Methods

Expression and Purification of full length BRCA2. The full length cDNA (10.3 kb) of human BRCA2 was cloned into phCMV1 (Genlantis San Diego, CA) along with two tandem repeats of the maltose binding protein (MBP) tag located at the N-terminus of BRCA2. All cloning steps were sequence verified (MCLab) and the final construct was verified by utilizing 15 primers spanning 700 b.p. regions of the full length BRCA2 cDNA. A PreScission Protease (GE Life Sciences) site was engineered in between the second MBP sequence and the start of the BRCA2 ORF such that both MBP tags could be cleaved by incubation with the PreScission Protease enzyme.

To express this construct, human 293TD cells (a gift from Rachel Litman, University of Massachusetts Medical School, Worcester, MA) were transiently transfected using TurboFect (Fermentas) and cells were harvested 31 hours post-transfection. Typically, twenty 15 cm plates containing 70% confluent 293T cells were used for purification. Cells were re-fed with fresh media, DMEM + 10% FBS (Invitrogen), before transfection and 16 hours post-transfection. Cells were harvested in 'buffer H': 50 mM HEPES (pH 7.5), 250 mM NaCl, 5 mM EDTA, and 1 mM DTT with the addition of 1% Igepal CA-630, 3 mM $MgCl_2$, 1 mM ATP, 1 mM PMSF and Protease Inhibitor Cocktail (Roche). The cell suspension was rotated for 20 minutes and then spun down at 10,000 g for 15 minutes in Sorval centrifuge (Oakridge tubes) and the supernatant was incubated overnight with 1 mL of amylose resin (New England Biolabs) per 50 mL of cell lysate, (washed extensively with buffer H before addition to the supernatant). The amylose resin was then spun down at 2,000 g in a swinging bucket rotor (JS 5.3 Beckman), washed one time with lysis buffer, and then poured into a disposable plastic column (Pierce) and

washed extensively with buffer H. The protein was then eluted with 10 mM maltose in Buffer 'HG': 50 mM HEPES (pH 7.5), 250 mM NaCl, 0.5 mM EDTA, 10% glycerol, and 1 mM DTT. These fractions were then pooled and loaded onto a HiTrap Q (GE Life Sciences), washed with buffer HG, and eluted with buffer HG containing 450 mM NaCl (final storage buffer for BRCA2). The full length BRCA2 protein was verified by western blot with antibodies to both the C-terminus of BRCA2 (Ab-2, EMD) and to the N-terminal MBP tag (anti-MBP, Zymed). The concentration of 2XMBP-BRCA2 was determined using an extinction coefficient at 280 nm of 365,160 $M^{-1}cm^{-1}$. The final concentration was adjusted by subtracting the contributions from contaminants, β-tubulin (which ranged from ~10-25% in multiple preparations) and truncated BRCA2 polypeptide (see Fig. 1a, which ranged from ~5-15%), based on SyproOrange quantification. The proteins in the Coomassie-stained gel shown in Fig 1a comprised full-length protein (~85%), truncated BRCA2 (~5%) and tubulin (~10%). Some preparations contained a trace amount of HSP70 as determined by mass spectrometry; however, this contaminant was minimized by the inclusion of 3 mM $MgCl_2$ and 1 mM ATP in the lysis buffer. We were unable to detect DSS1 in our preparation; based on comparison to known amounts of purified human DSS1 (a gift from Dr. Wolf Heyer, University of California, Davis), we estimate by Coomassie quantification that the amount of endogenous DSS1 bound to our purified BRCA2 protein is less than 2% (mole DSS1/mole BRCA2). Typical purification yields from twenty 15 cm plates ranged from 50-100 μg. Contaminant bands were cut out as gel slices from Coomassie stained gradient (4-15%) SDS-polyacrylamide gels and analyzed by mass spectrometry (UC Davis Proteomics Core Facility).

Immunodetection of BRCA2. Cell lysates or purified fractions generated from 293TD cells transfected with 2XMBP-FL BRCA2 were run on 6% SDS- polyacrylamide gels, transferred to PVDF membranes overnight, blocked in 5% milk with 1X TBS-T, and incubated with the primary antibody, Ab-2 (EMD), overnight. For immunoprecipitations (IP's), lysates were quantified for protein content by Bradford method and 1 mg total protein was used in an immunoprecipitation reaction containing 20 µL anti-BRCA2 (Ab-1, EMD) antibody and 40 µL protein G+ agarose (Santa Cruz Biotechnology). IP's were rocked for 2 h at 4 °C and then washed with buffer H followed by resuspension in 15 µL sample buffer. The samples were heated at 55 °C for 4 minutes, loaded onto 6% SDS-polyacrylamide gels, and processed for western blotting as described above. A secondary antibody, anti-mouse or anti-rabbit horse radish peroxidase (hrp) conjugated (Santa Cruz Biotechnology) was incubated on the membranes for 40 minutes. Blots were then washed 4 times and incubated with ECL Plus (Amersham GE healthcare) for 5 minutes before visualization on a Storm PhosphorImager.

Generation of Stable Cell Lines and Clonogenic Survival Assay. The MBP-BRCA2 and 2XMBP-BRCA2 constructs were stably transfected into VC8 (gift from Malgorzata Zdienicka, Leiden University Medical Center, The Netherlands) BRCA2 mutant hamster cells, using FuGene6 (Roche) transfection followed by selection in HAM's F10 media (Invitrogen) plus 10% FBS (Invitrogen) containing 1 mg/mL G418. To verify expression of BRCA2, total RNA was isolated from VC8 stable cell clones using TRIzol (Invitrogen). 1 µg of total RNA was used in each RT-PCR (Titanium One-step RT-PCR, Clontech)

reaction containing either MBP primer set (RJ-5'MBP2XN/RJ-3'MBP2XN) to amplify the MBP tag (1.1 kb) or C-terminal primer set (RJ-5'8269/RJ-3'AGEIBRCA2) to amplify the last 0.9 kb of the BRCA2 open reading frame. RT-PCR reactions were run out on 1% agarose gel and visualized with ethidium bromide staining on an Alpha Innotech UV imager. To confirm expression of 2XMBP- or MBP-BRCA2 in VC8 cells at the protein level, an immunoprecipitation (IP)/western using Ab-1 (EMD) as the IP antibody and Ab-2 (EMD) as the western antibody was performed. We consistently observed higher expression of the 2XMBP-BRCA2 protein compared to MBP-BRCA2 both in stable VC8 clones, as well as in transient transfections of both VC8 and human 293T cells. Clones positive for expression were tested for complementation by clonogenic survival response to mitomycin C. Cells were seeded at plating density of $5x10^5$ cells in 6 cm dishes. Cells were 50-70% confluent at time of drug treatment. Mitomycin C (Sigma) stock was a 1.5 mM stock solution. For treatment of cells, mitomycin C was diluted in 2 mL HAM's F10 media with no serum at the following concentrations: 0.1 μM, 0.25 μM, and 0.5 μM and placed on cells for one hour. After one hour incubation, the media was aspirated off, cells were washed with PBS, then trypsinized and resuspended in 2 mL of HAM's F10 + 10% FBS. Cells were counted using a hematocytometer, serially diluted, and plated out in triplicate into 6 well plates. Cells were re-fed with media containing penicillin/streptomycin (Hyclone) to prevent any contamination during 8 days of cell growth. After 8 days, the cells were removed from the incubator, washed with 0.9% NaCl (saline solution), and stained with crystal violet. Plates were dried overnight and colonies containing 50 or more cells were counted on each plate and the surviving fraction was determined for each drug treatment.

Protein Affinity Pull-downs. Prior to pull-down assays, amylose resin (NEB) was equilibrated with binding buffer 'B': 50 mM HEPES (pH 7.5), 250 mM NaCl, 0.5 mM EDTA, and 1 mM DTT. Purified 2XMBP-BRCA2 (2.4 μg) was incubated with 1 μg purified RAD51, RPA, SSB, RecA, yRad51, DMC1, or RAD52 for 30 minutes at 37 °C and then batch bound to 30 μL of amylose resin for one hour at 4 °C. RAD51 and RPA were purified as described previously [21]. SSB, RecA, and yRad51 were purified as described [50-52], respectively. RAD52 was a kind gift from Alex Mazin (Drexel University), and the purification of DMC1 (Amitabh Nimonkar) will be described elsewhere. As controls for non-specific binding to the amylose resin, candidate proteins (1 μg) were incubated with amylose resin in the absence of 2XMBP-BRCA2. The complexes were then washed with buffer B containing 0.1% Igepal CA-630 and resuspended in protein sample buffer, heated at 54 °C for 4 minutes, and loaded onto a 4-15% gradient SDS-polyacrylamide gel (Bio-Rad TGX gel). The gel was run for 1 hour at 100 Volts and stained with SyproOrange (Invitrogen) or Coomassie (Bio-safe, Bio-Rad). The protein bands were quantified by ImageQuant software on a Storm 860 PhosphorImager (Molecular Dynamics). The amount of RAD51 pulled down with 2XMBP-BRCA2 in Fig. 1E was determined using standard curves generated from known concentrations of RAD51 and 2XMBP-BRCA2 run in parallel in the same gel. The total input amount of 2XMBP-BRCA2 in each pull-down reaction was 64 nM and the total input amount for RAD51 ranged from 85 nM to 2 μM. The analyses to determine the ratio of RAD51 to 2XMBP-BRCA2 was derived from a fit to a segmented linear regression (GraphPad Prism 5.0b).

Electrophoretic Mobility Shift Assays. Oligonucleotide substrates were obtained from either Sigma or IDT (Ultramers) and were purified by polyacrylamide gel electrophoresis (PAGE). The following oligonucleotides were utilized: RJ-167-mer (5'-CTG CTT TAT CAA GAT AAT TTT TCG ACT CAT CAG AAA TAT CCG TTT CCT ATA TTT ATT CCT ATT ATG TTT TAT TCA TTT ACT TAT TCT TTA TGT TCA TTT TTT ATA TCC TTT ACT TTA TTT TCT CTG TTT ATT CAT TTA CTT ATT TTG TAT TA TCC TTA TCT TAT TTA-3'); RJ-5'TAIL-167-mer (5'-ATT TAT TCT ATT CCC TTT ATT TTC TCT GTT TAT TCA TTT ACT TAT TTT GTA TTA ATT TCC TAT ATT TTT TAC T TG T AT T TC T TA T TC A TT T AC T TAT TTT GTA TTA TCC TTA TTT ATA TCC TTT CTG CTT TAT CAA GAT AAT TTT TCG ACT CAT CAG AAA TAT CCG-3'); RJ-PHIX-42-1 (5'-CGG ATA TTT CTG ATG AGT CGA AAA ATT ATC TTG ATA AAG CAG-3'); RJ-Oligo1 (5'-TAA TAC AAA ATA AGT AAA TGA ATA AAC AGA GAA AAT AAA G-3'); RJ-Oligo2 (5'-CTT TAT TTT CTC TGT TTA TTC ATT TAC TTA TTT TGT ATT A-3'). To generate the 3' tailed DNA substrate, RJ-167-mer was radio-labeled with ^{32}P at the 5'-end and then annealed at a 1:1 molar ratio to RJ-PHIX-42-1. To generate the 5' tailed DNA substrate, RJ-5'TAIL-167-mer was radio-labeled with ^{32}P at the 5'-end and annealed at 1:1 molar ratio to RJ3'PHIX-42-1. The dsDNA was generated by radio-labeling RJ-Oligo1 with ^{32}P at the 5'-end and annealing it to RJ-Oligo2. The ssDNA substrate was RJ-167-mer radio-labeled with ^{32}P at the 5'-end.

2XMBP-BRCA2, at the indicated concentrations, was incubated with 0.2 nM (molecules) radio-labeled DNA substrate for 30 min at 37 °C in DNA strand exchange buffer (25 mM TrisOAc (pH 7.5), 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 μg/μL BSA, 2 mM ATP, and 1 mM DTT). The reactions were resolved by electrophoresis on a 6%

polyacrylamide gel in TAE (40 mM Tris-acetate (pH 7.5), 0.5 mM EDTA) buffer for 70 minutes at 60 V. The gel was then dried and exposed to a PhosphorImager screen overnight. The screen was scanned on a Molecular Dynamics Storm 840 PhosphorImager and bands quantified using ImageQuant software. The percentage of protein-DNA complexes was calculated as the free radio-labeled DNA remaining in a given lane relative to the protein-free lane, which defined the value of 0% complex (100% free DNA). Where BRCA2 was preincubated with RAD51, the two proteins were mixed at the indicated concentrations at 37 °C for 15 minutes before addition of the radiolabeled DNA substrate, which was followed by an additional 30 minute incubation at 37 °C.

DNA Strand Exchange Assays. DNA substrates were generated as described above for the EMSA analysis except that RJ-167-mer and RJ-5'TAIL-167-mer were not radio-labeled. The dsDNA donor was generated by first radio-labeling RJ-Oligo1 with ^{32}P on the 5'-end and annealing it to RJ-Oligo2 at a 1:1 molar ratio. The assay buffer contained: 25 mM TrisOAc (pH 7.5), 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 μg/μL BSA, 2 mM ATP, and 1 mM DTT. All pre-incubations and reactions were at 37 °C. The DNA substrates and proteins were at the following concentrations unless otherwise indicated in the figure legend: RPA (0.1 μM); RAD51 (0.22 μM); (3' tail, 5' tail, or ssDNA (4 nM molecules); and dsDNA (4 nM molecules). Unless a time course was shown, the reaction time was 30 minutes. Where proteins were omitted, storage buffer was substituted. The protein storage buffer contributed an additional 68 mM NaCl and 4% glycerol to the assay buffer used in the reactions. RecA (0.22 μM) reactions contained 3 mM ATPγS instead of ATP and were performed in 10 mM $MgCl_2$ in the absence of $CaCl_2$. Reactions utilizing SSB (0.1 μM)

contained 5 mM $MgCl_2$. The product yield using RecA alone or hRAD51 alone was 59% and 37%, respectively. The reaction was terminated with Proteinase K/0.5% SDS for 10 minutes. The reactions were loaded on a 6% polyacrylamide gel in TAE buffer and electrophoresis was at 60 V for 70 minutes. The gel was then dried and exposed to PhosphorImager screen overnight. The percentage of DNA strand exchange product was calculated as labeled product divided by total labeled input DNA in each lane.

ATP Hydrolysis Assays. The assay was carried out essentially as described, [21]. Briefly, BRCA2 at concentrations 0-100 nM was preincubated with 3' tail DNA (0.9 μM nucleotides, nt) in 10 μL of buffer containing 20 mM TrisHCl (pH 7.5), 4 mM $MgCl_2$, 1 mM DTT, 0.5 mM ATP, and 20 μCi/ml [γ^{32}P] ATP. The reaction was started by adding RAD51 (0.3 μM) or storage buffer, and further incubated at 37 °C for 90 min. 2 μL aliquots were spotted onto a polyethyleneimine (PEI) thin layer chromatography (TLC) plate (EMD Chemicals) at each time point. The spots were air-dried and the plates were developed in 1 M formic acid and 0.5 M LiCl. The amount of ATP hydrolyzed was determined from dried plates using a Molecular Dynamics Storm 840 PhosphorImager. The percentage of ATP hydrolysis was quantified using ImageQuant software and any residual signal from the BRCA2-only lanes was subtracted from the RAD51+BRCA2 lanes. The results were plotted using GraphPad Prism 5.0b.

Single-Stranded DNA Annealing Assays. Cold 167-mer (RJ-167mer, IDT Ultramer, PAGE purified) at 8 nM (molecules) and 5' radio-labeled 40-mer (RJ-Oligo1, Sigma, PAGE purified) at 4 nM (molecules) were each incubated separately in 10 μL reactions

containing 25 mM TrisOAc (pH 7.5), 1 mM $MgCl_2$, and 1 mM DTT for 5 minutes with or without RPA (100 nM). The 40-mer is complementary to the 167-mer at the 3' end. All incubations were at 37 °C. The oligonucleotides were then incubated with either BRCA2 (40 nM), RAD52 (100 nM), or protein storage buffer for 5 minutes. The two separate reactions were then mixed and incubated for 1, 5, 15, or 30 minutes to allow for annealing. At the indicated time points aliquots were removed and added to stop buffer (4 mg/mL Proteinase K, 1% SDS, and 0.4 μM unlabeled 40-mer (RJ-Oligo2)) complementary to RJ-Oligo1 for 15 minutes. Loading dye was then added to the samples and they were run on 6% polyacrylamide gels in TAE buffer for one hour at 60 V. The gels were dried onto DEAE (Whatman) paper and exposed to a PhosphorImager screen overnight. The screens were scanned on a Storm 860 system (Molecular Dynamics) and bands quantified using ImageQuant. The percentage of annealed product was calculated as the radio-labeled product divided by the total radio-labeled input DNA in each lane.

Biotinylated DNA pull-down assay. An oligonucleotide substrate composed of the same sequence as RJ-PHIX-42-1 but containing a 3' biotin modification (Biotin-TEG) was obtained from Eurofins MWG Operon. The 3' biotin modified oligonucleotide was purified by polyacrylamide gel electrophoresis (PAGE) and annealed at a 1:1 molar ratio to RJ-167-mer to create the biotinylated 3' tailed DNA substrate. RAD51 was preincubated with or without BRCA2 for 15 minutes at 37 °C in Buffer 'S': 25 mM TrisOAc (pH 7.5), 1 mM $MgCl_2$, 0.1 μg/μL BSA, 2 mM ATP, and 1 mM DTT. The proteins were then added to a mixture of both the biotinylated 3' tailed DNA and a heterologous 90 b.p. duplex DNA (PAGE purified Oligo#90: 5'-CGGGTGTCG GGGCTGGCTTAACTATG

CGGCATCAGAGCAGATTGTACTGAGA GTGCACCATATGCGGTGT GAAATACCGCACAGATGCGT-3' annealed 1:1 to PAGE purified Oligo#60: 5'-ACGCATCTGTGCGGTATTTCACACCGCATATGGT GCACTCTCAG TACAATCTGCTCTGATGCCGCATAGTTAAGCC AGCCCCGACACCCG-3') derived from the pUC19 plasmid sequence. The incubation was continued for 5 minutes at 37 °C followed by capture of the DNA-protein complexes by adding 2.5 µL of pre-washed MagnaLink Streptavidin magnetic beads (SoluLink) in buffer S supplemented with 0.1% Igepal CA-630. After binding to the beads at 25 °C for 10 minutes, the bead complexes were washed with buffer S containing 0.1% Igepal CA-630 and resuspended in 15 µL protein sample buffer, heated at 54 °C for 4 minutes, and loaded onto a 4-15% gradient SDS- polyacrylamide gel. The amount of RAD51 protein bound and eluted from the biotinylated DNA substrate was determined by western blot using a monoclonal antibody specific to human RAD51 (14B4, Novus).

Supplemental References

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