of the mitotic spindle are rapidly formed. At metaphase, the nuclear envelope breaks down, enabling the spindle to capture the chromosomes. These findings suggest the existence of a centrosome checkpoint that functions to monitor the coordination between centrosome duplication and nuclear division.

A number of proteins that regulate the cell cycle have been reported to localize to the centrosomes; cyclin E [13], p53 [14], PARP [15] and so on. BRCA1, which is another gene responsible for hereditary breast cancer and binds to BRCA2, also has been reported to be associated with the centrosomes [16]. In this paper, by using immunofluorescence microscopy and biochemical analysis of not only whole cells but also isolated centrosomes, we demonstrate that BRCA2 also localizes to the centrosome to ensure the appropriate duplication and migration of the centrosome. Our results suggest that BRCA2 may play an important role in the regulation of the centrosome cycle.

Materials and methods

Cell lines. NB1-RGB (normal human skin fibroblasts) and HeLa S3 cells were purchased from the RIKEN GeneBank. MCF7 (human breast cancer cell line) and Capan-1were purchased from ATCC.

Antibodies. The following antibodies were used in this study: BRCA2 (Ab-1)-mouse monoclonal antibody (mAb) (Oncogene); BRCA2 (H-300 and H299)-rabbit polyclonal antibody (Santa Cruz); BRCA2 (Ab-2)-rabbit polyclonal antibody (NEOMARKERS); FLAG (F3165)-mAb (Sigma–Aldrich, Co.); γ-tubulin rabbit IgG of antiserum (Sigma–Aldrich Co.); γ-tubulin (C-11)-mAb (Santa Cruz); α-tubulin mAb (Sigma–Aldrich Co.); β-actin (MAB1501R)-mAb (Chemicon); centrin2 (N-17)-goat polyclonal antibody (Santa Cruz); PCNA-mAb (MBL).

Plasmids and constructs. Wild-type and mutant GFP–CLS constructs were created by annealing double-stranded oligonucleotides into the expression vector, pcDNA3.1/NT-GFP-TOPO (Invitrogen). For the construction of wild-type GFP–CLS, the oligonucleotides 5′-TATTTACCATCACGTGCACTAACAAGACAGCAAGTTCGTGCTTTGCAAGATGGTGCAGAGA-3′ and 5′-CTCTGCACCATCTTG -3′ were annealed and ligated into pcDNA3.1/NT-GFP-TOPO. For the construction of mutant GFP–CLS (A), the oligonucleotides 5′-TATTTACCAGCACGTGCACTAACAAGACAGCAAGTTCGTGCTTTGCAAGATGGTGCAGAGA-3′ and 5′-CTCTGCACCATCTTGCAAAGCACGAACTTGCTGTCTTGTTAGTGCACGTGCTGGTAAATAA-3′ were used. For construction of mutant GFP–CLS (AA), the oligonucleotides 5′-TATTTACCAGCAGCTGCACTAACAAGACAGCAAGTTCGTGCTTTGCAAGAT-GGTGCAGAGA-3′ and 5′-CTCTGCACCATCTTGCAAAGCACGAACTTGCTGTCTTGTTAGTGCAGCTGCTGGTAAATAA-3′ were used. All the plasmids were verified by DNA sequencing. Full-length human BRCA2 cDNA, a gift from F.J. Couch (Mayo Clinic and Foundation), was subcloned into the pME18S-FL3 vector. The pME18S-FL3 vector was kindly provided by Dr. S. Sugano (The University of Tokyo). To generate FLAG-tagged BRCA2 protein, we fused the FLAG gene 3′ to the BRCA2 gene, such that the FLAG tag was fused to the carboxy terminus of the BRCA2 protein.

Immunoblot analysis and immunoprecipitation. Whole cell lysates were obtained by harvesting the cells in lysate buffer (20 mM Tris–HCl, pH 8.0; 100 mM NaCl, 1 mM EDTA, 0.1% NP40) containing protease inhibitors (completely EDTA free; Roche Applied Science, Indianapolis, IN). The extract was centrifuged at 12,000*g* for 30 min to remove cell debris, and γ-tubulin, α-tubulin or BRCA2 antibodies were added to the cell extract and incubated for 30 min at 4 °C with shaking. Protein G–Sepharose (20 μl of 50% suspension) was added and incubated for 1 h at 4 °C with shaking. The immunoprecipitates were washed five times with lysis buffer minus the protease inhibitors. They were analyzed by electrophoresis through SDS–PAGE gels and transferred electrophoretically onto 0.45-μm PVDF membranes (Millipore, Billerica, MD). The blots were probed with primary antibodies for 2 h at room temperature and then with secondary antibodies coupled to horseradish peroxidase (ECL anti-mouse or rabbit IgG; Amersham) for 1 h at room temperature. The blots were developed using the enhanced chemiluminescent reagent SuperSignal (Pierce, Rockford, IL) and exposed to Kodak X-OMAT film, according to the manufacturer's instructions.

Immunofluorescence microscopy. Cells were fixed with 3.7% formaldehyde in PBS for 10 min on ice and permeabilized sequentially with 50%, 75%, and 95% ethanol on ice for 5 min each. The slides were blocked with PBS-containing blocking solution for 30 min at room temperature and then incubated with primary antibody for 1 h at room temperature, and washed for 5 min with PBS three times. The cells were then incubated with Alexa Fluor 488- or 594-conjugated secondary antibody (Molecular Probes) for 1 h at room temperature, washed three times with PBS, and preserved in Vectashield (Vector Inc., Burlingame, CA). DNA was stained with 1 μg/ml bis-benzimide (Hoechst33258) in the final PBS wash. The samples were examined with an Olympus Power BX51 fluorescence microscope (Olympus, Co., Tokyo, Japan).

Time lapse microscopy. HeLa cells were grown on Lab-Tek chambered cover glasses (Nalge Nunc International, Rochester, NY). We examined cell division phenotypes using time-lapse phase-contrast microscopy by capturing one frame every 60 min over 20 h, starting 24 h after transfection. This procedure was performed with a Leica DMI 6000B microscope using Leica FW4000 software. The microscope was equilibrated in 5% CO_2 and maintained at 37 °C, and images were taken with a 40/0.6 PL Fluotar.

Results and discussion

BRCA2 has been reported to localize to discrete nuclear foci during S-phase [17]. When we examined the subcellular localization of BRCA2 at each phase of the cell cycle by immunofluorescence microscopy, we observed a strong focus of BRCA2 fluorescence that corresponded to the position of the centrosome, in addition to the nuclear dot pattern of BRCA2 fluorescence throughout G1- and S-phase. Based on this finding, we hypothesized that BRCA2 might localize to the centrosomes and play a vital role in the function of the centrosome checkpoint. To confirm co-localization of the BRCA2 protein with the centrosome, we conducted immunofluorescence microscopy in HeLa cells and normal fibroblasts using an anti-BRCA2 antibody (H-300) in combination with an antibody against γ-tubulin (C-11), an essential centrosomal component and centrin2 as a marker for centrioles (Fig. 1). Fluorescent spots corresponding to BRCA2 and γ-tubulin were detected close to the nucleus in both cells. The merged image showed that BRCA2 localized to the region of the centrosome and that in addition the signal localized to nuclear foci. Two other BRCA2 antibodies, H299 and Ab-2, were also shown to recognize a region corresponding to the centrosome and to foci within the nucleus (Fig. 1). Similar results were obtained using MCF7 breast cancer cells (data not shown). To verify the specificity of the BRCA2 antibodies for immunolocalization studies, we labeled the pancreatic cancer cell line, Capan-1, which has a naturally occurring 6174delT mutation in one BRCA2 allele accompanied by

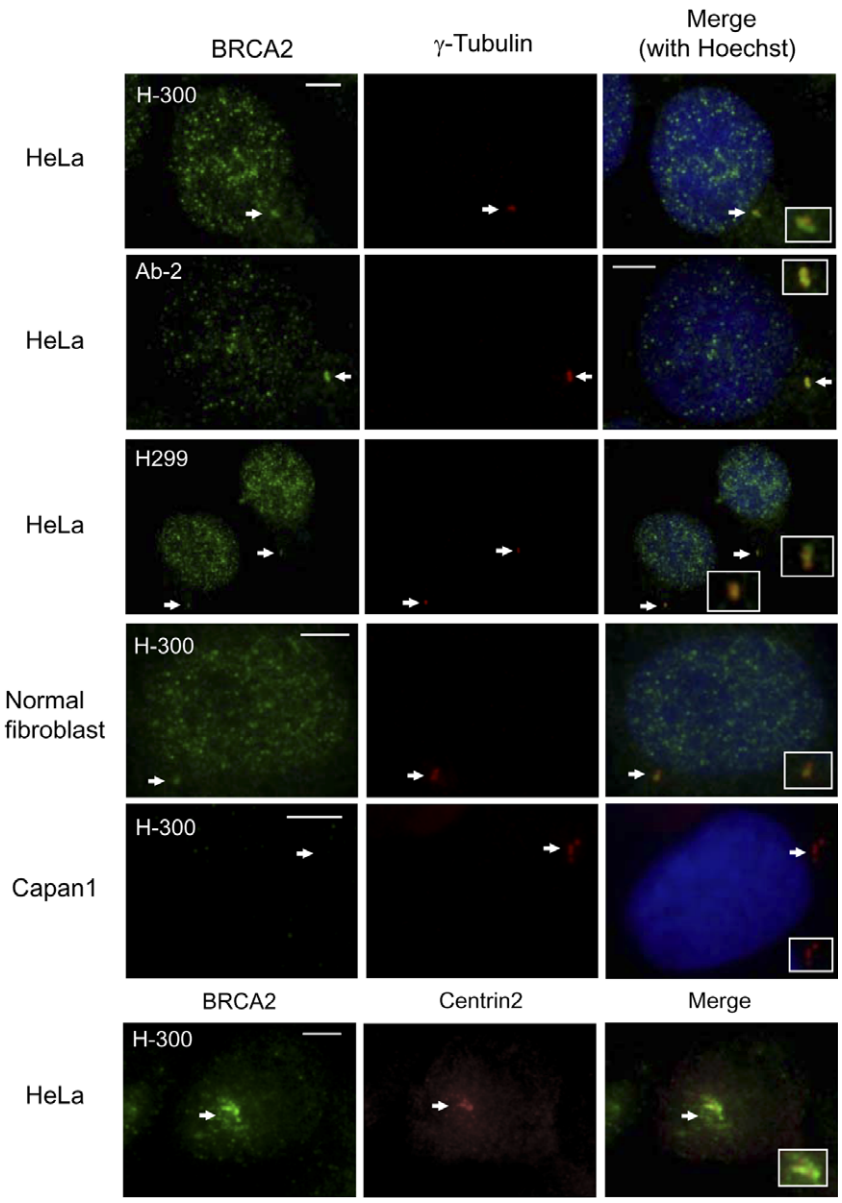

Fig. 1. BRCA2 localizes to the centrosome and binds to γ-tubulin. Exponentially growing HeLa, human normal fibroblasts and Capan-1 cells were subjected to immunofluorescence analysis with anti-BRCA2 antibody (H-300, H299, and Ab-2; green), anti γ-tubulin antibody (C-11; red), anti centrin2 antibody (N-17; red), and Hoechst33258 (blue). The magnified area represents the region of the main image around the centrosome denoted by the arrowheads. The yellow signal in the merged images denotes co-localization of the antibodies against BCRA2 and γ-tubulin. Scale bar, 5 μm.

the loss of the wild-type allele. The truncated BRCA2 in Capan-1 cells does not contain the epitope recognized by the H-300 antibody. Consequently, the H-300 antibody did not give rise to an immunofluorescent signal in Capan-1 cells (Fig. 1). These results demonstrate that H-300 specifically detects endogenous BRCA2.

We next investigated whether the centrosomal localization of BRCA2 is regulated during the cell cycle. A typical centrosomal staining pattern observed in the tested cell lines with the anti-BRCA2 antibody was one or two dots in the interphase cells. The HeLa cells synchronized in S-phase (0 h after release), which were characterized by positive staining for PCNA, showed the clear co-localization of BRCA2 with the centrosomes (Fig. 2A) in addition to the distinct BRCA2-foci within the nucleus. In G1-phase cells (14 h after release) characterized by negative or very little staining of PCNA, definite co-localization of BRCA2 with centrosomes was not detected (Fig. 2A).

To investigate whether BRCA2 localizes to centrosomes during M-phase, exponentially growing HeLa cells at various stages of mitosis were immunostained for BRCA2. The results indicated that BRCA2 was localized to the centrosomes during prophase, but that it did not exhibit centrosomal localization after prophase (Fig. 2B). These results strongly suggest that BRCA2 may play a role in regulating the centrosomes during interphase and early M-phase of the cell cycle. We then hypothesized that BRCA2 might function in collaboration with γ-tubulin, a

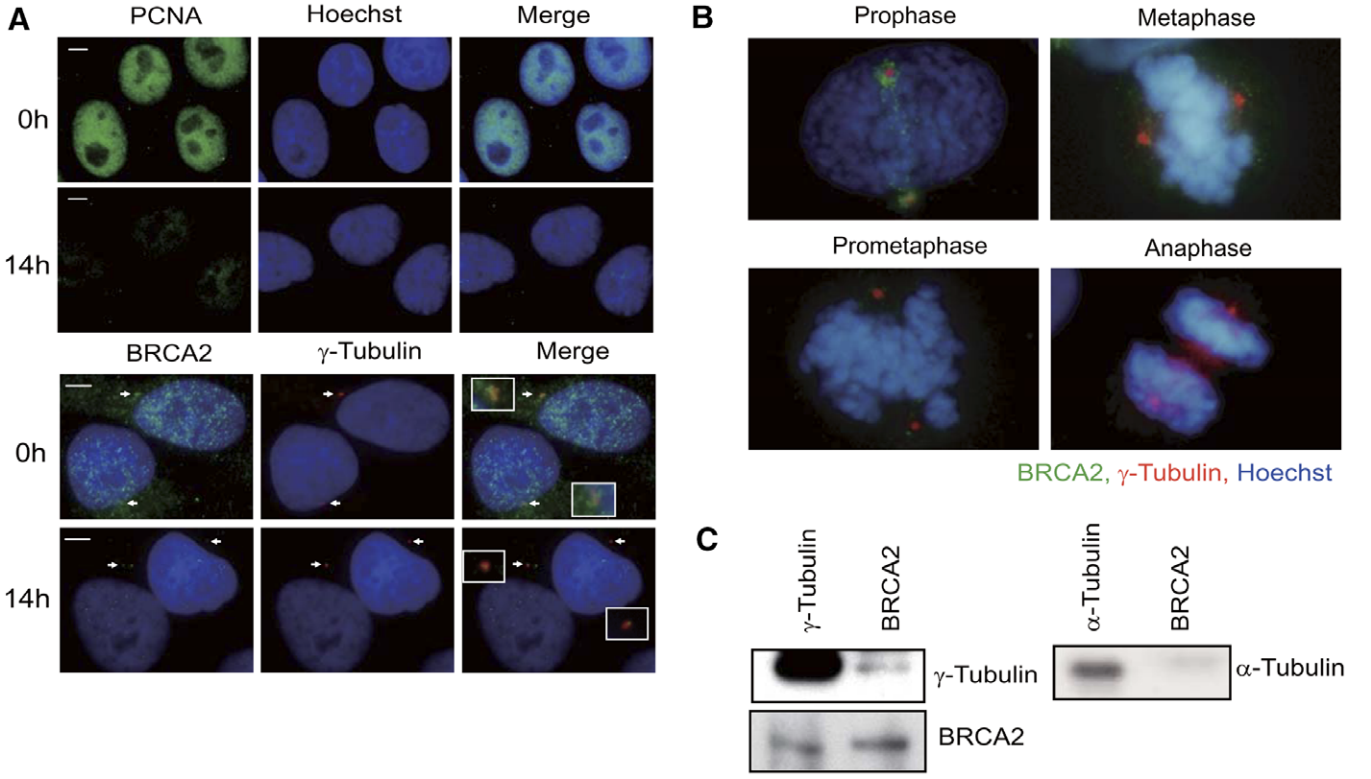

Fig. 2. Verification of centrosome localization of BRCA2. (A) HeLa cells were synchronized at S-phase with aphidicolin treatment. Then HeLa cells were released in aphidicolin-free medium and harvested for preparation of immunostaining. The subcellular localization of BRCA2 in HeLa cells synchronized in the cell cycle S-phase (0 h after release) or G0/G1 phase (14 h after release) was determined by immunofluorescence microscopy using antibody Ab-2 in combination with an antibody against γ-tubulin. Control stainings of cells in S-phase were performed with antibody recognizing the nuclear replication protein PCNA. Scale bar, 5 μm. (B) Immunofluorescence microscopy of BRCA2 and γ-tubulin localization during M-phase. HeLa cells were incubated with anti-BRCA2 antibody (H-300; green), anti γ-tubulin antibody (C-11; red), and Hoechst33258 (blue). Co-localization of green and red spots was observed in prophase, but not in other phases. (C) BRCA2 interacts with γ-tubulin proteins. Lysates from HeLa cells were subjected to immunoprecipitation (IP) with anti-BRCA2 or anti-γ-tubulin. Immunoprecipitates were analyzed by immunoblot (IB) analysis with anti-γ-tubulin (upper panel) or anti-BRCA2 (middle panel). The co-immunoprecipitation experiment was performed using anti-BRCA2 and anti-α-tubulin antibodies as a negative control (lower panel).

critical component of the centrosome. We thus performed a co-precipitation analysis that showed that BRCA2 associates with γ-tubulin. BRCA2 was immunoprecipitated from HeLa cells, and analysis of anti-BRCA2 immunoprecipitates with anti-γ-tubulin demonstrated the presence of γ-tubulin in the immunoprecipitated complexes (Fig. 2C, left panel). In reciprocal experiments, a similar immunoblot analysis of anti-γ-tubulin immunoprecipitates for the presence of BRCA2 confirmed the interaction of the two proteins. On the other hand, BRCA2 did not bind to α-tubulin (Fig. 2C, right panel). Similar results were obtained in analogous studies in normal fibroblasts (data not shown). These results indicate that there is a specific direct or indirect interaction between γ-tubulin and BRCA2.

To corroborate our observation that BRCA2 localizes to the centrosomes, we analyzed the composition of centrosomes isolated from HeLa cells using sucrose density gradient centrifugation [18]. The anti-γ-tubulin antibody detected γ-tubulin in fractions 7 and 8, indicating that these fractions contained the centrosomes (Supplementary Fig. 1A). Analysis of proteins contained within these fractions using the BRCA2 antibody demonstrated the presence of BRCA2. The centrosomes present in fraction 8 were fixed and exposed to antibodies against γ-tubulin and BRCA2 (Supplementary Fig. 1B). The immunofluorescent signal from the γ-tubulin antibody overlapped with that of the BRCA2 antibody, providing further evidence that BRCA2 associates with centrosomes.

We next transfected a plasmid encoding FLAG-tagged (octapeptide; N-terminus DYKDDDDK C-terminus) BRCA2 into HeLa cells to evaluate further the centrosomal localization of BRCA2. The full-length BRCA2–FLAG fusion product was recognized by the FLAG antibody (Supplementary Fig. 1C). Immunofluorescence microscopy using anti-FLAG and anti-γ-tubulin antibodies revealed fluorescent spots close to the nucleus, and the merged image (the two red spots corresponding to γ-tubulin were surrounded by green spots corresponding to BRCA2) provided evidence for the localization of exogenous BRCA2 to centrosomes with nuclear foci at S-phase (Supplementary Fig. 1D, upper panel). The FLAG antibody did not give rise to an immunofluorescent signal in cells that were not transfected with the BRCA2–FLAG construct (Supplementary Fig. 1D, lower panel).

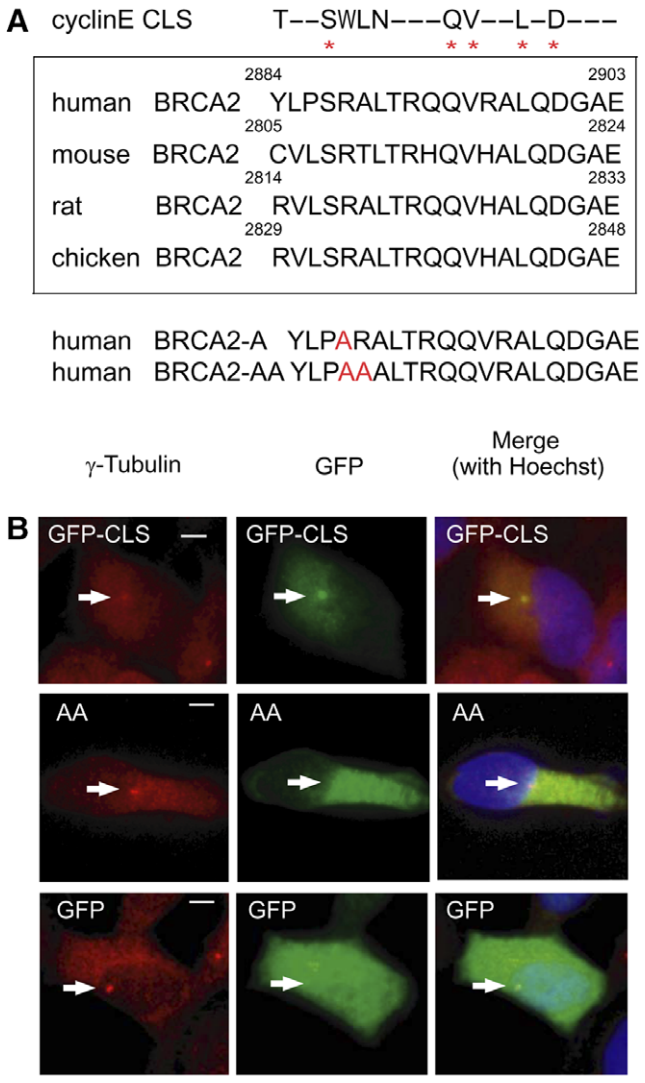

Fig. 3. The CLS is responsible for localization of BRCA2 to the centrosome. (A) Alignment of the 20 amino acid CLS motif in cyclin E with a homologous motif identified in BRCA2 sequences. Species abbreviations (with accession numbers in parentheses): human, *Homo sapiens* (U43746); mouse, *Mus musculus* (U65594); rat, *Rattus norvegicus* (U89653); chicken, *Gallus gallos* (AB066374). Identical residues are indicated by an asterisk. The red letters indicate substitutions in BRCA2-A and BRCA2-AA. (B) GFP–CLS localizes to the centrosome. HeLa cells were formaldehyde-fixed and incubated with anti-γ-tubulin antibody 8 h after transfection with plasmids expressing GFP–CLS, GFP–CLS (AA) or GFP. The arrows indicate the centrosome. Only the GFP–CLS localized to the region of centrosome (upper panels). Scale bar, 5 μm.

Next, we examined the mechanism through which BRCA2 localizes to centrosomes. One possibility is that BRCA2 contains a centrosome-targeting domain. Cyclin E has a modular centrosome-targeting domain termed the centrosomal localization signal (CLS) [13]. We thus aligned the cyclin E CLS sequence with a 20-amino acid region of homology within human, mouse, rat, and chicken BRCA2 (Fig. 3A). We found that 56% of the residues in this region were identical. We next sought to determine whether the CLS domain of BRCA2 is responsible for targeting of the protein to the centrosome. We first transfected into HeLa cells a plasmid encoding a green fluorescent protein (GFP)-tagged CLS. To examine the localization of the GFP–CLS fusion protein, we performed immunofluorescence microscopy on the transfected cells using the anti-γ-tubulin antibody and Hoechst. The co-localization of the fluorescent signals derived from GFP and the anti-γ-tubulin antibody suggested that the CLS targeted the GFP–CLS fusion protein to the centrosomes (Fig. 3B, upper panels). However, a similar analysis of cells expressing a fusion protein containing mutations in two conserved residues of the CLS domain (S2887A and R2888A, designated BRCA2-AA) demonstrated that mutation of the CLS abrogated localization of the fusion protein to the centrosome (Fig. 3B, middle panels). Mutation of a single conserved residue within the CLS domain of the fusion protein (S2887A, designated BRCA2-A) had no effect on centrosome localization (data not shown). To evaluate further the requirement for the BRCA2 CLS motif in the centrosome localization of BRCA2, we performed the expression of a recombinant BRCA2–FLAG in HeLa cells (Supplementary Fig. 2A). In addition to the wild type of BRCA2–FLAG, we also created a mutant of BRCA2–FLAG in which the S2887 and R2888 residues of the CLS were substituted with alanine (designated BRCA2 (CLSAA)-FLAG). Although FLAG-tagged BRCA2 (BRCA2–FLAG) localized to centrosomes, BRCA2 (CLSAA)–FLAG did not co-localize with γ-tubulin, but continued to localize to nuclear foci (Supplementary Fig. 2A, lower panels). These results suggest that the CLS may be sufficient to target BRCA2 to the centrosome.

To determine in greater detail the function of CLS in BRCA2, we performed immunofluorescence microscopy on HeLa cells expressing GFP–CLS using antibodies against GFP and BRCA2. We observed that the centrosomal localization of endogenous BRCA2 was disrupted in cells expressing GFP–CLS (Fig. 4A). In addition, several centrosomal abnormalities were observed in HeLa cells transfected with the plasmid expressing GFP–CLS, including altered centrosomal positioning (Fig. 4B, upper panels) and replication (Fig. 4B, middle panels; enlargement of centrosome, lower panels; abnormality of the number of replicates). These effects were not observed in cells expressing the fusion protein containing the double mutation in the CLS (GFP–CLS [AA]). These results suggest that the expression of the GFP–CLS fusion protein may prevent or interfere with the localization of the endogenous BRCA2 protein, due to competition with the expressed GFP–CLS for binding to the centrosome. Aberrations in centrosome duplication have been reported to result in the formation of monopolar or multipolar spindles, and it has been proposed that such aberrations may cause multinucleation. We thus asked whether similar effects might occur in the GFP–CLS-expressing HeLa cells, due to the mislocalization of endogenous BRCA2. We indeed observed the generation of multinucleation in these cells (Supplementary Fig. 2B, upper panels). A significant fraction (mean frequency, $10.5\% \pm 1.0\%$ ($n = 1000$)) of the

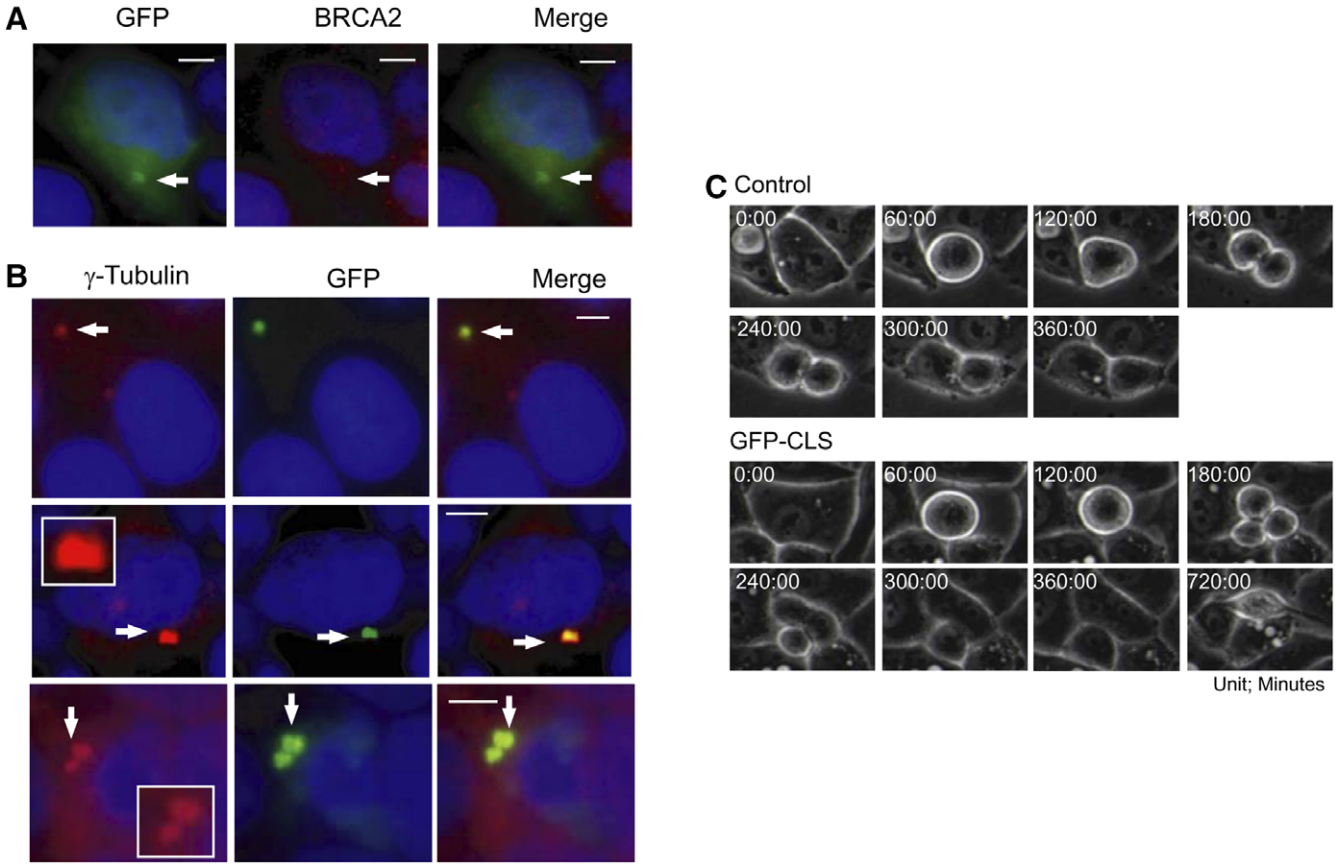

Fig. 4. Abnormal localization and replication of centrosomes due to the forced expression of recombinant GFP–CLS in HeLa cells. (A) Expression of GFP–CLS prevents endogenous BRCA2 from localizing to the centrosome. Eight hours after transfection with plasmids expressing GFP–CLS, HeLa cells were evaluated by fluorescence microscopy for GFP expression and centrosomal localization of BRCA2 (red) antibody and the DNA dye Hoechst (blue). The arrows indicate the centrosomes. Scale bar, 5 μm. GFP–CLS prevents endogenous BRCA2 from localizing to the centrosome (middle panel). (B) Twenty-four hours after transfection with plasmids expressing GFP–CLS, HeLa cells were evaluated by fluorescence microscopy for GFP expression and centrosomal localization of γ-tubulin (red) antibody and the DNA dye Hoechst (blue). The arrows indicate the centrosomes. The magnified area represents the region of centrosomes. Scale bar, 5 μm. The centrosome containing γ-tubulin and GFP–CLS is located far from the nuclear surface (upper panels). The centrosome is enlarged (middle panels), and three or more replicates appeared (lower panels). (C) Representative time-lapse images of HeLa cells transfected with either control or GFP–CLS. Time frames from time-lapse videomicroscopy show phase contrast images of cells. Times indicated are in minutes.

GFP–CLS-expressing cells were multinucleated, whereas a much smaller fraction ($3.1\% \pm 0.61\%$) of cells expressing the mutant GFP–CLS (AA) were multinucleated (Supplementary Fig. 2C).

We next obtained time-lapse images to further explore the mitotic failure and the progression of cell division in GFP–CLS-transfected HeLa cells. HeLa cells were first transfected with plasmids expressing either GFP–CLS or GFP alone as a control. Phase-contrast time-lapse microscopy of transfected cells ($n = 14$) was initiated 24 h later and continued for 24 h. A representative series of frames from time-lapse videomicroscopy showed that the cells expressing GFP–CLS ($n = 4$) were induced into M-phase and divided into three or more daughter cells, of which two or more fused, thereby resulting in the formation of multinucleated cells and proceeding to catastrophic cell death (Fig. 4C). The cells expressing GFP alone were induced into M-phase and divided into two daughter cells normally (Fig. 4C). Depletion of BRCA2 by small interfering RNA (siRNA) in HeLa cells was also given rise to similar results (data not shown).

We presented here evidences demonstrating an association of BRCA2 with the centrosome during S and early M phases using immunofluorescence microscopy technique and biochemical analysis. Particularly the result obtained by the biochemical analysis of isolated centrosomes strongly supports the presence of BRCA2 in the centrosomes. Furthermore, co-immunoprecipitation of BRCA2 with γ-tubulin suggests an interaction between these two proteins. A recent two-hybrid screen using the conserved COOH-terminal residues of BRCA2 as bait resulted in the identification of a daughter centriole-associated protein called centrobin [19]. This result is consistent with the results of the current study, which demonstrate that BRCA2 localizes to the centrosome and might regulate the duplication and migration of the centrosome. Furthermore, we have shown that a reduction in the function of BRCA2 in the centrosome leads to failure of nuclear

division. Taken together, our results suggest that some of the BRCA2 proteins may function with centrosomes during S and early M phases. Abnormality of the number of replicates in cells expressing GFP–CLS (Fig. 4A) may be the result of overduplication by the interference with the centrosomal localization of the endogenous BRCA2, however it cannot be ruled out that the effects on centrosome numbers are due to an accumulation of centrosomes. BRCA2 is known to localize to the cleavage furrow during late mitosis and functionally involved in cytokinesis [6]. Therefore, the observed bi- or multinucleation is due to cytokinesis defects and a subsequent fusion of daughter cells.

We have shown that CLS (aa 2884–2903) in BRCA2 is required for centrosomal targeting. Expressed wild-type, but not mutant, BRCA2 localized on the centrosome. A central database named Breast Cancer Information Core (BIC) has been established in the National Human Genome Research Institute (NHGRI) to coordinate the information related with BRCA1 and BRCA2 research. According to the BIC database, the mutation of codon 2888 in BRCA2 is reported by five examples. Somatic mutation of BRCA2 has been thought to be rare in breast cancers, though common allelic deletions in the BRCA2 locus (13q12–q13) imply an important role of somatic mutation in these tumors. Although the cellular role of BRCA2 in sporadic cancers remains to be determined, the results presented here should help elucidate the molecular mechanism of sporadic breast cancer.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.01.100.

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