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Human BRCA2 protein promotes RAD51 filament formation on RPA-covered single-stranded DNA

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BRCA2 is a tumor suppressor that functions in homologous recombination, a key genomic integrity pathway. BRCA2 interacts with RAD51, the central protein of recombination, which forms filaments on single-stranded DNA (ssDNA) to perform homology search and DNA strand invasion. We report the purification of full-length human BRCA2 and show that it binds to ~6 RAD51 molecules and promotes RAD51 binding to ssDNA coated by replication protein A (RPA), in a manner that is stimulated by DSS1.

Germline mutations in the *BRCA2* gene confer a highly elevated lifetime risk of developing breast, ovarian and other cancers[1]. The tumor suppressor function relates to a role of BRCA2 protein in homologous recombination[2], but the mechanistic analysis of human BRCA2 has been impeded by difficulties purifying this large (3,418-amino-acid) protein. BRCA2 contains eight conserved BRC repeats and a C-terminal region that bind RAD51 with varying affinity[3,4] and is required for RAD51 localization to sites of DNA damage[5]. RAD51 filament formation on ssDNA is inhibited by the binding of the ssDNA-binding protein RPA to the substrate. Filament formation requires the action of mediator proteins[6], and studies of the much shorter fungal and nematode BRCA2 homologs and of isolated domains of human BRCA2 have led to a model in which BRCA2 nucleates RAD51 polymerization at DNA junctions to overcome the inhibitory effect of RPA[7–13]. However, this model has not been tested yet in the context of the full-length human BRCA2 protein.

To examine the function of human BRCA2 in homologous recombination, we have purified to near homogeneity small quantities of a full-length human BRCA2 fusion protein expressed in yeast. Using N-terminal glutathione S-transferase (GST) and C-terminal Flag epitope and His_{10} affinity tags, we selected specifically for full-length GST-BRCA2-Flag-His_{10} protein (**Fig. 1a**), with the presence of both tags verified by retention of the protein on the affinity resins (**Fig. 1b**) and by immunoblotting using anti-GST and anti-Flag antibodies (**Fig. 1c**). Antibodies directed to interstitial regions of human BRCA2 (BRC, CCR, VCT; **Fig. 1a**) confirmed the presence of these epitopes (**Fig. 1c**).

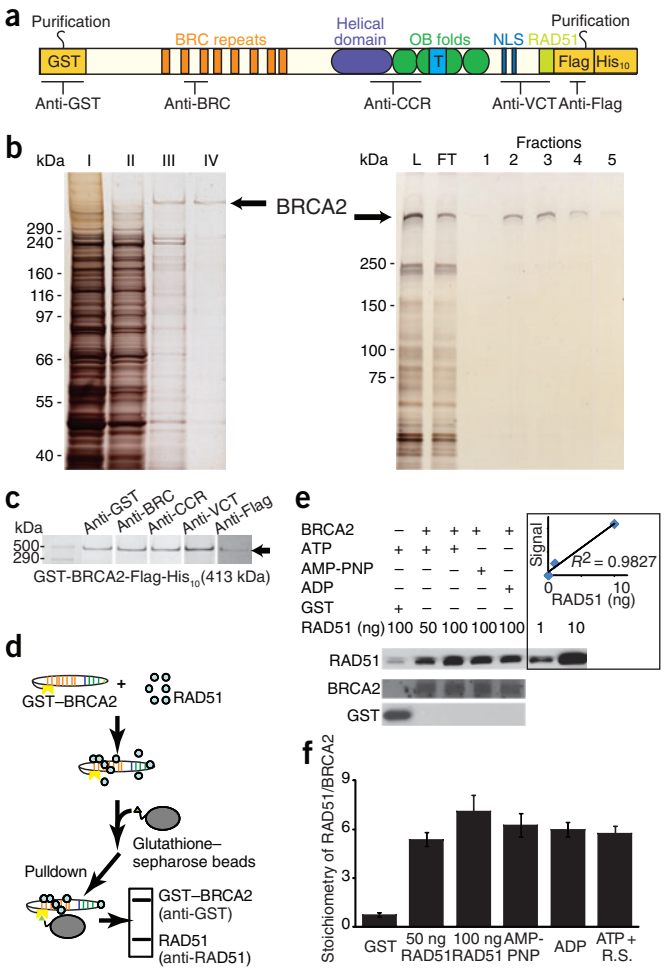

Figure 1 Purification of human BRCA2 and interaction with RAD51. (**a**) Schematic representation of purified human BRCA2 protein. OB folds, ssDNA-binding domains; T, Tower domain; NLS, nuclear localization sequence; RAD51, C-terminal RAD51-binding domain. (**b**) BRCA2 purification analysis. Left: silver-stained fractions from purification steps: I, lysate; II, pooled fraction after ammonium sulfate precipitation; III, pooled fraction of eluate from glutathione affinity chromatography; IV, fraction from anti-Flag column eluate. Right: analysis of Flag column load (L), flow-through (FT) and fractions by PAGE and silver staining. (**c**) Immunoblotting with antibodies spanning the full-length tagged BRCA2 protein as indicated in **a**. (**d**) GST pull-down assay design. (**e**) Immunoblots and (**f**) quantification of the proteins in the pull-down assay; box shows RAD51 calibration. The reactions contained 4.65 nM (50 ng) or 9.30 nM (100 ng) RAD51; 0.1 nM (10 ng) or no BRCA2; and 1 mM adenylyl imidodiphosphate (AMP-PNP), ADP or ATP. Error bars, s.d. of $n = 3$. R.S., ATP regenerating system.

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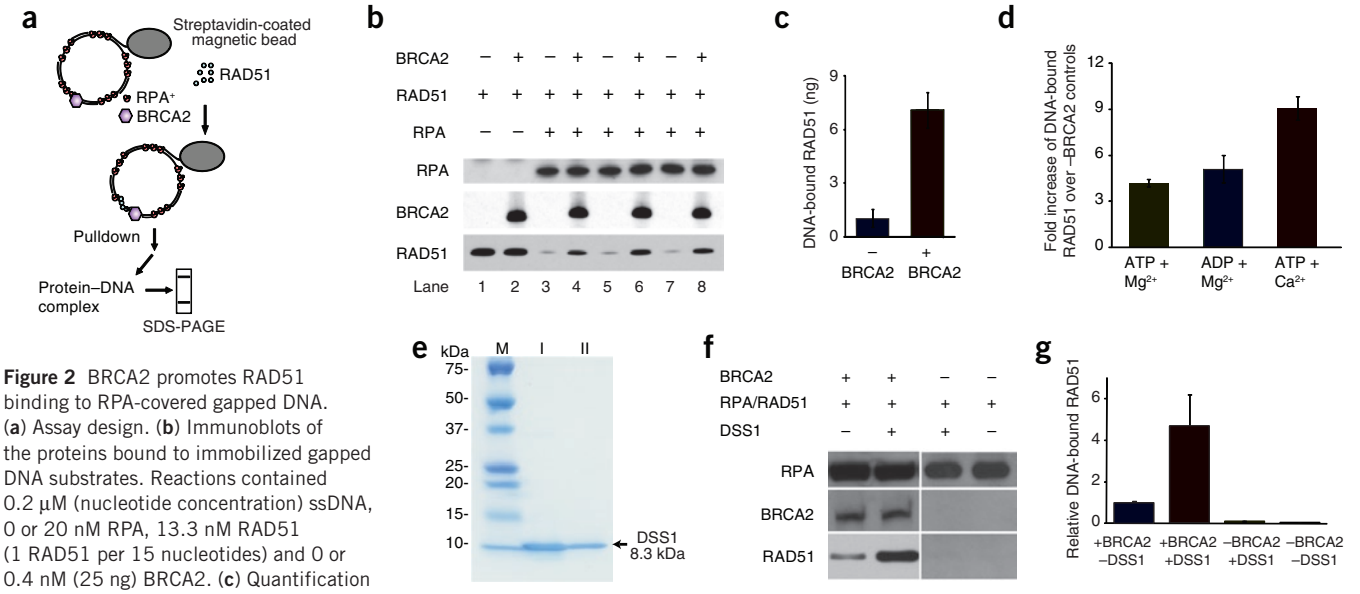

Figure 2 BRCA2 promotes RAD51 binding to RPA-covered gapped DNA. (**a**) Assay design. (**b**) Immunoblots of the proteins bound to immobilized gapped DNA substrates. Reactions contained 0.2 μM (nucleotide concentration) ssDNA, 0 or 20 nM RPA, 13.3 nM RAD51 (1 RAD51 per 15 nucleotides) and 0 or 0.4 nM (25 ng) BRCA2. (**c**) Quantification of DNA-bound RAD51 shown in **b**. (**d**) Quantification of DNA-bound RAD51 in the presence of different nucleotide cofactors. BRCA2 was 0.32 nM (20 ng); all other components were as in **b**. Plotted is fold increase over value in controls lacking BRCA2. (**e**) Purification of human DSS1 protein. I, pooled fraction of chitin column eluate; II, pooled fraction of Mono-Q (GE Healthcare) column eluate. (**f**) Immunoblots of the proteins bound to immobilized gapped DNA substrates. Reactions contained 0.2 μM (nucleotide concentration) ssDNA, 20 nM RPA, 13.3 nM RAD51 (1 RAD51 per 15 nucleotides), 0 or 4 nM DSS1 and 0 or 0.08 nM (5 ng) BRCA2. (**g**) Quantification of DNA-bound RAD51 shown in **b**. Plotted is fold increase over value in absence of DSS1. Error bars, s.d. of $n = 3$, and in one case $n = 2$.

By SDS-PAGE, purified full-length human GST-BRCA2-Flag-His_{10} migrated in a manner consistent with a predicted molecular weight of 413 kDa within the limit of resolution of these gels (**Fig. 1c**). To further confirm the integrity of the purified protein, we resequenced the entire gene from the overproduction yeast strain after protein purification and confirmed the absence of any rearrangements or mutations. From here on, we refer to GST-BRCA2-Flag-His_{10} as BRCA2.

Previous studies could not define the stoichiometry of the BRCA2–RAD51 interaction in humans, as the homologs from model organisms contain fewer BRC repeats (one BRC repeat in the case of the best-studied homolog, Brh2 from the fungus *Ustilago maydis*[13]) and the purified human domains or synthetic constructs did not contain all RAD51 binding sites or the proper structural context[7,9–12]. To determine the stoichiometry of the BRCA2–RAD51 interaction, we used a GST pull-down assay with excess RAD51 (**Fig. 1d**) and measured the amounts of pulled-down proteins by quantitative immunoblot (see **Supplementary Methods**). This analysis confirmed that BRCA2 bound human RAD51 protein in solution and indicated a stoichiometry of about 6 ± 1 RAD51 molecules per BRCA2 (**Fig. 1e**). This estimate hinges on the accuracy of the BRCA2 concentration, but it is consistent with an independent measurement using a different full-length human BRCA2 protein[14]. Although RAD51 can bind different nucleotide cofactors, we found the RAD51–BRCA2 interaction to be only marginally influenced by the nucleotide cofactor bound to RAD51 (**Fig. 1f**). Nucleation is the rate-limiting step for RAD51 filament formation, and single-molecule experiments have estimated that about 2 or 3 RAD51 protomers are sufficient for filament nucleation[15], although other estimates, at 4 or 5 protomers, are higher[16]. In either case, our results indicate that BRCA2 can bind sufficient RAD51 molecules to promote RAD51 filament nucleation.

The assembly of the RAD51–ssDNA filament is inhibited by RPA. Mediator proteins, including BRCA2, are thought to overcome this inhibition to facilitate the formation of RAD51 filaments on RPA-coated ssDNA[6]. To test this model with full-length human BRCA2 protein, we used a magnetic bead–based pull-down assay to measure the binding of RAD51 to RPA-coated ssDNA (**Fig. 2a**). In the presence of saturating amounts of RPA fully occupying the available ssDNA (1 RPA per 10 nt), we observed little RAD51 binding. Addition of BRCA2 resulted in a sevenfold stimulation of RAD51 binding to RPA-coated ssDNA (**Fig. 2b**,**c**). Use of the purified, full-length BRCA2 protein was critical, as preparations containing BRCA2 truncation or degradation products inhibited rather than stimulated RAD51 binding to RPA-coated ssDNA (data not shown). BRCA2-mediated stimulation of RAD51 binding to RPA-coated ssDNA occurred at a substoichiometric ratio of 1 BRCA2 molecule to 33 RAD51 protomers. Nonspecific binding of RAD51 to double-stranded DNA or to the bead can be ruled out, as the BRCA2 effect was dependent on the presence of RPA (**Fig. 2b**; lanes 1, 2). The amount of RAD51 bound to ssDNA is not expected to lead to an appreciable decrease in bound RPA, because of the small amounts of BRCA2 and RAD51 present in the reactions and the limited cooperativity of RAD51. BRCA2-mediated stimulation of RAD51 binding to RPA-coated ssDNA was highest with ATP + Ca^{2+}, a condition that stabilizes the active, ATP-bound form of RAD51 on ssDNA[17] (**Fig. 2d**). This is consistent with previous data showing that a peptide covering the fourth BRC repeat of human BRCA2 inhibits the RAD51 ATPase activity[11], thereby maintaining the active form of RAD51 (ref. 17).

Previous studies have suggested that BRCA2 orthologs show a preference for binding single-stranded/double-stranded DNA junctions[8]. However, when we compared the binding of BRCA2 to an ssDNA circle or a gapped DNA substrate containing nine junctions (five 5′ and four 3′ junctions), we saw no significant difference (**Supplementary Fig. 1**), consistent with findings in ref. 14. It is possible that other proteins (PALB2, RAD51 paralogs) impart junction specificity.

BRCA2 associates with the small (70-amino-acid) DSS1 protein, which is required for recombinational repair but whose mechanism has been enigmatic[18–20]. Addition of purified human DSS1 (**Fig. 2e**) alone did not stimulate RAD51 binding to RPA-covered ssDNA (**Fig. 2f**,**g**). In contrast, in the presence of BRCA2, RAD51 binding to RPA-covered

ssDNA was stimulated about fivefold by DSS1 above that observed with BRCA2 alone. These results indicate a direct, positive role of DSS1 in recombinational DNA repair and are consistent with genetic data in human cells and model organisms showing that DSS1 is required *in vivo* for recombinational DNA repair, RAD51 focus formation and genomic stability[19,20].

Purification of full-length human BRCA2 (see also ref. 14) and DSS1 sets the stage for structural and functional studies of this important human tumor suppressor complex in its native context and will allow direct examination of the molecular defects associated with BRCA2 or DSS1 polymorphisms or mutations.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

J.L. designed, performed and analyzed all experiments and helped write the manuscript. T.D., J.L. and B.G. purified BRCA2 and DSS1. W.-D.H. conceived the project, designed experiments, contributed to data analysis and wrote the manuscript with J.L., with contributions from all authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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