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Identification of Rad51 regulation by BRCA2 using *Caenorhabditis elegans* BRCA2 and bimolecular fluorescence complementation analysis

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Abstract

BRCA2 is involved in double-stranded DNA break repair by binding and regulating Rad51-mediated homologous recombination. Insights as to how BRCA2 regulates Rad51-mediated DNA repair arose from *in vitro* biochemical studies on fragments of BRCA2. However, the large 400-kDa BRCA2 protein has hampered our ability to understand the entire process by which full-length BRCA2 regulates Rad51. Here, we show that CeBRC-2, which is only one tenth the size of mammalian BRCA2, complemented BRCA2-deficiency in Rad51 regulation. CeBRC-2 was able to bind to mammalian Rad51 (mRad51) and form distinct nuclear foci when they interacted. In our bimolecular fluorescence complementation analysis (BiFC), we show that the strength of the interaction between CeBRC-2 and mRad51 increased markedly after DNA damage. The BRC motif of CeBRC-2 was responsible for binding mRad51, but without the OB fold, the complex was unable to target damaged DNA. When *CeBRC-2* was introduced into BRCA2-deficient cells, it restored Rad51 foci after DNA damage. Our study suggests a mode of action for BRCA2 with regard to DNA repair.

Keywords: BRCA2; *C. elegans*; Rad51; CeBRC-2; DNA repair; BiFC

Germ-line mutations in *BRCA2* predispose to early-onset breast cancer [1]. Targeted disruption of the BRCA2 allele in mice leads to lethality at embryonic day 7.5 [2,3]. A hypomorphic allele truncating BRCA2 in exon 11, the frequently deleted region in human cancers, overcomes the embryonic lethality, and a few mice survive to birth and succumb to thymic lymphoma in 12 weeks after weaning [4,5]. Studies of these hypomorphic allele ($Brca2^{Tr}$) mice revealed that BRCA2 is involved in double-stranded DNA break repair via binding to Rad51—cells isolated from $Brca2^{Tr/Tr}$ mice exhibit chromatid breaks, chromosome breaks, tri-radials, and quadri-radials, hallmarks of defects in double-stranded DNA break repair [6].

The crystal structure of the 800-amino acid C-terminus of BRCA2 (amino acids 2373–3173) revealed that it has 3 oligonucleotide-binding (OB) folds and a helix-turn-helix (HTH) motif [7], capable of binding to double-stranded DNA. Importantly, OB folds found in BRCA2 are very similar to OB folds of RPA [7,8], which is capable of binding single-stranded DNA. Another crystal structure showed that the BRC repeats, in the conserved exon 11, bound to Rad51 and inhibited self-oligomerization of Rad51 by mimicking a motif in Rad51 that served as an interface for Rad51 oligomerization. As a consequence, BRCA2 is capable of controlling the assembly of the Rad51 nucleoprotein filament that is essential for strand-pairing and invasion during homologous DNA recombination [9]. At the extreme C-terminus of exon 27, another Rad51 binding domain is present [10]. Studies using *in vitro* assays showed that this domain bound to Rad51 oligomers and not to monomers (unlike BRC repeats), and facilitated efficient nucleation of Rad51 oligomers on DNA [11]. When this domain bound to oligomerized Rad51 on single-stranded DNA, it stabilized the nucleofilament against disassembly by monomeric BRC peptides.

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Taken together, these studies suggest a mechanism for how BRCA2 regulates Rad51-mediated recombinational DNA repair. Yet, the manner in which full-length BRCA2 functions *in vivo* in response to DNA damage has yet to be elucidated.

Bioinformatic analyses have identified BRC repeat-containing proteins in *Caenorhabditis elegans* [12]. Subsequent studies in *C. elegans* mutants demonstrated that *C. elegans* BRCA2 (CeBRC-2), which possesses a single BRC motif and 1 OB fold at the C-terminus, was capable of mediating DNA double-strand break repair [8]. Because CeBRC-2 is approximately one-tenth the size of mammalian BRCA2, we posited that it might be useful in studying the *in vivo* function of BRCA2 in mammalian cells, which has been hampered by the prohibitively large size of mammalian BRCA2.

In order to study the regulatory role of BRCA2 in Rad51-mediated DNA repair *in vivo*, we used a recently developed technique that enabled the visualization of direct protein–protein binding in a localized cellular compartment in response to various cellular stimuli, such as DNA damage. This approach is based on complementation between fragments of fluorescent protein [13,14]. In this fluorescence complementation (BiFC) analysis, only the direct bimolecular protein–protein interaction is visualized as fluorescence. Changes in fluorescence intensities in BiFC represent the strength of binding in a given cell under different stimuli, facilitating the direct measurement of binding affinity in a localized cellular compartment *in vivo*.

In this study, we demonstrate that *CeBRC-2* complements BRCA2-deficiency in regulating Rad51. Our results show that regulation of Rad51 by BRCA2 is conserved from worms to humans.

Materials and methods

Plasmids and antibodies. Expression plasmids were constructed by inserting sequences encoding 6XMyc-CeBRC-2 into pcDNA 3.1 (Invitrogen), and mouse Rad51 into 3XFlag-pcDNA3.1. The plasmid encoding GST-CeBRC-2 was generated by inserting sequences encoding CeBRC-2 into pGEX4T-1 (Amersham). EGFP-CeBRC-2 was constructed by PCR and subsequent ligation into EGFP-N1 (Invitrogen).

The BiFC (bimolecular fluorescence complementation) plasmid Venus N-terminal 1–173 (VN173) and Venus C-terminal 155–238 (VC155) were generous gifts of Dr. C.-D. Hu (Purdue University, USA). Full-length CeBRC-2, N-terminus CeBRC-2 (containing the BRC motif and first NLS), and C-terminus CeBRC-2 (containing the OB fold and second NLS) were subcloned into VN173, and mouse Rad51 was subcloned into VC155.

The following antibodies were purchased from the vendors indicated: anti-GST, Upstate Biotechnology; and anti-Flag (M2), Sigma–Aldrich; anti-Rad51, Santa Cruz. In immunoprecipitation experiments with anti-HA or anti-myc, we used 12CA5 hybridoma or 9E10 culture supernatants, respectively.

Treatment with DNA damaging agents. For induction of DNA damage, cells growing on coverslips were exposed to γ-irradiation (5, 10, 15, 20 Gy), then allowed to grow for 4 h, and analyzed by BiFC. For chemical-induced DNA damage, normally growing cells were treated with 10 μM mitomycin C (MMC) or 2.4 μM doxorubicin (adriamycin), and analyzed 6 h later by BiFC.

BiFC (bimolecular fluorescence complementation) analysis and image acquisition. For the BiFC assay, cells were fixed with 4% paraformaldehyde and permeabilized in PBST (PBS with 0.5% Triton X-100), then mounted on microscope slides with DAPI-containing VECTASHIELD. The images were acquired using a CoolSnap HQ cooled CCD camera on a DeltaVision Spectris Restoration microscope built around an Olympus IX70 stand, with a 100×/1.35 NA lens.

Results

C. elegans BRCA2 directly binds to mouse Rad51 in vitro and in vivo

To explore the evolutionarily conserved function of BRCA2, we cloned BRCA2 from *C. elegans* by genomic PCR, and confirmed it by sequencing (data not shown). This ortholog of BRCA2 from *C. elegans*, named CeBRC-2 [8], was then subcloned into various vectors for further study.

Binding between CeBRC-2 and mouse Rad51 (mRAD51) was tested by GST-pulldown assay. Purified GST-CeBRC-2 bound to Sepharose 4B beads were incubated with cell lysates prepared from Flag-tagged *mRAD51*-transfected cells, washed, and analyzed by Western analysis. Flag-tagged mRAD51, as well as endogenous Rad51, was shown to bind to recombinant CeBRC-2 (Fig. 1A).

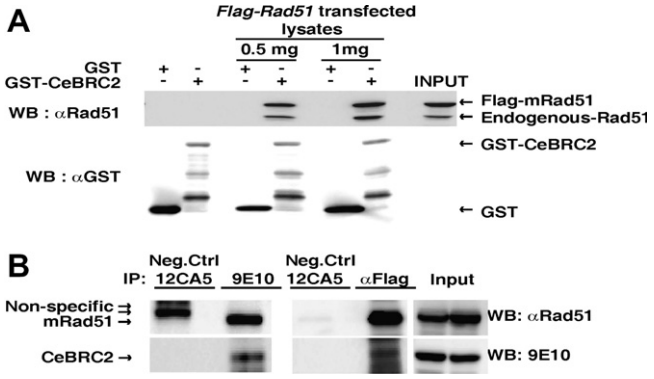

Fig. 1. CeBRC-2 binds to mouse Rad51 *in vitro* and *in vivo*. (A) Purified recombinant GST-CeBRC-2 (4 μg) or GST (1.3 μg) bound to Sepharose 4B beads were incubated with 0.5 mg or 1 mg of cell lysates prepared 48 h after transfection with 5 μg of *Flag-mRad51*-encoding plasmids. Samples were subjected to Western blot (WB) analysis with anti-Rad51 antibodies. Endogenous mRad51, as well as transfected Flag-tagged Rad51, was detected in the Western blot. One-twentieth of the total cell lysate (input) was included for control. The same blot reprobed with anti-GST antibodies shows the expression levels of purified GST or GST-CeBRC-2 used in the GST-pulldown assay. (B) Interaction between CeBRC-2 and mRad51 was confirmed by co-immunoprecipitation followed by Western analysis. 293T cells were cotransfected with 5 μg of expression plasmids encoding *Flag-mRad51* and *myc-CeBRC-2*. Forty-eight hours posttransfection, cell lysates were subjected to immunoprecipitation (IP) with anti-myc antibody (9E10), and WB with anti-Rad51 antibodies (left panel). IP with anti-HA antibody (12CA5) was used as a negative control. One-twentieth of total cell lysates was included for transfection and expression control (input). Reciprocal immunoprecipitation with anti-Flag antibodies, and WB with 9E10 confirmed the interaction between CeBRC-2 and mRad51 (right panel).

Next, we asked whether CeBRC-2 could bind to mRAD51 *in vivo* under physiological conditions. Although it had been shown previously that CeBRC-2 and Rad51 from *C. elegans* are associated [8], whether CeBRC-2 can bind to mammalian Rad51 and regulate it has never been documented. To this end, myc-tagged *CeBRC-2* in a mammalian expression vector was cotransfected with *Flag-mRAD51*-encoding plasmids into 293T cells, and subjected to immunoprecipitation followed by Western analysis. Our results showed that mRad51 precipitated with the CeBRC-2 immune complex, and reciprocally, CeBRC-2 was present in the mRAD51 immune complex (Fig. 1B).

The BRC motif in CeBRC-2, responsible for binding Rad51 in *C. elegans* [8,15,16] , has a 20% average identity with each human BRC repeat, whereas human and mouse BRC repeats share over 70% identity (Supplementary information). In the C-terminus, 1 OB fold is found in CeBRC-2, whereas BRCA2 has 3 OB folds and an HTH motif, to which it has very limited homology [7,8]. The extreme C-terminus of exon 27 of BRCA2, which is absent from CeBRC-2, is essential in the formation of Rad51 nucleoprotein filaments on damaged DNA and inhibits the disassembly of Rad51 oligomers by BRC repeats [11,17]. This raises the question of whether the function of CeBRC-2 is similar to that of BRCA2 in the regulation of Rad51-mediated HR [9,18–20]. That CeBRC-2, with its single BRC motif, bound to mRAD51 *in vitro* and *in vivo* allowed us to investigate further and ask whether CeBRC-2 complemented mammalian BRCA2.

BiFC analysis reveals the direct interaction between CeBRC-2 and mRad51 under physiological conditions

Due to the unmanageable size of mammalian BRCA2, the visualization of BRCA2 and Rad51 operation, however, has not been reported. Therefore, we asked whether, we could extend our understanding of the role of BRCA2 in regulating Rad51-mediated DNA repair by visualizing the interaction. With BiFC analysis, we were especially interested in determining the specific interaction of CeBRC-2 and mRad51; e.g., whether they formed the repair foci reminiscent of functional Rad51-mediated DNA recombinational repair, which depends on the presence of intact BRCA2 [9,18,21–24]; if DNA damage signals influenced the interaction; and how different domains of BRCA2 regulated Rad51 loading and filament formation at damaged DNA sites *in vivo*. For these questions, we utilized a recently developed BiFC system derived from Venus, in which the BiFC efficiency is 13-fold higher than YFP and requires less incubation time [25].

We wanted to understand the evolutionarily conserved role of BRCA2 in Rad51 regulation. Therefore, instead of using *C. elegans* Rad51, we adopted mRad51 for the BiFC assay with CeBRC-2 in 293T cells. When CeBRC-2-VN173- and mRad51-VC155-encoding plasmids (Fig. 2A) were expressed in 293T cells (Fig. 2B and C), fluorescence was readily observed in interphase cells in the form of punctuate nuclear foci (Fig. 2D). When the N-terminal fragment of VN173 and the corresponding VC155 BiFC vectors were transfected individually, they did not fluoresce (Fig. 2C), confirming that the assay was functional. Because the interaction between CeBRC-2 and mRad51, visualized as Venus fluorescence, was in the form of foci, we reasoned that the binding between CeBRC-2 and mRad51 was recapitulating the interaction in mammalian cells [18].

Next, we asked whether the strength, location, or the number of interactions between CeBRC-2 and mRad51 altered upon DNA damage. Cells transfected with the plasmids in Fig. 2A (Fig. 2A and B) were challenged with the radiomimetic drug doxorubicin (Fig. 2E), γ-irradiated (Fig. 2F), or treated with mitomycin C (MMC) (Fig. 2G). The results showed that the intensities of bimolecular fluorescence increased markedly upon all types of DNA damage, and they formed significantly larger nuclear complexes (Fig. 2E and F). When we exposed the cells to increasing doses of irradiation, the interaction affinity was not found to be dose-dependent (Fig. 2H).

The action of different CeBRC-2 domains mirrors mammalian BRCA2 in regulating Rad51-mediated DNA repair

Next, we wanted to understand the mode of interaction between BRCA2, Rad51, and damaged DNA. To achieve this aim, we made 2 deletion constructs of CeBRC-2: 1 with the BRC motif and the first NLS, and 1 with the OB fold and the second NLS (Fig. 3A). Using these 2 mutant constructs, including the full-length CeBRC-2, in BiFC assays, we asked which domain was responsible for mRad51 binding, and which domain was responsible for transporting Rad51 into the nucleus and to DNA.

Full-length CeBRC-2 and mRad51 interacted inside the nucleus as distinct foci (Fig. 3A(a)), as revealed by BiFC. The BRC motif-containing CeBRC-2 deletion mutant bound to mRad51 in the cytosol, but not inside the nucleus, despite the retention of 1 NLS (Fig. 4B). In comparison, the OB fold and the second NLS in CeBRC-2 did not interact with mRad51 (Fig. 3B(c)). It has been suggested that BRCA2 is required for Rad51 to be able to enter the nucleus—because Rad51 does not have an NLS—and localize to damaged DNA sites [7,9,11,15,17]. Our results confirmed the previous report and show further that with the BRC motif alone and 1 NLS, CeBRC-2 was not able to transfer Rad51 inside the nucleus; yet, this action required the OB fold and the second NLS for entry and targeting to DNA (Fig. 3(C)). This mechanism of interaction might mirror the behavior of mammalian BRCA2 in regulating Rad51.

With the information obtained on the interaction of the BRCA2-Rad51-DNA complex, we asked whether CeBRC-2 could complement the defects in mouse BRCA2-deficient cells, as it has been shown that BRCA2-deficient cells are devoid of Rad51-mediated recombinatorial foci after

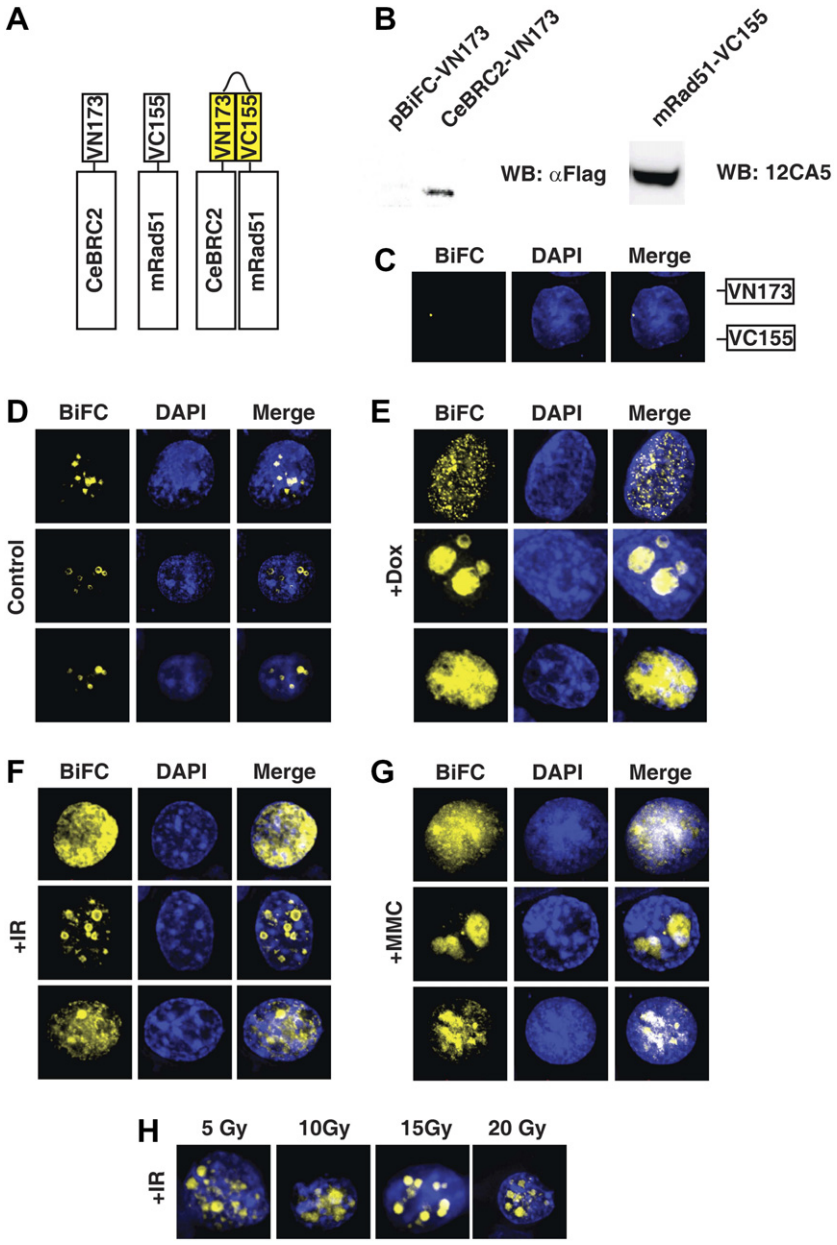

Fig. 2. BiFC analysis reveals that binding between CeBRC-2 and mRad51 is localized to distinct spots in the nucleus, and the binding affinity increases upon genotoxic insults. (A) Schematic illustration of the BiFC assay. Amino acids 1–173 of VN173 were fused to CeBRC-2 at the C-terminus, and amino acids 155–238 of the VC155 were fused to the C-terminus of mRad51. (B) Expression of CeBRC-2-VN173 and mRad51-VC155 were verified by Western blot analysis with anti-Flag or anti-HA (12CA5), respectively. VN173 plasmid has a Flag tag at the N-terminus, and VC155 has an HA tag at the N-terminus. (C) 293T cells transfected with the plasmids encoding VN173 and VC155 vector alone. (D) Interaction between CeBRC-2 and mRad51 was analyzed by cotransfection of CeBRC-2-VN173 with mRad51-VC155 plasmids in 293T cells. Binding between CeBRC-2 and mRad51 was confirmed with yellow fluorescence (control). (E–G) Association between CeBRC-2 and mRad51 after cells encounter DNA damage. Cells were treated with 2.4 μM adriamycin (E, +Dox) for 4 h; γ-irradiation (F, +IR, 15 Gy,); or 10 μM mitomycin C (G, +MMC) for 4 h. (H) BiFC analysis with increasing doses of DNA damage. Cells cotransfected with CeBRC-2-VN173 and mRad51-VC155 were challenged with increasing doses of γ-irradiation.

DNA damage [22,24,26]. To determine whether introducing a CeBRC-2 expression plasmid would restore Rad51 foci after DNA damage in BRCA2-deficient cells, we examined BRCA2-deficient mouse fibroblasts transformed with mutant p53 ($Brca2^{Tr/Tr}$-R273L) [27].

Before introducing CeBRC-2, $Brca2^{Tr/Tr}$-R273L cells did not show any Rad51-positive foci before or after 10 Gy of γ-irradiation (Fig. 4A). When *GFP-CeBRC-2*-encoding plasmids were transfected, Rad51-positive foci, immunostained with anti-Rad51 antibodies, were visible after irradiation in GFP-positive cells. Many Rad51 foci merged with GFP-CeBRC-2 after irradiation, albeit the Rad51 foci were noticeably smaller than the GFP-CeBRC-2 signals. This may be due to inefficiency of anti-Rad51 antibody immunostaining, or it might reflect the formation of CeBRC-2 homo-oligomers inside the cells

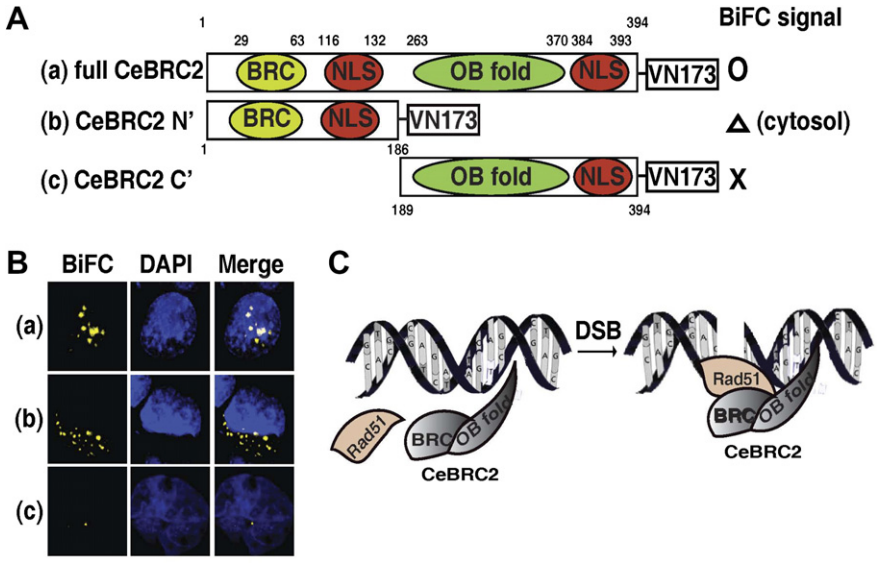

Fig. 3. Binding between CeBRC-2 and mRad51 at nuclear foci requires both the BRC and OB fold domain in addition to the NLS in CeBRC-2. (A) Schematic of CeBRC-2 deletion constructs for BiFC analysis with mRad51. Interaction between mRad51 and different deletion constructs are summarized at the right. (B) Result of BiFC analysis using the constructs. (C) Postulated model for the interaction between BRCA2 and Rad51 in relation to DNA damage.

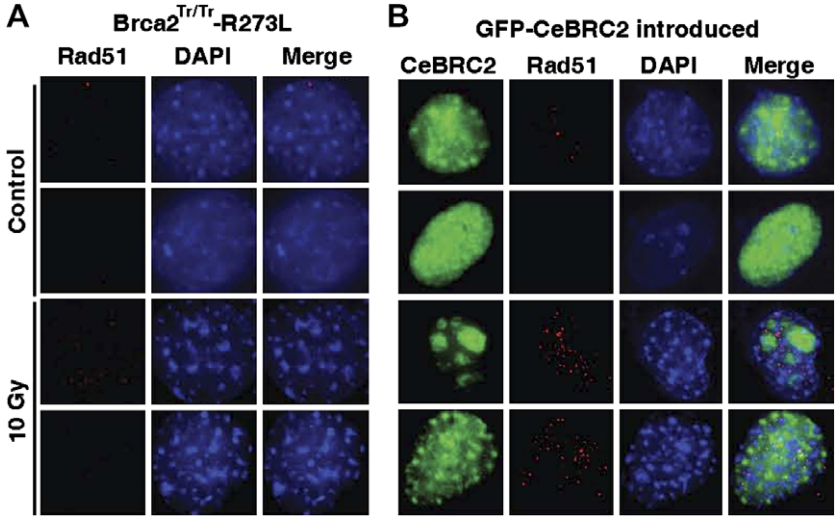

Fig. 4. Introduction of CeBRC-2 into BRCA2-deficient MEFs restores Rad51 foci upon γ-irradiation. Brca2$^{Tr/Tr}$-R273L cells were transfected with 5 μg of *GFP-CeBRC-2*-encoding plasmids. Cells were then either irradiated (10 Gy) or left alone for 8 h and subjected to immunostaining with anti-mRad51 antibodies. (A) Before transfection of *GFP-CeBRC-2*. (B) Introduction of *GFP-CeBRC-2* is detected as distinct green fluorescent spots in BRCA2-deficient MEFs. Rad51 foci are visible (red).

(Fig. 4B). These results strongly suggest that CeBRC-2, with a single BRC motif, can complement the function of mammalian BRCA2 in regulating Rad51-mediated DNA recombination.

Discussion

Petalcorin and colleagues [16] had shown recently that the conserved BRC motif in CeBRC-2 (amino acids 21–50) bound to monomeric Rad51, but the less-conserved sequences in the BRC motif (amino acids 60–89) bound and stabilized Rad51-DNA filaments, using peptides. In our assay, the first 186-amino acid N-terminal region of CeBRC-2 was not capable of entering the nucleus and localizing to DNA, although it contained all the sequences analyzed by Petalcorin and colleagues [16]. From our BiFC, we speculate that the OB fold may be required for targeting to DNA. We do not think that our data contradict the aforementioned report. Rather, combining our data and that of previous reports, we speculate that the conserved BRC motif binds monomeric Rad51, the second NLS shuttles the complex inside the nucleus, the OB fold recognizes single-stranded and broken DNA, and the less-conserved BRC motif (amino acids 60–89) binds to

Rad51-DNA and stimulates Rad51 oligomerization on DNA, inhibiting the disassembly of Rad51 by the conserved BRC motif. This sequence, mediated by different CeBRC-2 motifs with regard to Rad51 foci formation, might mirror the behavior of mammalian BRCA2 in the regulation of homologous recombination.

Then, what is the role of the OB fold in BRCA2? The OB fold is shared among many single-stranded DNA-binding proteins. Of those, RPA is the primary protein that binds to single-stranded DNA without sequence specificity [28]. It has been suggested from structural studies that BRCA2 replaces RPA; when DNA damage takes place, the analogous BRCA2 OB folds replaces RPA, then loads Rad51 on damaged DNA for error-free repair [7]. When this function is lost, legitimate repair can not take place, and mutations can arise, leading to cancer or death, hallmarks of BRCA2-deficient cells.

Our work has provided insight as to how full-length BRCA2 might operate in regulating Rad51 in response to DNA damage. With BiFC analysis, we demonstrated how BRCA2 and Rad51 cooperate inside and outside the nucleus in response to genotoxic stress *in vivo*. Most importantly, we have shown that Rad51-mediated repair foci can be restored when CeBRC-2 is introduced into BRCA2-deficient cells. This result strongly suggests that CeBRC-2 is indeed the ortholog of mammalian BRCA2. Future studies should explore whether CeBRC-2 is also involved in the regulation of cytokinesis [29,30], mitosis [27,31], and even unknown functions of human BRCA2.

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.2007.08.083.

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