The BRCA1 RING and BRCT Domains Cooperate in Targeting BRCA1 to Ionizing Radiation-induced Nuclear Foci*

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BRCA1 accumulates in nuclear foci during S-phase and reassembles into DNA repair-associated foci after DNA damage, reflecting its role in genome maintenance. BRCA1 comprises a RING domain at the N terminus and a BRCT domain at the C terminus, through which it associates with DNA repair proteins. The key sequences that target BRCA1 to DNA damage-induced foci have not been identified. Here, we mapped the BRCA1 foci-targeting domains of yellow fluorescence protein (YFP)-tagged BRCA1 in MCF-7 breast cancer cells exposed to ionizing radiation (IR). Cancer mutations specific to the BRCT domain, but not the RING domain, abolished BRCA1 recruitment to IR-induced foci. The YFP-BRCT domain itself, however, localized poorly at IR-induced foci, and the RING domain and other sequences were negative. We discovered that only when the RING and BRCT domains were combined was foci targeting restored to levels observed for wild-type BRCA1. The RING-BRCT fusion co-localized at foci with the MDC1 DNA damage response factor and inhibited entry of endogenous BRCA1 into nuclear foci. Our results explain why exon 11-deficient BRCA1 splice variants are targeted to IR-induced foci even though they are incapable of repairing DNA damage. We propose that both RING and BRCT domains together target BRCA1 to large focal assemblies at DNA double-stranded breaks.

The breast cancer susceptibility gene BRCA1 is a tumor suppressor gene involved in DNA maintenance and cell cycle control (1). Germ line mutations in BRCA1 (or in BRCA2) are found in ~50% of patients with inherited breast cancer and up to 90% of families with breast and ovarian cancer susceptibility (2, 3). BRCA1 is a large protein comprising 1863 amino acids and has been implicated in a variety of cellular functions including chromatin remodeling (4), DNA damage checkpoint activation (5), and various types of DNA repair (1, 6). BRCA1 contains two important protein interaction domains. The first is a zinc-binding RING motif at the N terminus, which forms an enzymatically active ubiquitin-protein isopeptide ligase (E3) when BRCA1 heterodimerizes with BARD1 through its N terminus (7), an activity abolished by RING cancer mutations (e.g., C61G) (8, 9). BRCA1 also comprises two BRCT motifs at the C terminus. The BRCT domain is common to DNA damage response proteins (10, 11) and was recently reported to facilitate interaction with phosphoserine peptides (12–14). BRCA1 binds in a BRCT-dependent manner to several proteins including p53 (15), CtIP (16), and the DNA helicase BACH1 (17). Cancer mutations in the BRCT domain impair BRCA1 interaction with some of these proteins (12, 13, 16, 18). The BRCT domain of BRCA1 also associates with the RNA polymerase II holoenzyme and activates gene transcription (19). Therefore, the RING and the BRCT domains contribute to multiple BRCA1 nuclear functions.

BRCA1 is implicated in different DNA repair pathways, including homologous recombination and non-homologous end joining, which facilitate repair of double-stranded DNA breakages (6, 20). Various in vitro biochemical assays and genetic studies revealed a direct influence of BRCA1 on DNA repair and showed that mutations in BRCA1, including those in the RING domain (e.g., C61G) and in the C-terminal BRCT domain, impair BRCA1 DNA repair function (1, 21, 22). Cellular BRCA1 is detected primarily in the nucleus and found to cluster in discrete nuclear “foci” during DNA replication at S-phase (23, 24). Immunostaining experiments showed that BRCA1 co-localizes with BARD1 and RAD51 in S-phase cells. Co-localization with the replication protein proliferating cell nuclear antigen was only observed in S-phase cells after treatment with the replication inhibitor hydroxyurea, suggesting that BRCA1/BARD1/RAD51/polymerase II homologus complexes can assemble at stalled DNA replication forks (23, 25). BRCA1 also responds to different types of DNA damage induced by chemicals or by ultraviolet and ionizing radiation (IR), which cause DNA dimerization and double-stranded breaks, respectively.

In IR-treated cells, BRCA1 redistributes into distinct nuclear foci that often co-stain with RAD51 or with RAD50/MRE11/NBS1 protein complexes that mediate homology-directed DNA repair or non-homologous end joining, respectively (6, 20). The first protein recruited to the DNA double-stranded break sites is the ataxia telangiectasia-mutated protein-phosphorylated histone subunit γH2AX (26), which signals the subsequent focal accumulation of other DNA damage and repair factors including the mediator of DNA damage checkpoint protein, MDC1 (27, 28), followed by the p53-binding protein, 53BP1 (29, 30), and BRCA1 (31, 32). These are then followed by the appearance of RAD50/MRE11/NBS1 (22) and RAD51, all of which all display at least partial co-localization with BRCA1 in nuclear foci.

The aim of this study was to identify the sequences required for BRCA1 accumulation in IR-induced nuclear foci. Despite predictions that the BRCT domain of BRCA1 is likely involved, 1 The abbreviations used are: IR, ionizing radiation; YFP, yellow fluorescence protein; PBS, phosphate-buffered saline.

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Targeting of BRCA1 to Nuclear Foci

The key targeting sequences have in fact not been delineated. Involvement of the RING region was suggested by our earlier finding that the N-terminal partner, BARD1, stimulated targeting of BRCA1 to nuclear foci after DNA damage by methyl methanesulfonate (33). The BRCT domain was implicated by studies showing that the BRCT mutant form of BRCA1 (5382insC) expressed in HCC1937 breast cancer cells did not form DNA damage-induced foci (21, 22, 34). Moreover, because the tandem BRCT domain from the PAX transactivating domain-interacting protein formed IR-inducible foci in transfected U2OS cells (12), one might predict that the BRCA1 BRCT domain is also sufficient to target BRCA1 to foci. In this study, we show that the BRCT region is sufficient to target BRCA1 to nuclear foci at modest levels in MCF-7 cells and that the RING domain enhanced the efficiency of targeting. This explains the previously reported staining at IR-induced foci of the murine BRCA1 exon 11 splice variant, which lacks the middle half of BRCA1 but retains the RING and BRCT domains (33, 35).

Materials and Methods

Plasmid Construction—All BRCA1 mutants and fragments tested in this study are shown in Figs. 2A and 3A. Yellow fluorescence protein (YFP)-tagged BRCA1 wild-type, Δ306–1312, and 1–304 plasmids, and the FLAG-tagged BARD1 plasmid were constructed as described previously (33, 36). Other YFP-labeled BRCA1 constructs were produced by inserting the YFP coding sequence into the NotI site upstream of the BRCA1 coding sequence in the FLAG-tagged constructs described previously (36). For the RING-BRCT fusion plasmids, BRCA1 DNA sequences (underlined) encoding the amino acids 1470–1866 (C939) and 1620–1862 (C243) were amplified using the PCR forward primers 5′-AGTCCGAGATTTCTGCGATAGTCTACC-3′ and 5′-AGTCGAGATCATGCTCTGCGGT-3′, respectively, and the reverse primer 5′-AGTCGAGATCATGCTCTGCGGT-3′. These BRCA1 fragments were inserted into the EcoRI (italicized) site at the 3′ end of the 1–304 sequence in the pYFP-BRCA1(1–304) plasmid. All of the ectopic proteins were expressed from the pFLAG CMV2 human expression vector (Sigma-Aldrich) except for the YFP-BRCA1 C243 plasmid, which was constructed by inserting the BRCA1 C243 fragment (amplified by PCR primers 5′-TGATGCGTGGACACTGAATCGTGGCTG-3′ and 5′-TGATGCGTGGACACTGAATCGTGGCTG-3′) into the SalI site of the pYFP-C1 vector (Clontech).

Cell Culture and Transfection—MCF-7 and T47D human breast cancer cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) at 37 °C in a humidified atmosphere of 5% CO2. For transfections, cells were seeded on glass coverslips and grown to ~50% confluence for each sample, a maximum of 12 μg of plasmid DNA was transfected using the Lipofectamine 2000 reagent (Invitrogen) as indicated by the manufacturer. At 6 h post-transfection, the transfection medium was removed and replaced with the Dulbecco’s modified Eagle’s medium as described above.

DNA Damage Treatment and Immunostaining—Approximately 44 h post-transfection, cells were either left untreated or exposed to 15 Gy of γ radiation from a cesium137 source (Gammacell 1000 irradiator, Atomic Energy of Canada) and then allowed to recover at 37 °C for 4 h. This dose and recovery time was determined to produce the most consistent IR response in BRCA1 and closely matches that used in previous BRCA1 studies (e.g. Refs. 12 and 35). Cells were fixed in 3.7% formaldehyde-phosphate-buffered saline (PBS) for 20 min and then permeabilized in 0.2% Triton-PBS for 10 min. To reduce nonspecific staining, cells were blocked in 3% bovine serum albumin-PBS for 45 min. We used mouse monoclonal antibody Ab-4 (Oncogene Research) to detect endogenous BRCA1 protein. For BARD1 and MDC1 staining, rabbit BARD1 antibody 59L (gift from Professor Richard Baer) and rabbit MDC1 BL578 antibody (Bethyl Laboratories) were used, respectively. Following 50 min of incubation of the primary antibody, the cells were stained with biotinylated secondary antibodies (Dako) and Texas Red-avidin D (Vector Laboratories) or fluorescein isothiocyanate-conjugated secondary antibodies (Santa Cruz Biotechnology). Cell nuclei were stained with Hoechst 33285 (Sigma).

Scoring of BRCA1 Nuclear Foci and Cell Imaging—Stained and transfected cells were observed and scored using an Olympus BX40 fluorescence microscope. Each BRCA1 construct was transfected in at least two separate experiments to obtain an average score, and at least 100 cells were counted for each sample. Ectopic BRCA1 foci were scored using a ×100 objective. BRCA1 nuclear focus formation was recorded as either no focus, 1–10 foci, or >10 foci. Cell images were captured by a Spot RT camera (Diagnostic Instruments) attached to an Olympus BX51 fluorescence microscope.

Western Blot Analysis—MCF-7 cells were transfected with various YFP-BRCA1 constructs, and total cell extracts were prepared after 48 h of expression for immunoblot analysis. Cells were resuspended in protein extraction buffer (20 mM Tris-Cl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40 supplemented with protease inhibitor mix (Roche Applied Science) and shock-frozen in liquid nitrogen. After a quick thaw at 37 °C, the cells were refrozen in liquid nitrogen, rethawed on ice for 20 min, and cleared of insoluble components by centrifugation at 13,000 rpm at 4 °C for 15 min. Cell extracts were then mixed (2:1) with sample buffer (100 mM Tris-Cl (pH 6.8), 20% glycerol, 0.01% bromphenol blue, 10% β-mercaptoethanol, and 5% SDS) and denatured at 95 °C for 5 min. Up to 200 μg of whole cell protein extract was loaded per lane, separated on a 6% or 10% SDS-polyacrylamide gel, and transferred onto nitrocellulose membranes (Millipore). The membranes were incubated in blocking solution (5% skim milk powder in PBST (PBS containing 0.2% Tween 20)) for 60 min at room temperature followed by incubation with primary antibody diluted in PBST for 2 h at room temperature. Incubation with secondary horseradish peroxidase-conjugated antibodies (Sigma, 1:10,000) was followed by detection by enhanced chemiluminescence (ECL, Amersham Biosciences) reaction. YFP-BRCA1 fusion proteins were detected using either an anti-green fluorescence protein monoclonal antibody (Roche Applied Science, 1:800 dilution), or the BRCA1 monoclonal antibody Ab1 (Oncogene Research, 1:100).

Results

Ectopic YFP-BRCA1 Forms Nuclear Foci That Co-localize with BARD1 and MDC1—Several studies have noted the redistribution of endogenous BRCA1 (22, 35) and of ectopic BRCA1 (37) after cellular DNA damage elicited by IR. To define these sequences responsible for the damage-dependent relocalization, we first compared the subcellular distribution patterns of transiently expressed YFP-BRCA1 with that of the endogenous BRCA1 protein before and after IR treatment. We examined endogenous BRCA1 in the T47D breast cancer cell line, which expresses a higher level of BRCA1 than MCF-7 cells. Transiently expressed YFP-BRCA1 wild type was assessed in both MCF-7 cells and T47D cells and was co-transfected with pFLAG-BARD1, which we previously showed enhances nuclear localization and focus formation of BRCA1 (33). In the majority of untreated cells, both endogenous and ectopic BRCA1 formed large foci (usually <10 per cell), which co-localized with BARD1 and with endogenous MDC1 (Fig. 1). When examined 4 h after a 15-Gy pulse of ionizing radiation, both the endogenous and ectopic BRCA1 had redistributed from large spots (presumably the S-phase foci) to a large number of small foci, which co-localized with BARD1 and the DNA damage response factor MDC1 (Fig. 1). YFP alone did not form nuclear foci with or without IR treatment (data not shown). These tests confirmed that overexpressed YFP-BRCA1 formed IR-responsive nuclear foci in a manner similar to endogenous BRCA1. Because YFP-BRCA1 displayed a clearer IR response in MCF-7 cells than in T47D cells (% cells with >10 foci increased from 9 to 22% in T47D and from 19 to 65% in MCF-7), we used MCF-7 cells to map the foci-targeting sequences of BRCA1.

Cancer Mutations in the BRCT Domain Disrupt BRCA1 Nuclear Focus Formation—To compare the effect of known cancer mutations on BRCA1 nuclear focus formation, we co-transfected different YFP-tagged BRCA1 mutant proteins with pFLAG-BARD1 into MCF-7 cells (Fig. 2). The majority (67%) of untreated transfected cells expressing wild-type BRCA1 exhibited <10 distinct nuclear foci. After IR treatment, the proportion of YFP-BRCA1-positive cells with >10 foci had increased from 19% (no treatment) to 65%. As we recently observed in cells exposed to methyl methanesulfonate (34), the BRCT-truncated mutant BRCA1–5382insC failed to cluster in nuclear foci before or after IR-induced DNA damage (2). Similarly,
three other frequently studied C-terminal BRCT mutants, P1749R, M1775R, and Y1853X, all displayed a dramatic decrease in foci localization compared with the wild-type protein. In contrast, the N-terminal RING domain mutant C61G, which is defective for ubiquitin ligase activity (8), was only modestly reduced in its overall ability to form foci and still exhibited a potent response to IR treatment (59% of cells with >10 foci). Note that only nuclear-stained cells were scored and that...
BARD1 can stimulate nuclear localization of all the BRCA1 mutants tested (33, 34). These findings indicate that cancer mutations in the BRCT domain, but not the RING domain, affect the recruitment of BRCA1 to the sites of DNA damage and repair.

The Combination of RING and BRCT Domains Results in Highly Efficient Targeting to IR-induced Nuclear Foci—We next mapped the critical foci targeting sequence(s) of BRCA1, employing the same co-transfection strategy as described in Fig. 2. In transfected MCF-7 cells, ectopic YFP-BRCA1 with a C-terminal truncation of both BRCT motifs (amino acids 1–1637) exhibited severe loss of foci in untreated and IR-treated cells (see Fig. 3). Deletion of the RING domain (amino acids 170–1863) also caused a marked reduction in focus formation but not as dramatic as the loss of the BRCT domain, with 53% of untreated cells displaying 1–10 foci and following IR treatment up to 18% of cells revealing >10 foci (Fig. 4A).

These results are generally consistent with results observed for the cancer point mutations (Fig. 2) and indicated that the C-terminus of BRCA1 may play a more significant role than the N terminus in nuclear focus formation.

We therefore tested three sequential (and slightly overlapping) BRCA1 subfragments comprising amino acids 1–304, 279–1315, and 1290–1863. Although the 1290–1863 fragment is less nuclear due to lack of nuclear import signals (33), it displayed sufficient nuclear staining for unambiguous detection of IR-inducible foci. Interestingly, all three individual subfragments lost the ability to form nuclear foci efficiently (+IR treatment). The 1290–1863 fragment contains the BRCT domain, and we were interested to know if the BRCT sequence alone would respond the same way, given that the BRCT fragment of the PAX transactivating domain-interacting protein was reported to form IR-induced nuclear foci (12). We therefore cloned and tested the core BRCT domain of BRCA1 (amino acids 1620–1862, named “C243”), which was observed to form some foci before IR (53% of cells with 1–10 foci) and after IR treatment (cells with >10 foci increased from 2 to 13%). The results of this core BRCT fragment were almost identical to those of the 170–1863 fragment. The small difference in nuclear distribution between these two BRCT-containing fragments and the 1290–1863 fragment may relate to differences in folding and/or a potential regulatory sequence in the region 1290–1620.

We showed previously that the BRCA1 splice variant Δ306–1312, which lacks central exon 11, can be escorted into the nucleus by BARD1 and forms foci in response to the DNA damaging agent methyl methanesulfonate (33). Here, we demonstrate that the intranuclear distribution of this naturally occurring form of BRCA1 also responded to IR (see Figs. 3 and 4), consistent with results seen in mice homozygous for the same form of BRCA1 (35). To further refine the critical focus-targeting region, we transiently expressed two fusion constructs that linked the N-terminal 1–304 fragment (N304) to either the C-terminal sequence from amino acids 1470 to 1862 (C393) or the sequence from amino acids 1620 to 1862 (C243, tandem BRCTs alone). These fusion proteins, like the splice variant, also formed IR-inducible foci (Figs. 3 and 4A). The N304-C243 protein, comprising only the RING/BARD1-binding domain and the BRCT sequences fused together, displayed the most efficient response to IR. This was despite the poor response of either sequence tested individually or when each sequence was co-transfected as a separate construct into MCF-7 cells (data not shown). In T47D cells, we observed a similar foci pattern following IR treatment for wild-type

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**FIG. 3.** Mapping of BRCA1 nuclear focus-forming regions. Diagram of BRCA1 constructs and images of nuclei from MCF-7 cells transfected with the YFP-tagged BRCA1 proteins. Cells were assessed before and after IR treatment. NLS, nuclear localization sequence.
BRCA1 and the RING-BRCT fusion (see Fig. 4B). Although the BRCT fragment C243 was detectable in nuclear foci in untreated cells, we did not observe a clear induction of C243-positive foci after ionizing radiation, which may reflect the overall reduced proportion of cells displaying >10 foci in this cell line. We confirmed the correct expression of all the YFP-BRCA1 fusion proteins by cell staining using antibodies that recognize the N terminus (Ab1), central exon 11 (Ab4), or the C terminus (Ab5, data not shown) and further validated the key constructs by Western blot using green fluorescent protein or BRCA1 antibodies (see Fig. 5). Collectively, these findings reveal that optimal targeting to IR-induced foci involves a combination of the N-terminal RING/BARD1-binding domain and the C-terminal BRCT domain.

Ectopic BRCA1 BRCT Sequences Co-localize with MDC1 Foci—BRCA1 has nuclear roles in transcription regulation, chromatin remodeling, and different types of DNA repair (6), and the foci in which it accumulates may vary accordingly (4, 22). In Fig. 1, we confirmed that wild-type YFP-BRCA1 formed IR-dependent foci that co-stained with MDC1, a major DNA repair site marker (27, 38). It was therefore important to test the IR-responsive BRCA1 subfragments for similar co-localization. Four YFP-tagged BRCA1 constructs, 5382insC, 170–1863, N304+C243, and C243, were examined with respect to MDC1 co-localization (Fig. 6). Cells transfected with the 5382insC plasmid do not form BRCA1 foci despite the presence of bright IR-induced MDC1 foci in those cells. Transient expression of the other BRCA1 constructs that do form foci, including the BRCT sequence alone, did not perturb MDC1 foci but did co-localize very well with MDC1 (Fig. 6), validating their targeting to sites of DNA repair.

The RING/BARD1-binding Domain, but Not the BRCT Domain, Disrupts Targeting of Endogenous BRCA1 to Foci—Recently, it was shown that overexpression of the MDC1 BRCT domain caused a reduction in IR-induced foci positive for cellular MDC1 and H2AX (39). These DNA damage response factors act upstream of BRCA1 (26, 27), which explains why overexpression of the BRCA1 BRCT domain did not impair formation of MDC1 foci after irradiation (Fig. 6). We were, however, interested to determine whether overexpression of the BRCA1 RING or BRCT-containing fragments would disrupt endogenous BRCA1 foci before or after IR (Fig. 7). We employed the BRCA1 Ab-4 antibody to stain for endogenous

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**Fig. 4. Quantification of IR-induced BRCA1 nuclear foci.** A, BRCA1 nuclear foci scoring results for each of the various YFP-BRCA1 sequences expressed in MCF-7 cells with or without 15 Gy of IR and 4 h of recovery. B, similar quantification of foci formed in T47D cells transfected with key BRCA1 constructs as shown. Average percentage of transfected cells (±S.E) are shown for no focus, 1–10 foci, and >10 foci. White bars represent untreated samples, and black bars represent irradiated samples.
BRCA1 in transected T47D cells, as Ab-4 does not detect the ectopic BRCA1 sequences. We first tested three N-terminal BRCA1 peptide fragments. The 1–70 sequence contains the BARD1-binding domain (amino acids 8–96 (40)), were predicted to compete well for the endogenous BARD1 protein (41). Most of the transfected cells (50–60%) expressing these two BRCA1 fragments displayed a lack of endogenous BRCA1 staining, and of those transfected (1–304 fragments that contain the BARD1-binding domain (Fig. 7). In contrast, the BRCA1 1–110 and 1–304 sequences consistently blocked formation of IR-stimulated BRCA1 foci. This is consistent with saturation of the N-terminal sequences and not the BRCT domain. Similar results were observed in three separate experiments. We conclude that overexpression of the BRCA1 N terminus competes out a saturable protein, likely to be BARD1, required for foci targeting. The lack of competition displayed by the C-terminal BRCT domain suggests that it does not compete out a saturable factor or that binding of multiple BRCT domains can occur simultaneously at nuclear foci target sites.

**DISCUSSION**

The BRCA1 tumor suppressor has been described as a caretaker of the genome (1, 6). It accumulates in nuclear foci during S-phase and reassembles into DNA repair-associated foci after DNA damage, reflecting its role in genome maintenance. BRCA1 subcellular localization is known to be regulated by specific targeting sequences that facilitate its nuclear entry (33, 42), nuclear export (36), and centrosomal association (43). And yet arguably the most important targeting signals, those that direct BRCA1 to DNA repair focal assemblies in response to DNA damage, have not been defined. In this study, we show for the first time that it is the combination of the RING and BRCT domains that is critical for accumulation of BRCA1 at damage-induced foci. Notably, these protein interaction domains have each been implicated in macromolecular assembly (44, 45). Our results explain why exon 11-deficient BRCA1 splice variants, which retain the RING and BRCT-containing ends of BRCA1, are targeted to IR-induced foci (35). These splice variants may thus act as competitive inhibitors of BRCA1-dependent DNA repair at nuclear foci.

The BRCT domain is common to DNA repair proteins and is targeted by some cancer mutations. Mutations in the BRCT domain of the nibrin protein (NBS1), which forms a DNA repair complex with RAD50/MRE11, has been shown to abolish IR-induced nibrin foci (46) and to increase predisposition to the cancer-prone Nijmegen breakage syndrome. Likewise, a mutation (5382insC) in the BRCT domain of BRCA1 correlates with lack of IR-induced BRCA1 foci in HCC1937 breast cancer cells (22, 37). Here, we confirmed that the BRCA1 (5382insC) mutant also failed to form IR-induced foci when overexpressed in MCF-7 cells. In fact, we discovered a clear foci-targeting defect for all of the C-terminal BRCT mutants tested before or after DNA damage by IR. The BRCT mutation in HCC1937 cells results in G1 checkpoint activation and hyper-
sensitivity to DNA damage agents (21) and aberrant chromosome structure (47). Certain BRCA1 mutations, such as P1749R and Y1853X, alter the stability and folding of the BRCT domain, which would interfere with BRCA1 binding to specific partners (48, 49). Other BRCT mutations such as M1775R affect surface residues but not the protein structure, thereby reducing binding affinity to BACH1 (49–51). These perturbations may explain why the BRCA1 BRCT mutants are not recruited to MDC1-positive DNA repair foci (see Fig. 2). On the other hand, the N-terminal RING mutation C61G, which has been shown to abolish ubiquitin ligase activity of the BRCA1-BARD1 heterodimer (8, 9), did not impair movement of BRCA1 into IR-induced foci in our experiments. This suggests that BRCA1 nuclear focus formation may be independent of ubiquitin ligase activity. In contrast, deletion of the N-terminal 170 amino acids (BARD1 binding site and RING) greatly reduced foci staining, supporting the hypothesis that BRCA1 nuclear focus formation is stimulated more by the binding of BARD1 (33) than by BRCA1/BARD1 ubiquitin ligase activity.

IR-induced double-stranded DNA breaks attract many DNA repair factors as determined usually by immunofluorescence microscopy. The key proteins required for IR-induced foci formation appear to be γH2AX, MDC1, and 53BP1. Although γH2AX is usually first to arrive at the damage sites, it is not required for the initial movement of BRCA1, 53BP1, or NBS1 to the break sites, but it is required for their subsequent retention and accumulation as foci at double-stranded breaks (52). Recent studies with sequence-specific small interfering RNAs or knock-out mice showed that silencing of γH2AX (31, 32), MDC1 (27), and 53BP1 (29) caused at least a 50% reduction in BRCA1 staining at IR-induced nuclear foci, and of those factors only 53BP1 knock-down seemed to specifically affect BRCA1 (29). Thus, these proteins act upstream of BRCA1 in the assembly of DNA repair foci at sites of double-stranded breaks. Moreover, they all contain C-terminal BRCT domains (10, 11), which have been identified as binding modules for phosphoserine peptides (12–14). Perhaps attributable to their sequence diversity, the BRCT domains of proteins such as BRCA1, BARD1, MDC1, and DNA ligase IV exhibit different phosphopeptide binding specificities (14).

The first confirmed phosphoserine-specific peptide target of BRCA1 is BACH1, and this DEAH helicase may contribute to BRCA1 foci recruitment through the BRCA1 BRCT domain (17, 51). Another candidate target is MDC1, which co-immunoprecipitates with BRCA1/BARD1 before and after IR treatment (53), although the interacting sequences have not been identified. 53BP1 also binds to BRCA1 in vivo; however, it does not bind after ionizing radiation and therefore is unlikely to di-
rectly anchor BRCA1 at focal assemblies (29). BRCA1 can potentially interact with several different DNA repair-associated factors through its BRCT domain (14), whereas of the known N-terminal binding partners, which include BARD1, estrogen receptor, c-Myc, and BAP1, only BARD1 was found to increase BRCA1 localization at nuclear foci (Ref. 33 and data not shown).

Our deletion mapping revealed the importance of the N-terminal RING domain and C-terminal BRCT domains for targeting BRCA1 to foci. A previously published deletion analysis of MDC1 reported that removal of the C-terminal BRCT domain, but not the N-terminal FHA domain, impacted on IR-induced focus formation (39). Given the importance of BRCT domains for the targeting of several proteins to IR-induced foci (39, 46, 54), their role in mediating specific protein-protein interactions during assembly of large DNA repair complexes seems reasonable. In the case of BRCA1, this is also supported by an in vitro study in which the BRCA1 BRCTs were found to multimerize and form foci (~10 monomers per focus) at the ends of DNA fragments (44). We found, however, that the BRCT domain of BRCA1 actually displayed very limited foci staining on its own. Moreover, in contrast to the effects of overexpressing the MDC1 BRCT domain, which supposedly reduced staining of MDC1, H2AX, and Chk2 at IR-dependent foci (39), overexpression of the BRCA1 BRCT domain had virtually no impact on cellular BRCA1 foci (Fig. 7). This was different from the effect of N-terminal BARD1-binding fragments, which prevented BRCA1 foci formation. BRCA1 is one of the downstream factors recruited to nuclear foci, and our combined data suggest that although the BRCT domain contributes to foci targeting of BRCA1, it does not compete out a saturating anchorage factor, but may instead synergize with the BARD1-binding region to increase the specificity or affinity of binding to the DNA repair complex.

We previously published that BARD1 increased BRCA1 trafficking to the nucleus and its ability to locate at methyl methanesulfonate-induced nuclear foci (33), and here we confirm a requirement of the BARD1-binding domain (~1–10 amino acids, overlapping the RING domain) of BRCA1. Like the BRCT domain, the RING domain of some proteins is implicated in formation of macromolecular complexes (45). In this context, it is intriguing to note that the RING domains of BRCA1 and BARD1 can assemble into a ring structure of ~12 monomers in vitro, and the BRCA1/BARD1 multimers act as a scaffold for and enhance ubiquitin oligomerization (45). In our study, the co-expression of BARD1 did not facilitate foci formation of YFP-BRCA1 (1–304); nevertheless, when this N-terminal fragment was linked to the BRCT domain, a profound enhancement was observed, suggesting that the RING region cooperates with the BRCT domain in some way. Indeed, the two domains had to be linked together to be targeted to foci, and their mere co-expression was insufficient to achieve this (data not shown). We therefore propose that BRCA1 is recruited to foci as part of a BRCA1/BARD1 heterodimer and that its integration into repair assemblies requires its BRCT-dependent interaction with quite possibly multiple phosphoserine-containing protein targets.

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