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Valine 1532 of human BRC repeat 4 plays an important role in the interaction between BRCA2 and RAD51

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## **Abstract**

The breast cancer susceptibility protein BRCA2 is essential for recombinational DNA repair. BRCA2 specifically binds to RAD51 via 8 BRC repeat motifs and delivers RAD51 to double-stranded DNA breaks. In this study, a mammalian two-hybrid assay and competitive ELISA showed that the interaction between BRC repeat 4 (BRC4) and RAD51 was strengthened by the substitution of a single BRC4 amino acid from valine to isoleucine (V1532I). However, the cancer-associated V1532F mutant exhibited very weak interaction with RAD51. This study used a comparative analysis of BRC4 between animal species to identify V1532 as an important residue that interacts with RAD51.

## **Key word**

BRCA2, RAD51, BRC repeat, homologous recombination, canine

## 1. Introduction

Mutations in the breast cancer susceptibility gene *BRCA2* are associated with a predisposition to breast and ovarian cancers [1,2]. The product of this gene, BRCA2 protein, is required for the homologous recombinational repair of DNA double-strand breaks (DSBs) [3,4]. Furthermore, it has been shown to associate with RAD51 recombinase, which is conserved from yeasts to mammals [5,6]. A primary outcome of BRCA2 deficiency is chromosomal instability, which results from a deficiency in the repair of DNA lesions by homologous recombination [7]. The direct interaction of BRCA2 with RAD51 is mediated by 8 highly conserved motifs called BRC repeats that are positioned in exon 11 in human BRCA2 [8,9]. A previous study determined the three-dimensional crystal structure of the human RAD51 (hRAD51)–BRC repeat 4 (BRC4) complex and revealed that the BRC repeats form the binding surface with RAD51 [10].

In female dogs, mammary tumors are the most frequent type of neoplasm [11,12]. It was recently reported that mammary tumor development in dogs is associated with BRCA2 [13]. The cloning of canine BRCA2 and RAD51 [14] facilitated the discovery of the interaction between BRC repeats and the C-terminus of canine BRCA2 and RAD51 [15-17]. Surprisingly, subsequent studies show that canine and feline BRC4 exhibit stronger interactions with RAD51 than human BRC4, which exhibits the strongest interaction among the 8 repeats (Fig. 1A) [18]. Only 4 canine BRC4 amino acids differ from human BRC4 (V1532I, K1533E, A1535T, and D1547E), suggesting that these amino acids could be responsible for strengthening the interaction with RAD51.

Although the human BRC4 amino acid residues 1524-FHTA-1527 and 1545-LFDE-1548 are critical for the interaction with RAD51 [9,19], the functions of other BRC4 residues have not been investigated. Structural studies suggest that V1532 plays a role in

the adherence of hBRC4 to RAD51 via hydrophobic contact with the M210 of RAD51 [10]; however, this has not been demonstrated by functional assays. Moreover, V1532F, a cancer-associated mutation with unknown functional implications, was identified from human breast cancer patients and recorded in the Breast Cancer Information Core Database (BIC; <http://research.nhgri.nih.gov/bic/>) [20].

In this study, we identified novel amino acids that influence the binding intensity between BRC4 and RAD51 by using a mammalian two-hybrid assay, competitive ELISA, and irradiation-induced focus formation of RAD51.

## 2. Materials and methods

### 2.1. Cell culture

HeLa (RIKEN Cell Bank) and COS-7 (American Type Culture Collection) cell lines were maintained in the presence of 5% CO<sub>2</sub> in air at 37°C in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal calf serum, streptomycin (38 mg·L<sup>-1</sup>), and penicillin (50,000 U·L<sup>-1</sup>).

### 2.2. Mammalian two-hybrid assay and binding interference assay

For the mammalian cell two-hybrid (MTH) assay, the coding regions of canine BRC4 (cBRC4, GenBank ID: NM\_001006653), feline BRC4 (fBRC4, GenBank ID: NM\_001009858) [21], human BRC4 (hBRC4, GenBank ID: NM\_000059), and canine RAD51 (cRAD51, GenBank ID: NM\_001003043) were cloned into pM and pVP16 plasmids (BD Biosciences). Amino acid substitution mutants of BRC4 were constructed using polymerase chain reaction (PCR) mutagenesis and subcloned into the pM vector. The MTH was performed as described previously [15]. VP16- and DBD-fused cRAD51 constructs were introduced into HeLa cells with the following interference constructs: wild-type hBRC4 (hBRC4wt), V1532I mutant (V1532I), V1532F, and other mutants (T1526A and G1529R) in NLS-pEGFP-C1 (BD Biosciences).

### 2.3. Enzyme-linked immunosorbent assay (ELISA)

Competitive ELISA experiments using BRC peptides, which are reported to disrupt the hRAD51-BRC4 interaction [9], were performed as outlined in *Supplementary Methods*.

### 2.4. RAD51 focus assay

The RAD51 focus assay using EGFP-fused BRC4 constructs was performed as outlined in *Supplementary Methods*.

## 2.5. *Crystal structure modeling*

We retrieved the crystal structure of the hRAD51-BRC4 complex from the Research Collaboratory for Structural Bioinformatics Protein Data Bank at <http://www.rcsb.org/> (PDB ID: 1N0W) and analyzed it using the University of California, San Francisco (UCSF) Chimera software (<http://www.cgl.ucsf.edu/chimera/>) [22].

### 3. Results

#### 3.1. Structure-based analysis of BRC4 and comparative analysis of the interaction between the BRC4 of 3 species and RAD51

Alignment of the BRC4 of several mammals and chicken revealed that residues 1532-IE-1533 were conserved specifically in the order Carnivora [8] (Fig. 1B). Feline and canine BRC4 have 3 and 4 amino acid residues that differ from hBRC4, respectively. Assessing the interaction between these 3 BRC4 and RAD51 varieties by using MTH revealed that cBRC4 and fBRC4 exhibited stronger associations with cRAD51 than hBRC4 (Fig. 1A).

#### 3.2. Mutation analysis of the interaction between BRC4 and RAD51

To identify the key residue to determine the differences in binding intensity, substitution mutants of canine and feline BRC4, which mimic human BRC4, were constructed by PCR-oriented mutagenesis; the substituted residues are shown in Fig. 2A. Luciferase activity was lower in hBRC4 than in cBRC4 or fBRC4 mutants (Fig. 2A right). A reciprocal analysis was conducted by substituting each of the 4 residues that differed between human and canine BRC4, namely V1532I, K1533E, A1535T, and D1547E (Fig. 2B). The V1532I substitution increased the luciferase activity by approximately 5 times (Fig. 2B), while the other substitutions did not affect the interaction. Immunoblots analyzing the protein expression levels of the hBRC4 and V1532I fusion proteins showed similar expression levels in transfected cells (Fig. 2B).

#### 3.3. The hBRC4 cancer-associated mutation V1532F affects the interaction with RAD51

To examine the influence of the residue substitution from 1532 Val to Phe isolated from individuals with breast cancer, the interaction between hBRC4 mutants and cRAD51 was

129 compared using MTH. The interaction between V1532F mutant and cRAD51 was as weak as  
130 those with T1526A and G1529R, for which functional deficiencies are reported (Fig. 3A) [23].  
131 To verify the results of the functional assay, the mutation tool in the UCSF Chimera software  
132 package was used to analyze the possible structural outcomes of these substitutions (Fig. 3B).  
133 The Rotamers tool allows amino acid side chain rotamers to be viewed and evaluated [22]. The  
134 best rotamers of Val, Ile, or Phe were selected according to their side-chain torsion as well as  
135 probability values in the rotamer library and in the context of the structural environment (Fig.  
136 3C). These calculations revealed that the substitution of V1532I increased the number of  
137 contacts for all kinds of direct interactions, whereas V1532F increased clashes (unfavorable  
138 interactions where atoms are too close together). V1532 showed 2 contacts with M210 of  
139 hRAD51 and no clashes, while I1532 showed 5 contacts with A190 and M210 of RAD51 and  
140 no clashes. On the other hand, F1532 showed 5 contacts with A190 of RAD51 and many  
141 clashes with H1525 or T1526 of hBRC4 (Fig. 3D–F). Three other substitutions—namely  
142 K1533E, A1535T, and D1547E—had minor effects on the interaction because these residues are  
143 opposite the RAD51 binding surfaces (Fig. 3B). The possible structural outcomes of the  
144 previously reported substitutions, T1526A and G1529R, demonstrate changes in the number of  
145 contacts in the  $\beta$ -hairpin structure of BRC4 (Supplementary Fig. 1A–D) [10] that may be related  
146 to the deficiencies of RAD51 binding.

#### 148 *3.4. Molecular mimicry of V1532I enhances the interference activity of both RAD51–RAD51* 149 *and BRC4–RAD51 interactions to a greater extent than that of hBRC4wt*

150 To measure the strength of interference in RAD51–RAD51 interaction, interference  
151 constructs were added to the MTH assay. V1532I inhibited cRAD51–cRAD51 interaction more  
152 significantly than hBRC4wt in a dose-dependent manner (Fig. 4A). On the other hand, the

BRC4 mutants that exhibited very weak interactions with RAD51 (i.e., V1532F, T1526A, and G1529R) did not inhibit cRAD51–cRAD51 interaction (Fig. 4A). To verify the cell biological methods used, we performed another *in vitro* assay: competitive ELISA (Fig. 4B). The mutant peptides containing substitution V1532I inhibited the interaction between plate-coated BRC4 and hRAD51 more strongly than hBRC4wt peptide. In contrast, the V1532F mutant peptide failed to competitively inhibit hRAD51-BRC4 interaction in the ELISA assay (Fig. 4C). In addition, to determine the functional importance of the V1532I substitution in the interaction between BRC4 and endogenous RAD51, we performed a RAD51 focus assay [24,25]. The RAD51 foci were more reduced by V1532I transfection than hBRC4wt transfection (Supplementary Fig. 2A and B).

#### 4. Discussion

This study used structural, cell biological, biochemical, and comparative analyses to investigate the effects of reciprocal amino acid substitutions on the interactions of human and canine BRC4 with RAD51. Although the structures of mammalian RAD51–BRC4 complexes—except that of humans—have not been clarified, RAD51 exhibits very high homology between mammals. For example, canine and human RAD51 differ by only 3 amino acids out of 339. Furthermore, these different residues are not located at an important position on the RAD51–BRC4 binding surface [10,14]. Therefore, referring to the crystal structure of the human RAD51–BRC4 complex is useful for analyzing the interaction between RAD51 and BRC4 in other mammals. The functional analysis of BRCA2 revealed that canine and feline BRC4 interact more strongly with cRAD51 than human BRC4. Two human BRC4 tetrameric motifs, namely 1524-FHTA-1527 and 1545-LFDE-1548, were recently reported to be accommodated in the binding pockets of RAD51 and are therefore essential for the interaction with the RAD51 recombinase [9]. An efficient chimera peptide of BRC repeats was recently constructed in an attempt to improve the anticancer properties of RAD51 inhibitors by selecting the best amino acid residue at each binding position of the BRC repeats [19]. However, V1532, which was identified in this study, had not been identified previously as an important residue for binding with RAD51. The single amino acid substitution of Val 1532 to Ile in BRC4, which is specifically conserved in Carnivora and differs by only one methyl group, remarkably enhanced the interaction between BRC4 and RAD51. Crystallographic analysis revealed that V1532 exists in the interacting interface of the BRC4–RAD51 complex and that this interface of BRC4 mimics the oligomerization motif of RAD51 [10,26,27]. Structural analysis of the substitution of V1532I using the UCSF Chimera program validated the direct interactions of V1532 and I1532 with M210 of RAD51 [10] and revealed an additional contact between I1532 and A190.

188 These results indicate enhanced interaction between V1532I and RAD51 as shown by the  
189 increase in the number of contacts with the amino acids of RAD51.

190 In this study, we investigated the interaction between BRC4 mutant and RAD51 by  
191 using modified MTH, competitive ELISA, and RAD51 focus assays. EGFP–V1532I interfered  
192 with RAD51–RAD51 interaction more strongly than EGFP–hBRC4wt. This result suggests that  
193 the V1532I substitution in BRC4 may potentiate the inhibition of RAD51 oligomerization by  
194 occupying hydrophobic pockets that would normally be occupied by an adjacent RAD51  
195 monomer in a filament [2,28]. Similarly, soluble V1532I BRC4 peptide inhibited the interaction  
196 between plate-coated BRC4 peptides and hRAD51 more strongly than hBRC4wt peptide. In  
197 addition, the RAD51 foci were readily formed in mock-transfected control cells and to a much  
198 lesser extent in cells transfected with EGFP–hBRC4wt or EGFP–V1532I. These data suggest  
199 that the strong interaction between the V1532I mutant and endogenous RAD51 inhibits the  
200 formation of RAD51 nucleofilaments in DSBs via endogenous BRCA2. This study  
201 demonstrates for the first time the role of Val 1532 of BRCA2 in its interaction with RAD51.

202 In addition, this study revealed the impairment of the interaction between the V1532F  
203 mutant of hBRC4 (V1532F) and RAD51, which was identified from human breast cancer  
204 patients. Although the risks of the V1532F single BRC missense mutation have not been  
205 investigated, V1532F might play an important role in the interaction between BRC repeats and  
206 RAD51. The structural analysis using UCSF Chimera revealed that the V1532F mutation  
207 induced some clashes against H1525 and T1526. These distortions in BRC4 conformation  
208 seemed to have caused the decrease in RAD51–BRC4 binding activity because these 2 amino  
209 acid residues are reported to be necessary for RAD51–BRC4 interaction [9,19]. Previous  
210 structural analyses of cancer-associated mutations affecting the BRC repeats revealed that  
211 weakening RAD51 affinity in one repeat is enough to increase breast cancer susceptibility

[10,29,30]. However, the restoration of BRCA2 function in BRCA2-deficient cells by the expression of the construct connecting a single BRC repeat and the replication protein A large subunit, which delivers RAD51 to single-stranded DNA [31], suggests that further analysis is necessary to confirm a causal relationship between this mutation and breast cancer onset.

Although the effects of random or cancer-associated amino acid substitutions in human BRC4 on the inhibition of RAD51 interactions are reported [23,30], there are no reports about the effect of amino acid substitutions among animal species. In this study, we found that the V1532I substitution strengthens the interaction with RAD51, whereas V1532F abolishes it; this indicates a novel, critical role of V1532 in BRC4–RAD51 interaction. Our findings from the comparative analysis of BRCA2 between animal species might be important for elucidating the general mechanism of DNA repair mediated by BRC repeats.

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229

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321

## **Figure legends**

### **Figure 1**

Interaction between BRC4 and RAD51, and multiple sequence alignment of BRC4. (A) The left panel depicts the positions of the BRC4 sequences: human BRC4 (hBRC4), canine BRC4 (cBRC4), and feline BRC4 (fBRC4). Sequence alignment of BRC repeats (bold letters indicate amino acids that differ from human BRC4). The two-hybrid constructs were introduced into HeLa cells to measure their interaction with canine RAD51 (cRAD51). Luciferase activity was measured by using a mammalian cell two-hybrid assay. DBD: GAL4-DNA-binding domain fusion protein. VP16: VP16 transactivation domain fusion protein. The results are expressed as the mean (SE) ( $n = 4$ ). All graphs in this report show luciferase activities on the top right column as 100%. (B) Sequence alignment of consensus motifs of BRC repeats and BRC4 of several mammals and chickens. The IE residues of BRC4 are boxed.

### **Figure 2**

Effect of amino acid substitutions in BRC4 on its interaction with RAD51. (A) The left panel depicts the constructs in which amino acids were substituted from canine to human BRC4 (cBRC4m) and from feline to human BRC4 (fBRC4m). (B) The left panel depicts the constructs in which single amino acids were substituted from hBRC4 to cBRC4. The GAL4-DNA binding domain fusion proteins of hBRC4wt or V1532I were transiently transfected into cells. The fusion proteins were detected by immunoblotting using anti-GAL4-DBD and peroxidase-labeled secondary antibodies: lane 1, intact cell lysate; lane 2, hBRC4wt transfectant; lane 3, V1532I transfectant.

### **Figure 3**

Effect of the V1532F mutation of hBRC4 on its interaction with RAD51. (A) The left panel depicts the single amino acid substitution constructs V1532F, V1532I, T1526A, and G1529R; the 2 tetrameric motifs in BRC4 that mediate its structural and functional interactions with cRAD51 [9] are underlined. The luciferase activity of hBRC4wt was set at 100%. (B) Crystal structure of the hRAD51-hBRC4 complex (PDB ID: 1N0W). The solvent-accessible surface of RAD51 is shown in transparent dark green except M210 (light blue), and the BRC4 peptide is depicted as a gold ribbon. The residues of hBRC4wt are highlighted in purple (i.e., F1526, V1532, K1533, A1535, F1546, and D1547). The V1532 substituted to Ile or Phe residues in the mutant BRC4 (V1532I or V1532F) are indicated in blue and yellow, respectively. (C) The probability values of the substitutions of amino acid residues on V1532 (Dunbrack backbone-dependent rotamer library). The contacts or clashes between the residue at position 1532 were calculated and are depicted (D), (E), and (F). BRC4 and RAD51 are depicted by gold and pink ribbons, respectively. Solid green lines signify stable contacts and solid red lines represent clashes, as determined by the UCSF Chimera software.

#### **Figure 4**

Overexpression of V1532I mutant interferes with RAD51–RAD51 interaction. (A) The schematic at the top illustrates the EGFP–BRC4 fusion product containing a BRC4 cDNA fragment translationally fused to an EGFP–nuclear localization signal cassette in a modified pEGFP-C1 plasmid, NLS-pEGFP/BRC4. The lower graph shows the strength of the interference caused by the expression of EGFP, wild-type EGFP–human BRC4 (EGFP–hBRC4wt), and hBRC4 with the canine amino acid substitution (EGFP–V1532I, V1532F, T1526A, and G1529R) on cRAD51–cRAD51 interaction by MTH. (B) Schematic of the ELISA assay used to detect BRC–RAD51 interaction and its disruption by soluble peptides.

(C) In a competitive ELISA assay, the V1532I peptide inhibited the solid-phase BRC4 and human RAD51 interaction the more strongly than 4BRC4wt; however the substitution of V1532F did not reconstitute the inhibitory behavior as much as hBRC4wt. Values are expressed as the mean (SE) absorbance at 490 nm for triplicate data sets normalized to the positive control without a soluble inhibitor.

### **Supplementary Figure 1**

Effects of amino acid substitutions of T1526A and G1529R on the interaction with RAD51. The contacts or clashes between the residues at positions 1526 and 1529 were calculated and depicted in (A), (B), (C), and (D). BRC4 and hRAD51 are depicted by gold and pink ribbons, respectively. Solid green lines signify stable contacts as determined by the UCSF Chimera software.

### **Supplementary Figure 2**

Overexpression of V1532I mutant reduces the formation of RAD51 foci after ionizing radiation. (A) The cell clones expressing EGFP and V1532I were visualized by EGFP autofluorescence. Immunostaining of COS-7 cells transfected with EGFP-hBRC4 fusion proteins as described previously [15]. *Arrowheads*, EGFP-positive cells containing RAD51 nuclear foci. *Arrows*, EGFP-V1532I-positive cells lacking hRAD51 nuclear foci. (B) Quantitative analysis of RAD51 focus-forming cells. The nuclear foci of EGFP-positive transfected cells were counted. EGFP-hBRC4 constructs were transfected at 5 or 50 ng. Cells containing at least 10 foci were determined to be positive for focus formation. At least 100 cells were counted for each data point. The results are expressed as the mean (SE) ( $n = 4$ ).

## Supplementary Methods

### 1. Enzyme-linked immunosorbent assay (ELISA)

To test the interference abilities of BRC4 peptides on RAD51–BRC4 interaction, we developed hBRC4wt (1521-LLGFHTASGKKVKIAKESLDKVKNLFDE-1548), V1532I (1521-LLGFHTASGKKIKIAKESLDKVKNLFDE-1548), and V1532F (1521-LLGFHTASGKKFKIAKESLDKVKNLFDE-1548) peptides (>95% purity, Scrum Inc.) to compete against the BRC4 peptide (in the solid phase) for recombinant full-length human RAD51 (Bio Academia). Ninety-six-well plates (Maxisorp, Nunc) were coated overnight at 4°C with 10 mg·L<sup>-1</sup> biotinylated BRC4 peptide in PBS. After discarding the peptide solution, the plates were blocked with PBS containing 3% bovine serum albumin (BSA) for 1 h at 37°C. After blocking, plates were washed 3 times with PBS containing 0.05% Tween 20 (PBS-T). RAD51 (0.5 mg·L<sup>-1</sup>) and inhibitor peptides at the indicated concentrations in ELISA buffer (PBS containing 0.5% BSA and 0.05% Tween 20) were incubated in a total volume of 50 µL in coated ELISA plates and incubated overnight at 4°C. The plates were then washed with PBS-T and incubated for 1 h at room temperature with anti-RAD51 antibody PC-130 (diluted 1:5000 with ELISA buffer). Plates were washed with PBS-T and incubated for 1 h at room temperature with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG antibody (diluted 1:5000 with ELISA buffer; Jackson ImmunoResearch Laboratories). After being washed with PBS-T, color reactions were performed with O-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich) and allowed to develop for 15 min at room temperature; the reaction was stopped by adding H<sub>2</sub>SO<sub>4</sub>. Absorbance was measured at 490 nm using a SpectraMax 340 microplate spectrophotometer (Molecular Devices).

### 2. Irradiation and immunostaining

418 COS-7 cell monolayers (80% confluent) cultured in LabTek chambers (Nunc) were  
419 transfected with 50 or 5 ng NLS-pEGFP-C1 vectors containing hBRC4wt or the V1532I mutant  
420 by using FuGENE6 (Roche). Forty-eight hours after transfection, the cells were irradiated with  
421 15 Gy X-rays using an MBR-1520R-3 irradiation device (Hitachi, Japan). The cells were  
422 returned to the tissue culture incubator immediately and fixed with 10% formalin 2 h after  
423 irradiation. After permeabilization with 0.2% Triton X-100 in PBS, the cells were incubated  
424 with a polyclonal antibody against human RAD51 (Santa Cruz) followed by Alexa Fluor  
425 647-conjugated goat anti-rabbit IgG (Molecular Probes). RAD51 foci were examined under a  
426 Nikon fluorescence microscope.  
427

Figure 1

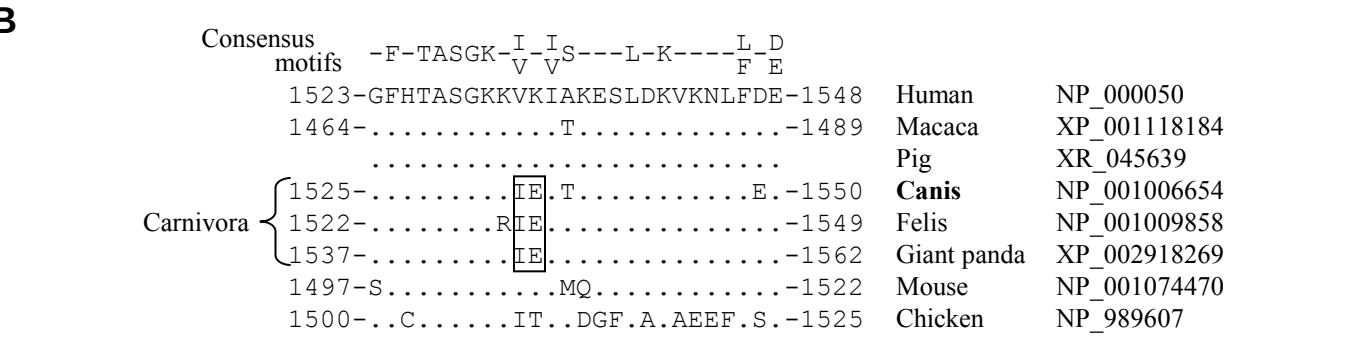
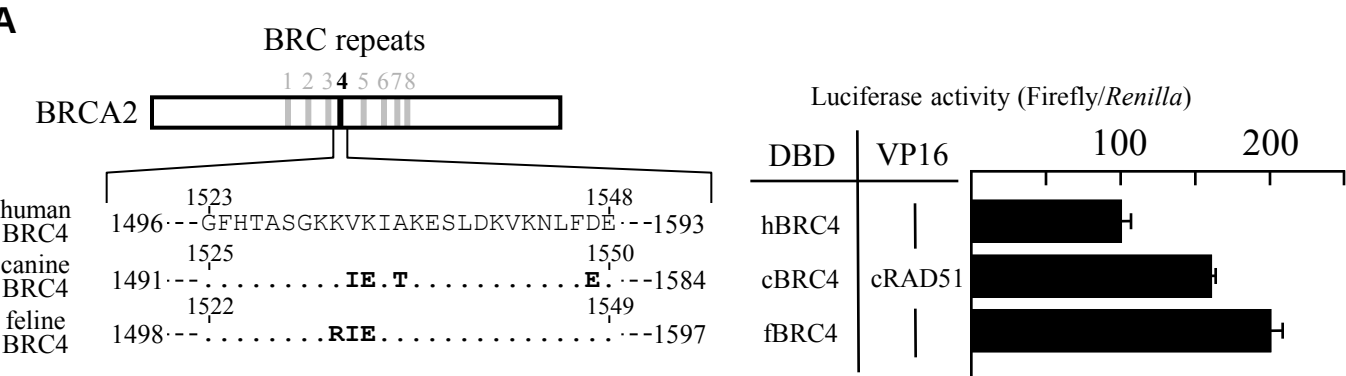


Figure 2

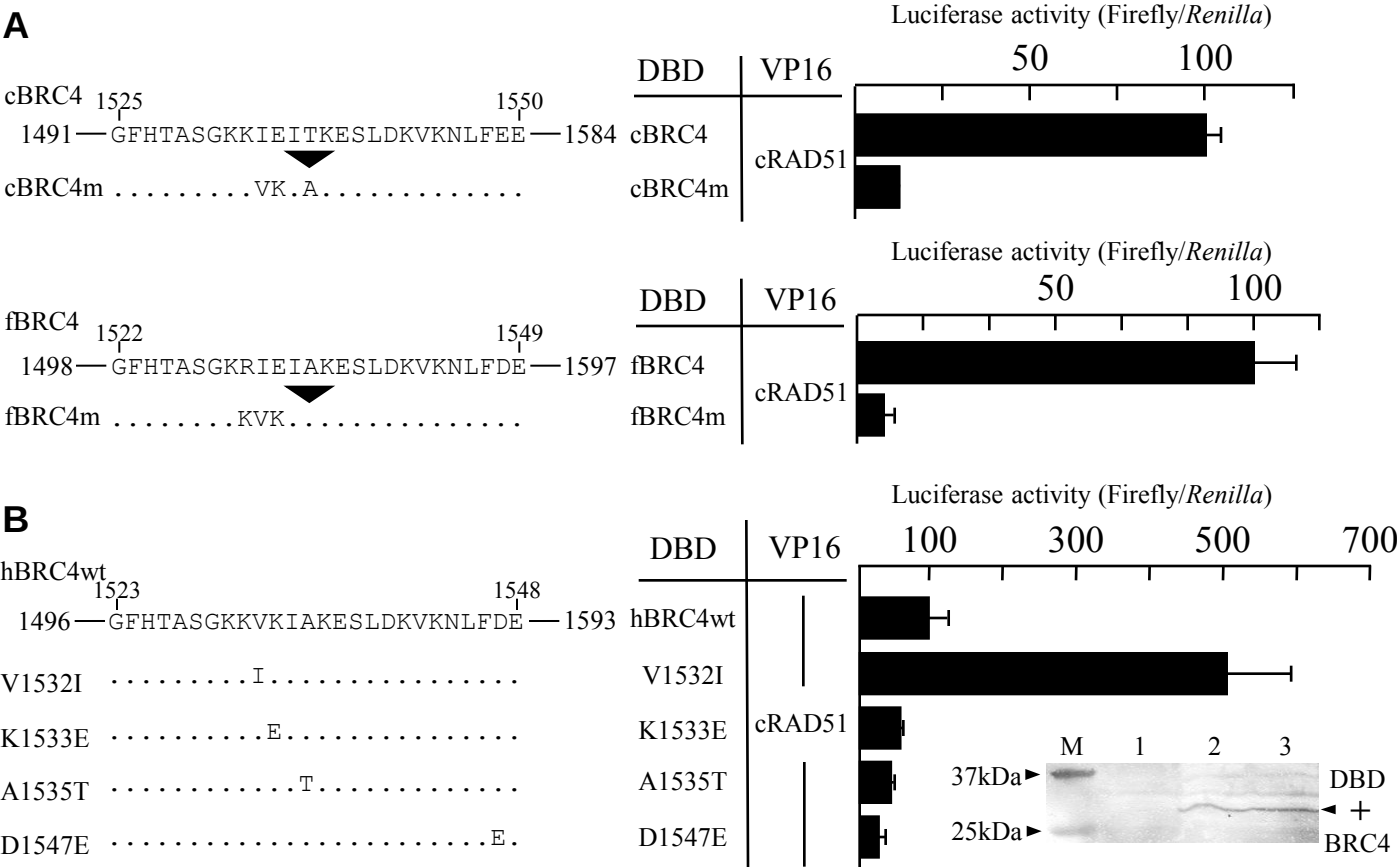


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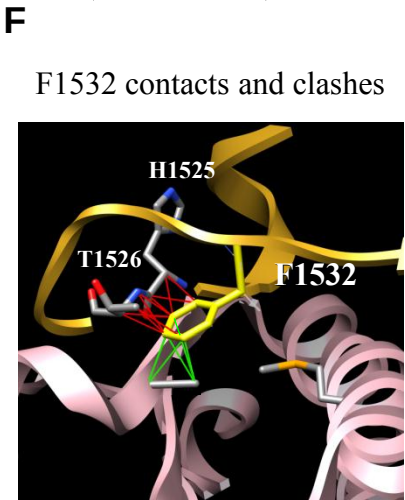
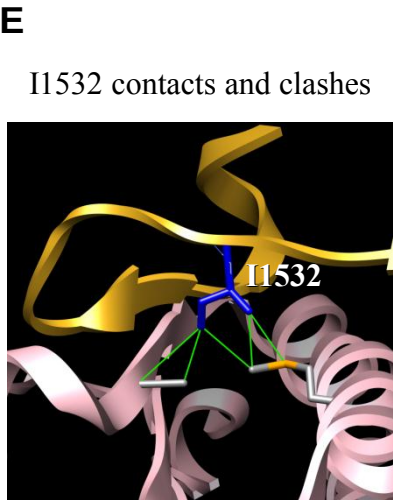
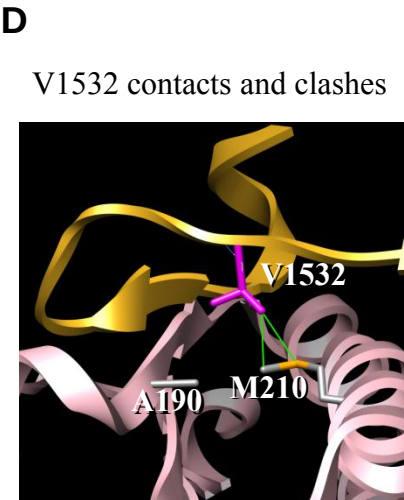
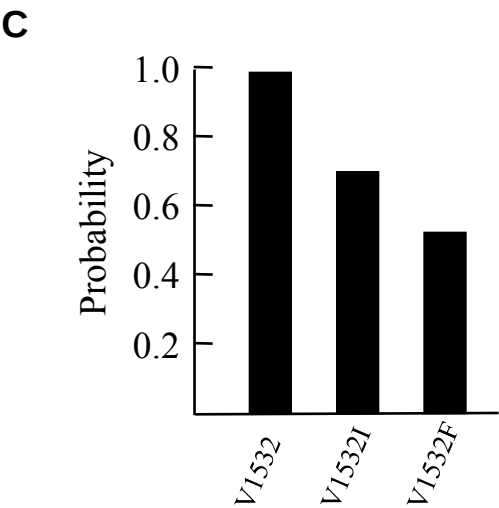
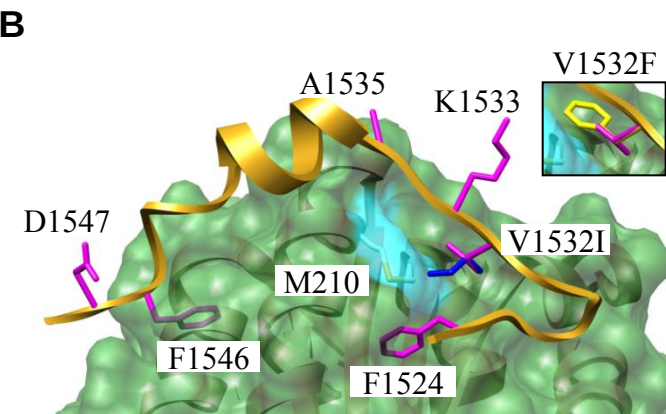
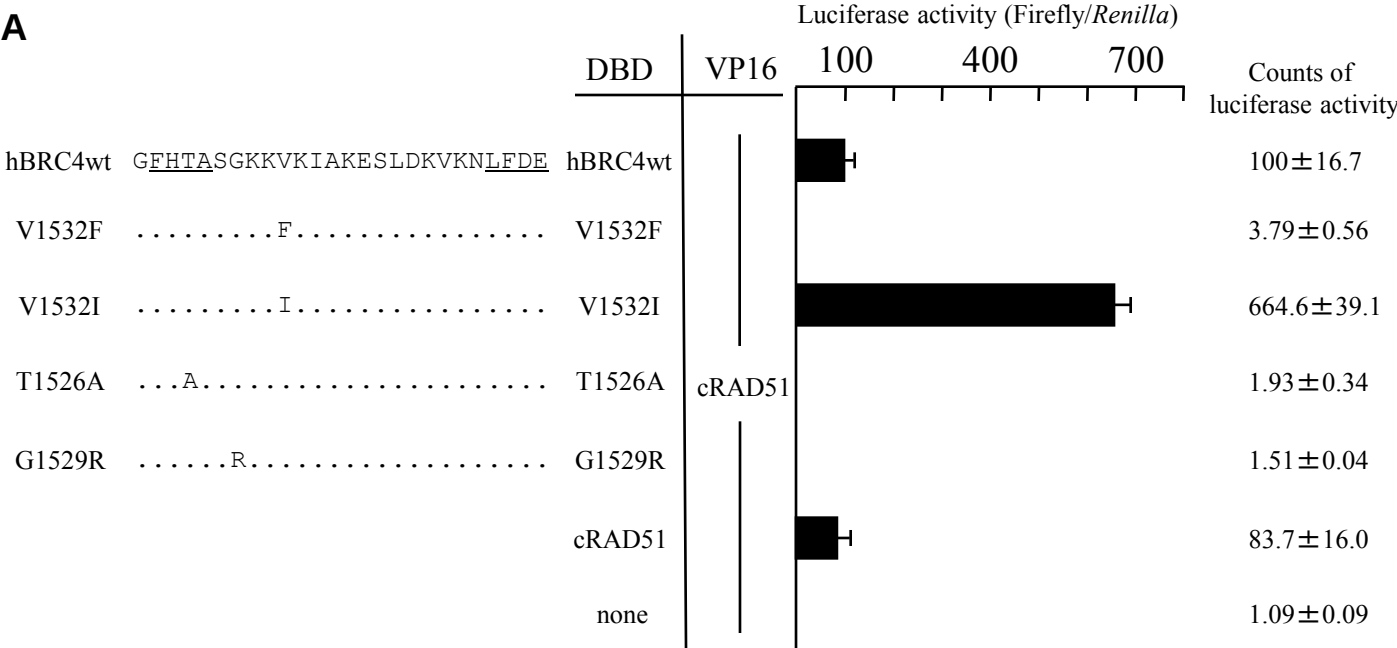
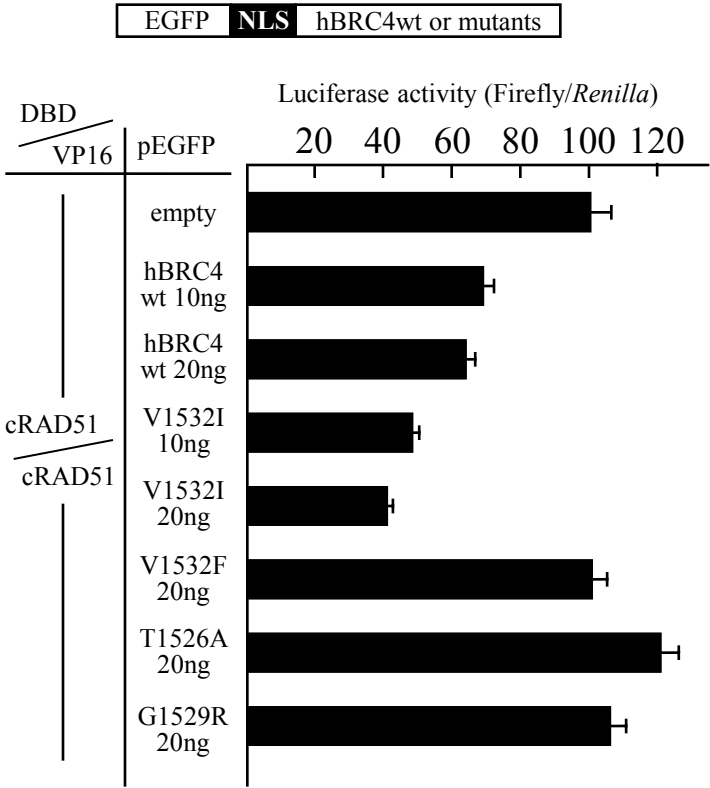
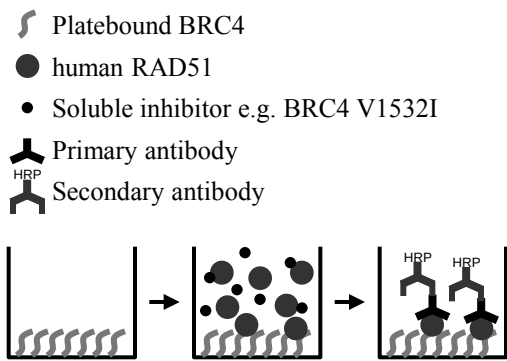


Figure 4

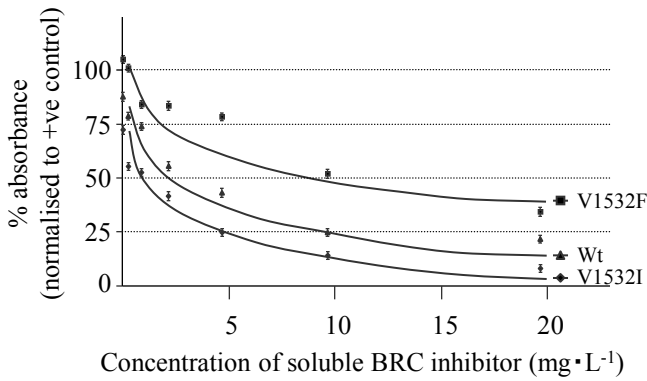
A



B



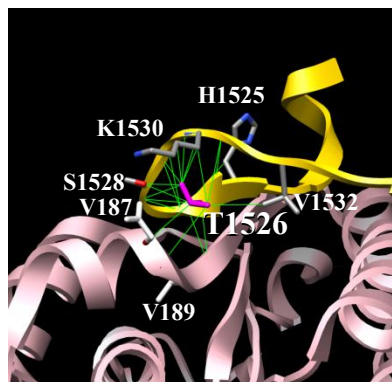
C



# Supplemental Figure 1

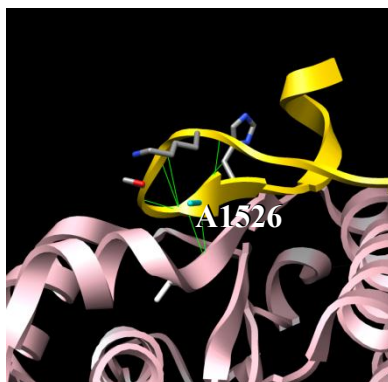
**A**

T1526 contacts



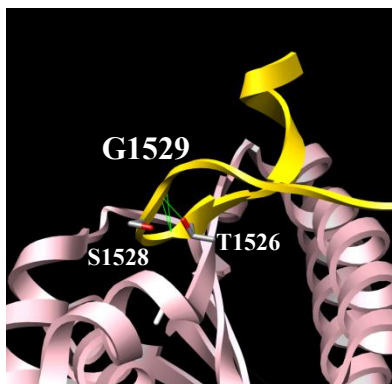
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A1526 contacts



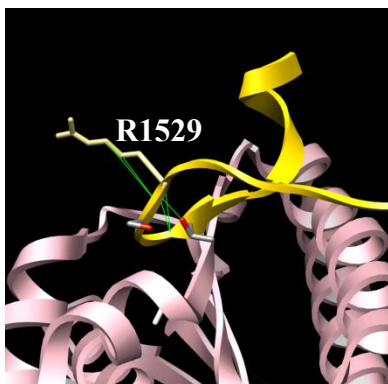
**C**

G1529 contacts



**D**

R1529 contacts



# Supplemental Figure 2

