BRCA2 C-terminal RAD51-binding Domain Confers Resistance to DNA-damaging Agents

Yasunaga Yoshikawa (✉ yyoshika@vmas.kitasato-u.ac.jp)

Kitasato University

Masami Morimatsu

Hokkaido University

Arisa Tanaka

Kitasato University

Ryo Morioka

Kitasato University

Koichi Orino

Kitasato University

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Abstract

Breast cancer type 2 susceptibility (BRCA2) protein is crucial for initiating DNA damage repair after chemotherapy with DNA interstrand cross-linking agents or X-ray irradiation. These treatments induce double-stranded breaks in genomic DNA. BRCA2 contains a C-terminal RAD51-binding domain (CTRBD) that interacts with RAD51-oligomer containing nucleofilaments. RAD51 nucleofilaments are essential for the homologous recombination repair of double-stranded DNA damage. In this study, we investigated the effects of expressing the CTRBD in cells exposed to X-ray irradiation and mitomycin C treatment. Surprisingly, expressing the BRCA2 CTRBD in HeLa cells increased their resistance to X-ray irradiation and mitomycin C. To determine the ability of the BRCA2 CTRBD to mediate DNA damage repair, the endogenous BRCA2 was depleted by shRNA. No significant differences were observed between the sensitivities of the BRCA2-depleted cells with or without expressing BRCA2 CTRBD. Thus, the resistance to X-ray irradiation conferred by the exogenous CTRBD required endogenous BRCA2 expression. To the best of our knowledge, our study is the first to report the ability of the BRCA2 functional domain to confer resistance to X-ray irradiation and mitomycin C treatment. This peptide may be useful in the protection of cells against X-ray irradiation or chemotherapeutic agents.

Introduction

Tumor therapy includes X-ray irradiation or chemotherapy, both of which induce double-strand breaks (DSBs) in DNA. Although these treatments are directed toward tumor cells, they often have harmful side effects on normal cells. Thus, improved tumor therapies aim to increase tumor cell death and reduce normal cell death^(1,2). To modulate these undesirable side effects, radiation and chemotherapy sensitizers and protectors have been developed. Preventing or enhancing the DNA repair pathways is a popular attribute used for developing sensitizers or protectors, respectively. DSBs are repaired by two major DNA repair pathways, homologous recombination (HR) repair and non-homologous end joining (NHEJ), which maintain the integrity of genomic DNA³. While NHEJ is error-prone, HR repair is a high-fidelity, template-dependent repair mechanism, that is, it uses the intact genome information from the sister chromatids replicated during the S phase. Moreover, HR can contribute to DNA interstrand crosslinks (ICLs), which can be induced by certain antitumor agents, such as cisplatin or mitomycin C (MMC)⁴. Therefore, controlling HR repair efficiency has potential application in developing chemotherapy sensitizers or protectors, as some chemicals that decrease or increase DNA repair efficiency have been developed in this regard previously^{5,6}.

The DNA recombinase RAD51 and its regulator BRCA2 participate in HR repair⁷. When the function of either protein is impaired, cells become highly susceptible to DSB- and ICL-inducing treatments⁸⁻¹⁰. To regulate the HR repair activity of RAD51, BRCA2 carries two RAD51-binding domains: one central domain featuring eight BRC repeats and a C-terminal RAD51-binding domain containing a phosphorylation site^{11,12} (Fig. 1A). Although the RAD51-binding ability varies among these eight BRC repeats, the interaction between any BRC repeat and a RAD51 monomer facilitates the loading of RAD51 onto single-stranded

DNA, thereby forming a nucleofilament—a complex composed of a RAD51 oligomer and single-stranded DNA—representing the first step in HR repair¹². The C-terminal RAD51-binding domain functions in HR repair by stabilizing the RAD51 oligomers and nucleofilaments^{13,14}. The RAD51-binding activity of this domain is controlled by phosphorylation at Ser3291 and Thr3387, that is, a phosphorylated BRCA2 C-terminus does not interact with RAD51 unlike a dephosphorylated domain^{12,15}.

Notably, BRC repeats have been reported to inhibit RAD51 nucleofilament formation in cells and *in vitro* and is thus considered to be a promising drug target candidate^{16–18}. However, the potential therapeutic application of the C-terminal RAD51-binding domain remains unknown.

We hypothesized that the expression of the exogenous C-terminal RAD51-binding domain, which interacts with the RAD51 oligomer, would effectively interfere with HR repair. In this paper, we first assessed peptides around the BRCA2 C-terminus strongly that interacted with RAD51. Then, we tested the sensitivity to DNA-damaging treatments, particularly in X-ray irradiation and MMC treatments of cells expressing the BRCA2 C-terminal RAD51-binding domain. Unexpectedly, the BRCA2 C-terminal RAD51-binding domain conferred resistance to X-ray irradiation and MMC treatment, and the BRCA2 C-terminal RAD51-binding domain-expressing cells exhibited an increased HR repair efficiency. These results indicate that BRCA2 C-terminal RAD51-binding domain peptides that increase HR repair efficiency could be utilized as protective agents against radiation- or chemotherapy-induced DNA damage.

Results

Effects of phosphorylation sites and C-terminal flanking region of C-terminal RAD51-binding domain on RAD51 interaction

A few studies have reported the key residues that are important in the interaction between the BRCA2 C-terminal RAD51-binding domain and RAD51^{12,15,22}. Moreover, the interaction of the C-terminal RAD51-binding domain with RAD51 is known to be regulated by Ser3291 and Thr3387^{12,15}. However, there are currently no integrated reports on this interaction. To identify candidate sensitizer peptides, we reassessed the mutations that influenced the RAD51-binding activity. We tested the RAD51-interacting activity of the BRCA2 C-terminal region (3260–3418 aa) containing the C-terminal RAD51-binding domain by coimmunoprecipitation. The BRCA2 C-terminal region interacted with RAD51, and this interaction was enhanced by the T3387D or T3387E phosphorylation-mimicking mutants. However, this finding was not observed with the T3387A mutation, which disturbed the phosphorylation process (Fig. 1B). Then, we examined the effect of the C-terminal region flanking region of the C-terminal RAD51-binding domain. The immunoprecipitated peptides of the shortened C-terminal RAD51-binding domain had a lower yield than those of the C-terminal region, but the yields of the coimmunoprecipitated RAD51 were comparable (Fig. 1C). Thus, the RAD51-interacting activity of the shortened peptide was stronger than that of the whole C-terminus. Using the mammalian two-hybrid assay, we then assessed three mutants of the C-

terminal RAD51-binding domain in which S3291 was either substituted by cysteine (S3291C) or glycine (S3291G) or was deleted (delS3291) to prevent phosphorylation on this site (Fig. 1D). The P3292L mutation was used as the positive control because this mutation was reported to abolish the phosphorylation motif for cyclin-dependent kinase (CDK) and the constitutive interaction with RAD51²². RAD51-interacting activity was abolished in all three S3291 mutants, like in the phosphorylation-mimicking S3291E mutant, which was reported to have no interaction with RAD51. The P3292L mutant interacted with RAD51, but its interacting activity was slightly weaker than that of the wildtype as previously reported²². We also performed coimmunoprecipitation assay to investigate the characteristics of the P3292L mutant (Fig. 1E). The expression of the P3292L mutant was low compared to that of the wildtype or the S3291E mutant; however, its interaction with RAD51 persisted.

C-terminal RAD51-binding domain, but not BRCA2 C-terminus, confers resistance to X-ray irradiation and MMC treatment

We then tested the effects of exogenously expressed C-terminal RAD51-binding domain on the sensitivity of cells to X-ray irradiation and MMC treatment using HeLa cells that stably expressed the FLAG-HA-fused C-terminal RAD51-binding domain. The expression of the C-terminal RAD51-binding domain containing the P3292L mutation was lower than that of the wildtype and the S3291E mutant (Fig. 2A). Compared to empty vector-transduced HeLa cells, wild-type and P3292L-mutated C-terminal RAD51-binding domain-expressing cells exhibited a significantly increased resistance to X-ray irradiation and MMC treatment, but the increased resistance was not observed in the S3291E mutation-expressing cells (Fig. 2B and C). This resistance to X-ray irradiation was also exhibited by HepG2 cells (Supplementary Fig. S1)

We also tested the sensitivity of BRCA2 C-terminus-expressing HeLa cells to X-ray irradiation and MMC treatment (Fig. 2D–F). Cell survival analysis showed that the sensitivity to the treatments was comparable between the BRCA2 C-terminus- and FLAG-HA-tag-expressing cells (Fig. 2E and F).

Resistance to X-ray irradiation conferred by C-terminal RAD51-binding domain depends on endogenous BRCA2

The BRCA2 C-terminal RAD51-binding domain with the P3292L peptide exhibited the strongest interacting activity among all peptides assessed. However, the expression of this mutant was too low and was difficult to increase. Wild-type BRCA2 C-terminal RAD51-binding domain-expressing cells exhibited a phenotype that was comparable with that of P3292L peptide-expressing cells. Thus, we decided to use the wild-type peptide of the BRCA2 C-terminal RAD51-binding domain for subsequent experiments. We then investigated whether the C-terminal RAD51-binding domain alone was capable of conferring resistance to DNA damage, or whether endogenous BRCA2 was required. An shRNA knockdown of BRCA2 was performed in empty vector-transduced HeLa cells and HeLa cells expressing wild-type or S3291E-mutated C-terminal RAD51-binding domain (Fig. 3A), and the sensitivity of these cells to X-ray irradiation or MMC treatment was evaluated. Compared to scramble shRNA and empty vector-transduced

HeLa cells, wild-type C-terminal RAD51-binding domain-expressing HeLa cells exhibited a significantly increased resistance to X-ray irradiation and MMC treatment, but the cells expressing the S3291E-mutated C-terminal RAD51-binding domain did not (Fig. 3B and C). Both BRCA2-knockdown strains—with or without the overexpression of wild-type or S3291E-mutated C-terminal RAD51-binding domain—exhibited a decreased resistance to X-ray irradiation and similar sensitivity levels (Fig. 3B and C).

Effects of exogenous expression of BRCA2 C-terminal RAD51-binding domain on HR efficiency

The exogenous expression of the BRCA2 C-terminal RAD51-binding domain, together with endogenous BRCA2, decreased DNA damage sensitivity. Thus, we expected the efficiency of HR to be increased by the expression of exogenous BRCA2 C-terminal RAD51-binding domain. We tested the HR efficiency using a GFP-based assay²⁶. We generated HeLa cells harboring a DR-GFP construct that expressed wild-type BRCA2 or S3291E-mutated C-terminal RAD51-binding domain, which was reported to lack the capacity to interact with RAD51 (Fig. 4A). We confirmed that the expression of the wild-type C-terminal RAD51-binding domain protected cells against MMC treatment (Fig. 4B). Moreover, HR repair efficiency was increased in wild-type C-terminal RAD51-binding domain-expressing cells, but not in S3291E mutant-expressing or empty vector-transduced cells. To further clarify the mechanism underlying the increased efficiency of HR repair, we performed the RAD51 foci formation assay, which is a popular assay for measuring the recruitment efficiency of RAD51 to DNA damage sites. After X-ray irradiation, wild-type C-terminal RAD51-binding domain-expressing cells generated RAD51 foci, similar to empty vector-transduced cells or C-terminal RAD51-binding domain-expressing cells (Fig. 5).

Discussion

We hypothesized that the BRCA2 C-terminal RAD51-binding domain, which interacts with the RAD51 oligomer and nucleofilaments, would interfere with HR repair-like peptides derived from BRC repeats. However, we found that the exogenous expression of the BRCA2 C-terminal RAD51-binding domain conferred resistance to X-ray irradiation and MMC treatment in the presence of endogenous BRCA2. One of the mechanisms involved was the enhancement of HR efficiency by the expression of the BRCA2 C-terminal RAD51-binding domain. The BRCA2 C-terminal RAD51-binding domain interacts with the RAD51 oligomer or RAD51 nucleofilament, forming the HR initiation complex consisting of single-stranded DNA wrapped around the RAD51 oligomer^{13,14}. Although the C-terminal RAD51-binding domain enhances RAD51 nucleofilament formation *in vitro*, the mechanism underlying the regulation of RAD51 nucleofilament formation and maintenance *in vivo* remains unclear. Wild-type C-terminal RAD51-binding domain-transduced cells exhibited similar RAD51 foci formation activity as empty vector-transduced cells. We speculated that the expression of the C-terminal RAD51-binding domain in cells with intact BRCA2 would improve the efficiency of RAD51 nucleofilament formation and HR repair after X-ray irradiation or MMC treatment, resulting in resistance to these treatments. HeLa and HepG2 cells expressing the S3291E mutant, whose RAD51-interacting activity was abolished, exhibited no resistance

to MMC treatment, and this result is consistent with our hypothesis. When the expression of endogenous BRCA2 was downregulated, the sensitivity to X-ray irradiation did not differ between empty vector-transduced and wild-type C-terminal RAD51-binding domain-transduced HeLa cells. Thus, the C-terminal RAD51-binding domain alone is insufficient to recruit a critical number of RAD51 molecules to DSB sites and mediate DNA repair.

We also determined the C-terminal RAD51-binding domain expression in HeLa cells. Comparing the wildtype and mutants (P3292L or S3291E), the P3292L mutant that induced a greater increase in RAD51-interacting activity than the wildtype, exhibited a lower expression of the C-terminal RAD51-binding domain than the wildtype, whereas the S3291E mutant without RAD51-interacting activity exhibited a higher expression. These results showed that the quantity and quality of significant RAD51-interacting activity were harmful to cells and that moderate RAD51-interacting activity was required to resist DNA damage. This implication should be an important consideration in the application of such a peptide to molecular drug development.

The full-length BRCA2 C-terminus (3260–3418 aa), which includes the C-terminal RAD51-binding domain, had no effect on the sensitivity of the cells to X-ray irradiation and MMC treatment. The RAD51-interacting activity of the BRCA2 C-terminus was lower than that of the shortened peptide (3260–3331 aa), suggesting that the extended region at the C-terminus, which possesses a phosphorylation site (Thr3387) that is activated by the checkpoint kinases Chk1 or Chk2 ¹⁵, does not directly interact with RAD51, but it reduces the affinity of BRCA2 for RAD51. In a previous study, the RAD51-interacting activity was decreased by phosphorylation or phosphorylation-mimicking mutations at Thr3387. However, unexpectedly, we detected an increased RAD51-interacting activity in the presence of the phosphorylation-mimicking mutations T3387D and T3387E, but not T3387A. The greatest difference between previous studies and ours lies in the transfected cell lines involved. In a previous study, the researchers used breast cancer-derived MCF7 cells; here, we used cervical cancer-derived HeLa cells. The difference in the origin of the cancer cells may have affected the role of phosphorylation at Thr3387 in the C-terminal RAD51-binding domain in the cell lineage ¹⁵. Although we did not investigate further, we surmised that this process involving phosphorylation at Thr3387 is likely the mechanism by which the C-terminal RAD51-binding domain is regulated by the C-terminal region of the domain.

The interaction between RAD51 and the C-terminal RAD51-binding domain is known to be regulated by the phosphorylation status at Ser3291 ¹². Thus, we assessed Ser3291 mutations that eliminated phosphorylation, resulting in a continuous interaction with RAD51. Previous reports have indicated that glycine or alanine substitutions can abolish RAD51-interacting activity ¹². Here, we also explored serine deletion and substitutions with cysteine, which is often used as a non-phosphorylated structural analog of serine. None of the mutations we investigated led to RAD51 interaction. Hence, Ser3291 on the C-terminal RAD51-binding domain is a crucial amino acid residue mediating the interaction between the C-terminal RAD51-binding domain and RAD51.

In this study, we unexpectedly found that expressing the BRCA2 C-terminal binding domain in the presence of intact BRCA2 increased HR efficiency and increased resistance to X-ray irradiation and MMC treatment. Although our work is currently limited to *in vitro* data, it provides substantial evidence for the potential applications of the BRCA2 C-terminal RAD51-binding domain in cell protection during radiation therapy and/or chemotherapy as a molecular drug that enhances HR activity.

Materials And Methods

Cell culture, antibodies, and generating cell lines

HeLa, HepG2, and HEK293T cells (RIKEN, Tokyo, Japan) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The following antibodies were used for western blotting: anti-RAD51 (dilution 1:500 H-92; Santa Cruz Biotechnology, Dallas, TX, USA) anti-BRCA2 (dilution 1:250 OP-95; Merck, Darmstadt, Germany), anti-Lamin B1 (dilution 1:1000 PM064; MBL, Nagoya, Japan), anti-FLAG (dilution 1:1000 M2; Merck), anti- α -Tubulin (dilution 1:1000 M175-3; MBL), and anti-GFP (dilution 1:1000 598; MBL).

FLAG-HA- or FLAG-HA-EGFP-fused BRCA2 C-terminus (3260–3418 aa) and FLAG-HA-fused C-terminal RAD51-binding domain (3260–3331 aa) were stably expressed using the pOZ-N plasmid¹⁹. shRNA-mediated knockdown of BRCA2 was achieved by expressing the target sequence 5'-TACAATGTACACATGTAACAC-3' in the pLKO.1 vector, which was donated by David Root (Addgene plasmid no. 10878) and scramble shRNA, which was donated by David Sabatini (Addgene plasmid no. 1864)^{20,21}. Transfections of plasmid DNA into HEK293T cells were conducted using FuGENE HD (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Immunoprecipitation

Immunoprecipitation of the FLAG tag-fused peptides was performed as previously reported²². In brief, HeLa cells that stably expressed the BRCA2 C-terminus or C-terminal RAD51-binding domain were washed with phosphate-buffered saline, harvested by scraping, and centrifuged for 5 min at 500 $\times g$. The pelleted cells were resuspended at 4°C with an equal volume of Benzonase buffer (20 mM Tris-HCl, pH 8.0; 50 mM KCl; 2 mM MgCl₂; 0.5% Triton X-100; 10% glycerol; and 200 U/ml Benzonase) to digest the genomic DNA. The resuspended cells were subsequently treated with a 10 $\times$ IP buffer (20 mM Tris-HCl, pH 8.0; 150 mM KCl; 2 mM MgCl₂; and 10% glycerol) to extract the proteins. The extracts were centrifuged at 17,000 $\times g$ for 10 min at 4°C, and the supernatants were combined with FLAG M2 affinity agarose (Merck) and incubated for 3 h at 4°C. The immunoprecipitates were recovered by centrifugation for 5 min at 500 $\times g$ and washed twice with the IP buffer. Finally, the samples were eluted into 50 μ l of 1 $\times$ LDS-loading buffer (Thermo Fisher Scientific, Waltham, MA, USA).

Mammalian two-hybrid assay

Mammalian two-hybrid assay was performed as previously described²³. Briefly, coding regions of the BRCA2 C-terminal RAD51-binding domain and RAD51 (GenBank ID: NM_000059 and NM_002875) were cloned into pM and pVP16 plasmids (Clontech, Mountain View, CA, USA), respectively. Approximately 2×10^5 cells were seeded in 24-well plates and co-transfected with 50 ng pVP, 50 ng pM, 100 ng pGluc, and 10 ng pRL-TK (Promega). The cells were harvested at 48 h after transfection, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The transfection efficiency was normalized to the measured Renilla luciferase activity using pRL-TK. The methods for generating the luciferase reporter plasmid pGluc have been described previously²⁴.

Clonogenic assay

To evaluate the effects of X-ray irradiation, cells were irradiated using an MX-80Labo (mediXtec, Chiba, Japan) at a dose of 1–6 Gy. Then, 100–4,000 cells per well were seeded in 6-well plates. For MMC treatment, 200–400 cells per well were seeded in 6-well plates and treated with 0.25–4 ng/mL MMC the following day. Colony formation was assessed after 10–14 days using 1% (w/v) crystal violet in methanol.

HR assay

HeLa cells were transfected with the pDRGFP plasmid for HR efficiency quantification. The transfected cells were treated with 2 µg/mL puromycin and cloned to cell lines that become GFP-positive after transfection of pCBAScel, the plasmid encoding I-SceI endonuclease. The pDRGFP and pCBAScel plasmids were donated by Maria Jasin (Addgene plasmid nos. 26475 and 26477, respectively)^{25,26}.

The C-terminal RAD51-binding domain or pOZ vector-transduced DR-GFP-harboring HeLa cells were generated using the method described above. These cells were seeded in 6-well plates and were co-transfected with 900 µg of pCBAScel and 100 µg of the DsRed monomer-encoding plasmid (Takara Bio, Shiga, Japan). The same batch of plasmid mixture was used throughout. Four days after transfection, the numbers of GFP-positive and DsRed-positive cells were determined by flow cytometry using CytoFLEX (Beckman Coulter, Brea, CA, USA). DsRed-positive cells were used to normalize transfection efficiency. For each sample, a minimum of 20,000 cells was analyzed.

Immunostaining and microscopy

HeLa cells were cultured on coverslips (Matsunami Glass, Osaka, Japan) and were irradiated using an MX-80Labo (mediXtec) at a dose of 10 Gy. After 6 and 24 h, the irradiated cells were harvested and fixed with 4% paraformaldehyde. After permeabilization with 0.3% Triton X-100 in phosphate-buffered saline, the cells were incubated with anti-RAD51 antibody (dilution 1:500 H-92; Santa Cruz Biotechnology), followed by the Alexa Fluor-568-conjugated goat anti-rabbit IgG (dilution 1:1000; Molecular Probes). Cell nuclei were stained with Hoechst 33342 (Molecular Probes); RAD51 foci were examined under a fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Cells with more than five RAD51 foci were considered RAD51 foci-positive cells.

Statistical analysis

Statistical analyses of the mammalian two-hybrid assay and HR assay were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test or Tukey's HSD test using the GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). Statistical analyses of the clonogenic assay and RAD51 foci formation assay were performed using an F-test followed by Student's *t*-test with Holm's correction using Excel software (Microsoft, Redmond, WA, USA). Statistical significance was set at $p < 0.05$ for all statistical analyses.

Declarations

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Authors' contributions

YY, MM, and KO conceived and designed the experiments. YY, AT, and RY participated in material preparation, data collection, and analysis. YY wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Availability of materials and data

The datasets used and analyzed in the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

RAD51-interacting activity of BRCA2 C-terminal RAD51-binding domain. (A) Schematic diagram of the BRCA2 protein and its C-terminal RAD51-binding domain. BRCA2 has two major sites to interact with RAD51, the BRC repeats and the C-terminal RAD51-binding region. Three nuclear localization signals and two phosphorylation sites (S3291 and T3387) are also indicated. (B) RAD51 interaction with wild-type BRCA2 C-terminus (3260–3418 aa) and its phosphorylation-mimicking (T3387D and T3387E) and

phosphorylation-eliminated (T3387A) mutants were assessed by coimmunoprecipitation. Lysates from these BRCA2 C-terminus-expressing HeLa cells were immunoprecipitated with an anti-FLAG antibody. The immunoprecipitated samples were analyzed by western blotting using anti-FLAG, anti-RAD51, and anti-LaminB antibodies (anti-LaminB antibody was used as the negative control). Asterisks indicate IgG light chain bands. (C) RAD51-interacting activity of BRCA2 C-terminus (3260–3418 aa) and C-terminal RAD51-binding domain (3260–3331 aa) was compared by coimmunoprecipitation. BRC repeat 4 was used as the positive control. Lysates from BRCA2 C-termini-expressing HeLa cells were immunoprecipitated with an anti-FLAG antibody. The immunoprecipitated samples were analyzed by western blotting using anti-FLAG, anti-RAD51, and anti-LaminB antibodies (the anti-LaminB antibody was used as the negative control). Asterisks indicate IgG light chain bands. (D) Interactions between mutations (S3291C, S3291G, Δ S3291, S3291E, and P3292L) in the BRCA2 C-terminal RAD51-binding domain (3260–3331 aa) and RAD51 were evaluated using a mammalian two-hybrid assay. Lysate luciferase activity was determined 48 h after transfection. Results are represented as the mean, and the error bars indicate the standard deviation ($n = 3$). Significance was examined by one-way analysis of variance and Dunnett's tests. Asterisks indicate significant differences between wild-type C-terminal RAD51-binding domain and the mutants (*: $p < 0.01$). (E) RAD51-interacting activity of wild-type BRCA2 C-terminal RAD51-binding domain (3260–3331 aa) and its phosphorylated mutants (S3291E and P3292L) was assessed by coimmunoprecipitation. Lysates from C-terminal RAD51-binding domain-expressing HeLa cells were immunoprecipitated with an anti-FLAG antibody. The immunoprecipitated samples were analyzed by western blotting using anti-FLAG, anti-RAD51, and anti-LaminB antibodies (the anti-LaminB antibody was used as the negative control). Asterisks indicate nonspecific bands.

Figure 2

BRCA2 C-terminal RAD51-binding domain confers resistance to X-ray irradiation and MMC treatment but not BRCA2 C-terminus. (A) Expression of exogenous FLAG-HA-fused BRCA2 C-terminal RAD51-binding domain (3260–3331 aa) and its mutants (S3291E and P3292L) was evaluated by western blotting using an anti-FLAG antibody. Long exp., long exposure; Short exp., short exposure. (B and C) A clonogenic survival assay of FLAG-HA-fused BRCA2 C-terminal RAD51-binding domain (3260–3331 aa)-expressing

cells treated with X-ray irradiation or mitomycin C is shown. Results are presented as the mean at each X-ray or MMC dose, and the error bars indicate the standard deviation ($n = 3$). Significance was examined by F-test, followed by Student's t-test with Holm's correction. Asterisks indicate significant differences between empty vector-transduced HeLa cells and FLAG-HA-fused wild-type or P3292L-mutated BRCA2 C-terminal RAD51-binding domain-expressing cells (*: $p < 0.05$, **: $p < 0.01$). CTRBD; C-terminal RAD51-binding domain. (D) Expression of exogenous FLAG-HA-EGFP-fused BRCA2 C-terminus (3260–3418 aa) was determined by western blotting using an anti-GFP antibody. (E and F) A clonogenic survival assay of FLAG-HA-EGFP-fused BRCA2 C-terminus- and FLAG-HA-EGFP-expressing cells was performed after treatment with X-ray irradiation or mitomycin C. Results are presented as the mean at each X-ray or MMC dose, and the error bars indicate the standard deviation ($n = 3$). Significance was examined by F-test, followed by Student's t-test with Holm's correction. n.s., not significant; CT, C-terminus.

Figure 3

Resistance to X-ray irradiation conferred by C-terminal RAD51-binding domain expression depends on endogenous BRCA2. (A) Expression of endogenous BRCA2 in empty vector-transduced cells and wild-type or S3291E-mutated C-terminal RAD51-binding domain-expressing cells was determined by western blotting using an anti-BRCA2 antibody in BRCA2-depleted HeLa cells generated by shRNA-mediated knockdown of BRCA2. (B and C) A clonogenic survival assay of BRCA2 depleted cells after X-ray

irradiation or MMC treatment is shown. Results are indicated as the mean at each X-ray or MMC dose, and the error bars indicate the standard deviation ($n = 3$). Significance was examined by F-test, followed by Student's t-test with Holm's correction. Asterisks indicate significant differences between empty vector-transduced HeLa cells and FLAG-HA-fused wild-type or S3291E-mutated BRCA2 C-terminal RAD51-binding domain-expressing cells (*: $p < 0.05$, **: $p < 0.01$). CTRBD; C-terminal RAD51-binding domain.

Figure 4

Expression of C-terminal RAD51-binding domain confers increased homologous recombination repair efficiency. (A) Expression of wild-type or S3291E-mutated C-terminal RAD51-binding domain (3260–3331 aa) in HeLa cells harboring DR-GFP, a GFP-based homologous recombination efficiency-assessing construct, was determined by western blotting using an anti-FLAG antibody. (B) A clonogenic survival assay of wild-type or S3291E-mutated FLAG-HA-fused BRCA2 C-terminal RAD51-binding domain-expressing cells treated with mitomycin C is shown. Results are presented as the mean at each MMC dose, and the error bars indicate the standard deviation ($n = 3$). Significance was examined by F-test, followed by Student's t-test with Holm's correction. Asterisks indicate significant differences between non-transfected HeLa cells or S3291E mutant and wild-type BRCA2 C-terminal RAD51-binding domain-expressing cells (*: $p < 0.05$, **: $p < 0.01$). (C) Homologous recombination efficiency was tested by GFP-based assay. The transfection efficiency was normalized by cotransfection of the DsRed monomer-expressing vector. Significance was examined by F-test, followed by Student's t-test with Holm's correction. Asterisks indicate significant differences (*: $p < 0.05$, **: $p < 0.01$). n.s.: not significant; CTRBD; C-terminal RAD51-binding domain.

Figure 5

Exogenous expression of BRCA2 C-terminal RAD51-binding domain does not affect RAD51 foci formation. Wild-type or S3291E-mutated C-terminal RAD51-binding domain (3260–3331 aa)-expressing cells were irradiated with X-ray at a dose of 10 Gy. These cells were harvested after 6 h and 24 h. Cells with more than five RAD51 foci were considered RAD51 foci-positive cells. Results are presented as the mean, and the error bars indicate the standard deviation (n = 3). Significance was examined by F-test,

followed by Student's t-test with Holm's correction. Results are indicated as the mean at each X-ray or MMC dose, and the error bars indicate the standard deviation ($n = 3$). n.s.; non-significant. CTRBD; C-terminal RAD51-binding domain.

Supplementary Files

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