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Supplemental Data

The BRC Repeats of BRCA2 Modulate the DNA Binding Selectivity of RAD51

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Protein expression and purification.

BRC4, ∆1524-30 peptides (Figure S1A) and T1526A mutant peptide were cloned into pGEX-6-1 (Amersham Biosciences), and the GST-tagged BRC4, ∆1524-30 and T1526A fusions were expressed in BL21 (DE3) cells. For each protein, about 8 g of cell paste from 3 liters of culture were suspended in 40 ml of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.3) supplemented with 1 mM PMSF. The cells were disrupted using a French press. The crude lysate was clarified by centrifugation in a Beckmann Ti 45 rotor 35,000 rpm, 60 min, and the cleared lysate was applied to a 5 ml GSTrap HP column (Amersham) equilibrated with PBS buffer. The GST-fusion protein was eluted with 10 mM reduced glutathione and 50 mM TrisHCl (pH 8.0). The protein sample was then dialyzed against a buffer containing: 20 mM TrisHCl (pH 7.5), 1mM EDTA, 1mM dithiothreitol (DTT), and 10% glycerol, and applied into a 6 ml Resource Q column (Amersham). The protein was eluted with a 60 ml 0 to 400 mM KCl gradient in the same buffer. The peptides were stored in buffer

containing: 20 mM TrisHCl (pH 7.5); 10% glycerol; 0.5 mM EDTA; 0.5 mM DTT; and 200 mM KCl, and the concentration was determined using an extinction coefficient at 280 nm of 40,920 $M^{-1}cm^{-1}$, Figure S1C. To produce untagged peptides, the eluate from the GSTrap column (Amersham) was cleaved with Pre-Scission protease and further purified using a HiTrap SP FF, 1 ml column (Amersham) equilibrated with: 20 mM HEPES buffer, (pH 7.0). The protein was eluted with a 20 ml gradient of 0 to 500 mM NaCl in the same buffer. Peptides were stored at -80 °C in the same buffer as above. Since the untagged peptides lack aromatic residues, the absorbance of the peptide bond was used to determine their concentration (μg/ml) from: 144(A_{215nm}-A_{225nm}) (Segel, 1976). $BRCA2_{BRC1-8}$ peptide was purified as previously described (Shivji et al., 2006).

Human RAD51 protein was expressed in a *recA*-deficient strain BLR (DE3) (Novagen) carrying plasmids pET15b-*RAD51* and pLysS, kindly provided by Dr. P. Sung (Yale). The protein was purified essentially as described (Sigurdsson et al., 2001) with the following modifications: Pooled fractions from Q-sepharose were resuspended in buffer K (20 mM KPO_4 (pH 7.3), 1 mM DTT, and 10 % glycerol) and applied to a 20 ml hydroxyapatite (HA) column (Bio-Rad); the protein was eluted using a gradient of 20 to 400 mM KPO_4. Pooled fractions from HA column were dialyzed against Resource Q loading buffer (20 mM TrisOAc (pH 7.5), 200 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 10% glycerol). The dialyzed fraction was loaded onto a 6 ml Resource Q column and RAD51 was eluted with a gradient of 120 ml 200 to 600 mM KCl. Final pooled fractions were dialyzed against storage buffer (50 mM TrisOAc (pH 7.5), 200 mM KCl, 1

mM DTT, 0.1 mM EDTA, and 50% glycerol). RAD51 was stored in aliquots at -80 ^{o}C. The concentration was determined using an extinction coefficient at 280 nm ($M^{-1}cm^{-1}$): 12,800.

Human RPA was purified from pET11d-tRPA, kindly provided by Dr. M. Wold (University of Iowa). The RPA was expressed in *E. coli* strain BL21 (DE3) pLysS and purified following a protocol kindly provided by Drs. Kendall Knight and Michael Meuse (University of Massachusetts Medical Center): 5.5 g of cell paste from three liters of culture were harvested by centrifugation at 5000 g for 15 min and resuspended in 40 ml of lysis buffer (25 mM Tris-HCl (pH 7.5), 0.25 mM EDTA, 0.01% Nonidet, 1 mM DTT, and 0.5 mM PMSF). The sample was incubated with lysozyme (0.2 mg/ml) at 4 ºC for 30 min. The cells were disrupted by passage through French press. The lysate was then centrifuged at 35,000 rpm for 1h in a Beckmann Ti 45 rotor and the supernatant was loaded onto a 45 ml Affigel Blue column (Bio-Rad) in buffer A (25 mM TrisHCl (pH 7.5), 1mM EDTA, 10% glycerol, and 1mM DTT) with 0.5 M NaCl. The column was washed with 0.5 M NaCl in buffer A, followed by 0.8 M NaCl in buffer A. RPA was eluted with 45 ml of 2.5 M NaCl in buffer A plus 40% ethylene glycol. The purest fractions were pooled and loaded onto a 30 ml ssDNA cellulose column (Amersham) equilibrated in buffer A with 0.5 M NaCl. The column was washed with 30 ml of 0.5 M and then 0.75 M NaCl in buffer A; RPA was eluted with 1.5 M NaCl in buffer A plus 50% ethylene glycol. The protein fractions were dialyzed against buffer A with 100 mM NaCl. Finally, the pooled fractions from the ssDNA-cellulose were loaded onto a 6 ml Resource Q column (Amersham) equilibrated

in buffer A with 100 mM NaCl. The same buffer was used to wash after loading the sample and the protein was eluted with a gradient of 100 to 400 mM NaCl. The purest fractions were pooled and dialyzed against storage buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 20% glycerol, 1 mM EDTA, and 1 mM DTT) and stored at -80 ºC. The concentration was determined using an extinction coefficient at 280 nm of 88,000 $M^{-1}cm^{-1}$.

Supplemental References

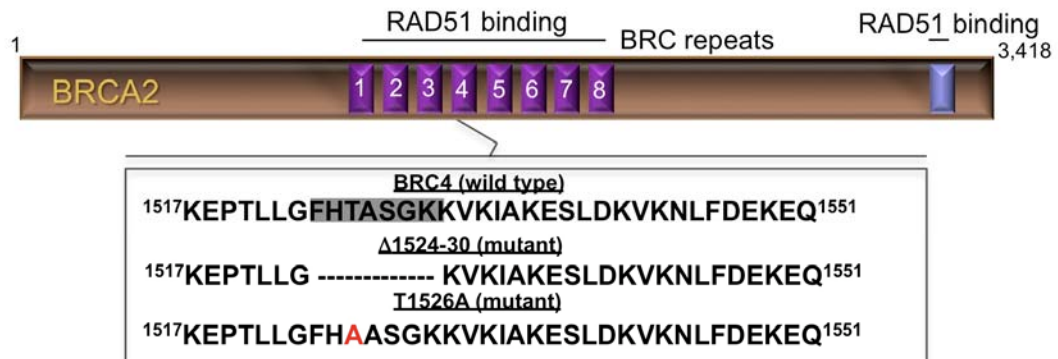
Segel, I.H. (1976). Biochemical Calculations: How to Solve Mathematical Problems in General Biochemistry, 2nd Edition edn (New York, Wiley).

Shivji, M.K., Davies, O.R., Savill, J.M., Bates, D.L., Pellegrini, L., and Venkitaraman, A.R. (2006). A region of human BRCA2 containing multiple BRC repeats promotes RAD51-mediated strand exchange. Nucleic Acids Res. *34*, 4000-4011.

Sigurdsson, S., Trujillo, K., Song, B., Stratton, S., and Sung, P. (2001). Basis for avid homologous DNA strand exchange by human Rad51 and RPA. J Biol Chem *276*, 8798-8806.

Supplemental Figures

A



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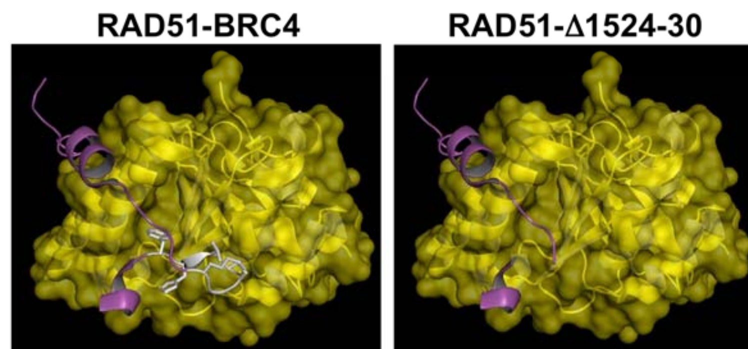

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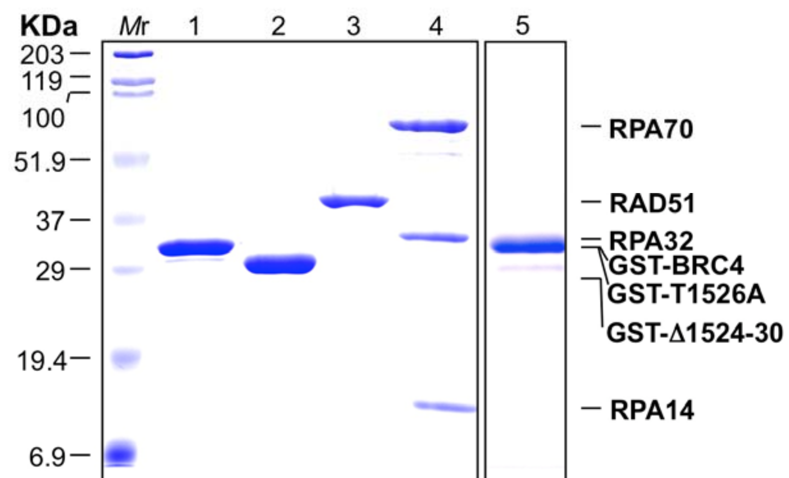

Figure S1. The BRC repeats of BRCA2 protein. (A) Schematic representation of BRCA2 protein indicating the BRC repeats and C-terminal domains that interact with RAD51. The amino acid sequence of the 35 amino acid peptide corresponding to the BRC4 repeat (amino acids 1517-1551) is indicated. The most conserved region of the BRC4 motif is shaded in gray. The deletion mutant peptide is also shown and the deleted amino acids are represented by a dashed line. The point mutation of mutant peptide T1526A is highlighted in red. (**B)** Structure of RAD51-BRC4 (pdb1n0w). RAD51 is represented by the yellow space-filling and ribbon model, and BRC4 by the magenta ribbon model, showing the highly conserved amino acids deleted in Δ1524-30 in gray. **(C)** Purified proteins analyzed by SDS-PAGE and stained with Coomassie blue: GST-tagged BRC4 (4 μg, lane 1), GST-tagged Δ1524-30 (4 μg, lane 2), RAD51 (4 μg, lane 3), heterotrimeric RPA (4 μg, lane 4), GST-tagged T1526A (4 μg, lane 5). M_r, molecular mass markers.

Figure S2. **Untagged BRC4 stimulates binding of RAD51 to ssDNA.** BRC4, at the concentrations indicated, and RAD51 (3 μM) were incubated for 15 min prior to addition of ^{32}P-labeled dT_{40}, and further incubated for 1 h at 37 °C, as in Figure 1A. As a control, the untagged Δ1524-30 peptide was used at 40 μM. The reaction products were resolved by electrophoresis using 6% polyacrylamide gels**.**

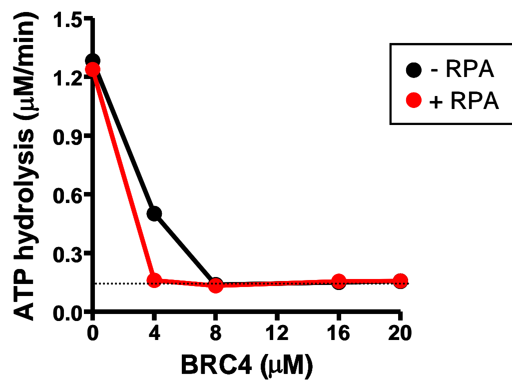

Figure S3. RPA does not affect the effect of BRC4 on the ssDNA-dependent ATPase activity of RAD51. RPA (1 μM) or RPA storage buffer was pre-incubated with dT_{40} for 5 min at 37 ºC in the presence of 0.5 mM ATP and 4 mM $MgCl_2$. This was followed by the addition of RAD51 (3 μM) and increasing concentrations of GST-BRC4, and further incubated for 1 h at 37 ºC: (black circle), absence of RPA; (red circle), presence of RPA. The dashed line represents the DNA-independent rate of ATP hydrolysis by RAD51 (~0.2 μM/min).

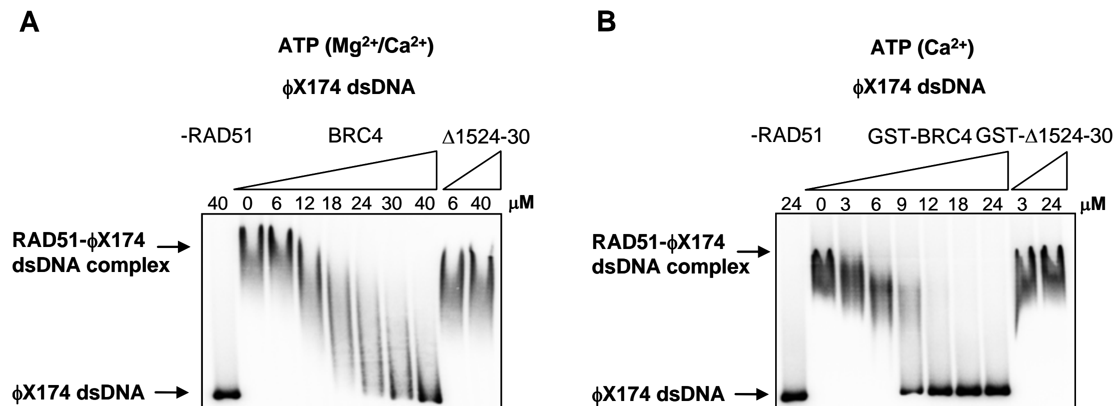

Figure S4. **Both tagged and untagged BRC4 inhibit binding of RAD51 to dsDNA. (A)** Untagged BRC4 inhibits binding of RAD51 to dsDNA in the presence of Mg^{2+} and Ca^{2+}. BRC4, at the concentrations indicated, and RAD51 (3 μM) were incubated for 15 minutes prior to addition of ^{32}P-labeled linear ϕX174 dsDNA (5 μM, nucleotides) and further incubated for 1 h at 37 °C in the presence of ATP, Mg^{2+}, and Ca^{2+} as in Figure 3A. The same reaction was carried out with the lowest and highest concentration of the untagged control peptide, Δ1524-30. **(B)** GST-BRC4 inhibits binding of RAD51 to dsDNA in the presence of ATP and Ca^{2+}. GST-BRC4, or the control peptide, GST-Δ1524-30, at the concentrations indicated, and RAD51 (3 μM) were incubated for 15 min prior to addition of ^{32}P-labeled ϕX174 linear dsDNA (5 μM, nucleotides) and further incubated for 1 h at 37 °C as in Figure 3A, in the presence of ATP and Ca^{2+}. Protein-DNA complexes were resolved by electrophoresis in a 0.5 % agarose gel, and analyzed by autoradiography.

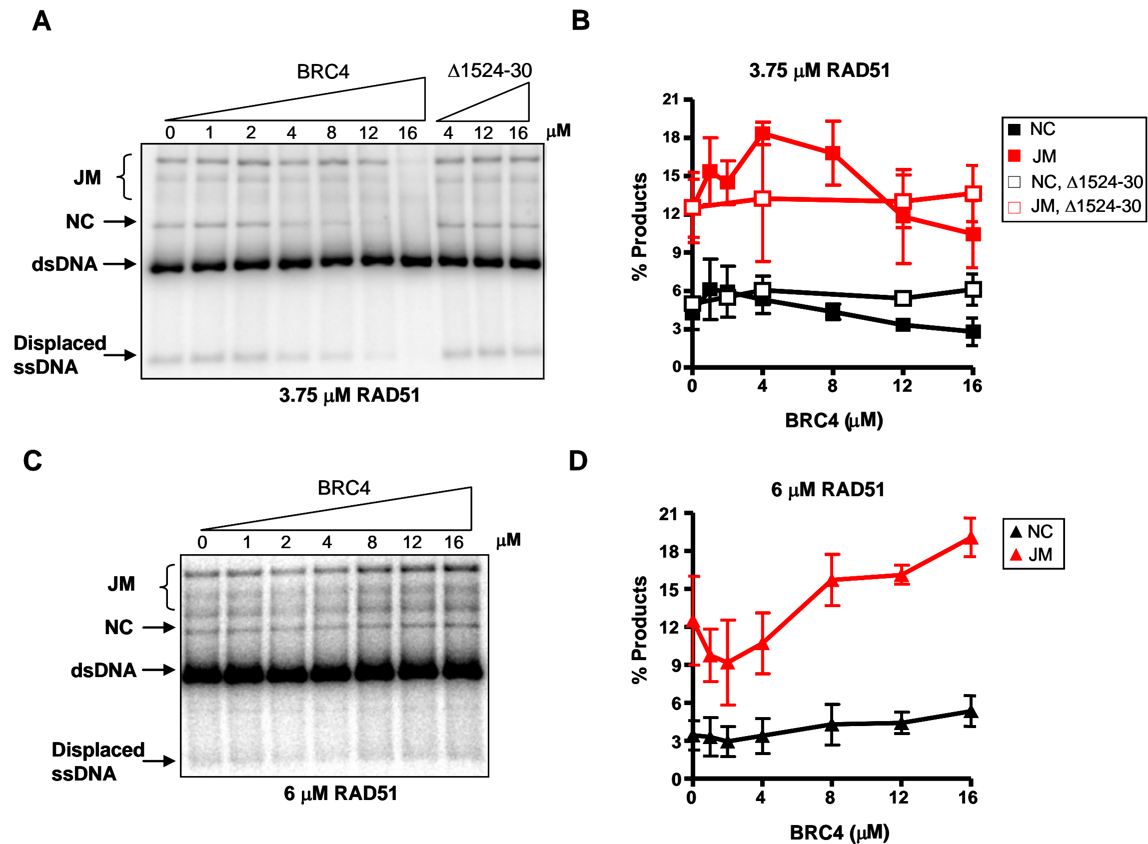

Figure S5. **Effect BRC4 or the control peptide, Δ1524-30, on DNA strand exchange promoted by RAD51. (A)** The DNA substrates were circular ϕX174 ssDNA and linear dsDNA (15 μM in nucleotides) and RAD51 was 3.75 μM. The reaction mixtures were incubated for 2 hours at 37 ^{o}C and stopped by de-proteinization for 15 min at 37 ^{o}C. The products of the reaction were analyzed by electrophoresis in a 1% agarose gel followed by autoradiography. **(B)** Quantification of the joint molecules (red square) and nicked circular dsDNA (black square) products using 3.75 μM RAD51. Open symbols correspond to GST-Δ1524-30; filled symbols correspond to GST-BRC4. **(C)** DNA strand exchange reaction performed as in (A), except using 6 μM RAD51. **(D)** Quantification of the joint molecules (red triangle) and nicked circular dsDNA

(black triangle) products using 6 μM RAD51. Error bars represent standard errors as calculated from at least three independent experiments.