Supplemental Information

Small Molecule Disruption of RAD52 Rings as a Mechanism for Precision Medicine in BRCA Deficient Cancers

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Supplemental Experimental Procedures

Dissociation of RAD52-ssDNA complexes

40 nM RAD52 WT, reaction buffer C and 10 nM ssDNA (RP316FAM) were mixed together in a total volume of 20 μl and incubated for 60 min at room temperature. 30 μM 6-OH-dopa was then added and FP was determined after 2 min using a using a Clariostar (BMG Labtech) plate reader.

EMSA

Initial hit validation. 120 nM RAD52 WT was pre-incubated with 60 μM compound then mixed with reaction buffer D (2.5 mM TrisHCl pH 7.5, 0.1 mM DTT, 3 mM NaCl, 0.001% NP-40, 0.1 mg/mL BSA, 0.5 mM $MgCl_2$, and 10% glycerol). 12 nM Cy3-conjugated ssDNA (RP334Cy3) was then added and reactions were incubated for 60 min on ice. Reactions were then resolved in non-denaturing 5% polyacrylamide gels. Cy3-conjugated ssDNA was visualized using a MultiImage III fluorescence imager (Alpha Innotech) using FluorChem Q software. Percent inhibition was determined by calculating the relative fluorescence intensity of shifted ssDNA bands.

ssDNA binding. 120 nM RAD52 WT or 400 nM RAD52 1-209 was pre-incubated with 60 μM 6-OH-dopa, then mixed with reaction buffer D and 12 nM Cy3-conjugated ssDNA (RP334Cy3) and analyzed as described above.

Dissociation of RAD52-ssDNA complexes. 120 nM WT RAD52 was incubated with 12 nM Cy3-conjugated ssDNA (RP334Cy3) for 60 min at room temp in reaction buffer D, then indicated amounts of 6-OH-dopa were added for an additional 30 min in a total volume of 20 μl. Reactions were then resolved as described above.

Competition Assay. 120 nM RAD52 WT in reaction buffer D was simultaneously mixed with 25 μM 6-OH-dopa and indicated amounts of Cy3-conjugated ssDNA (RP334Cy3) for 60 min in a total volume of 20 μl. Reactions were resolved as described above.

Agarose gel analysis. Indicated amounts of RAD52 WT were mixed with 20 nM Cy3-conjugated ssDNA (RP334Cy3) in 20 μl of reaction buffer D at room temp for 30 min. Reactions were fixed with the addition of 2 μl of 2% glutaraldehyde. Reactions were resolved in a 0.8% agarose gel containing 10% glycerol.

Single-strand annealing

75 nM RAD52 WT was pre-incubated with 60 μM 6-OH-dopa in 10 μl of reaction buffer E (50 mM TrisHCl pH 7.5, 1 mM DTT, 0.1 mg/mL BSA, 0.5 mM MgAc, 10% glycerol). 20 nM 5'-^{32}P radio-labeled ssDNA (RP40) was added, then after 5 min 20 nM complementary ssDNA (RP40C) was added. After 30 s annealing was quenched by the addition of 200 nM of cold (unlabeled) ssDNA (RP40). RAD52 was then degraded by the addition of 2 μl stop buffer (100 mM TrisHCl pH 7.5, 10 mg/mL proteinase K, 100 mM EDTA, and 1.5% SDS) and incubated for 15-30 min at 37° C. Radio-labeled DNA was resolved in 12% non-denaturing polyacrylamide gels and visualized by autoradiography. Reactions were performed at 37° C.

Gel filtration

4.4 μM RAD52 WT in the presence or absence of 44 μM 6-OH-dopa was incubated in 600 μl of buffer containing 20 mM TrisHCl pH 8.0, 200 mM NaCl, 1 mM EDTA, and 10% glycerol, then injected into a HiLoad 16/600 Superdex 200 pg column (GE Health Sciences) at 4° C using an AKTA L1 Pure (GE Health Sciences) with an automated fraction collector and multichannel peristaltic pump. Using a UV monitor detecting at 280 nM, protein peaks were plotted versus elution volume.

Native gel analysis

2.5 μM RAD52 WT or 3.5 μM RAD52 1-209 with indicated amounts of 6-OH-dopa, DL-o-tyrosine, or RU-0098062 was incubated in 20 μl of buffer containing 25 mM TrisHCl pH 8.8, 0.01% NP-40, 1 mM DTT, 0.675 M NaCl, 0.2% Tween-40, and 10% glycerol for 60 min at room temperature. Reactions were then resolved in a 4-

15% Mini-PROTEAN TGX native gel (Bio-Rad) in Tris-glycine buffer pH 8.9. Silver staining was used to visualize proteins.

Isothermal calorimetry

Isothermal calorimetry was performed using a MicroCal Auto-iTC200 (Malvern, Worcestershire, UK). Both components, RAD52 1-209 and 6-OH-dopa were prepared in identical buffer which consisted of 20 mM TrisHCl pH 7.0, 250 mM NaCl, 1 mM DTT, 10% glycerol, and 0.01% Igepal. The 200 µl reaction cell equilibrated at 25° C was loaded with 20 µM RAD52 1-209. The titration consisted of 16 injections of 2.5 µl of a 2 mM solution of 6-OH-dopa every 5 s. A reference titration of 6-OH-dopa was performed into the sample cell loaded with reaction buffer to account for the heat of dilution and was carried in the same experimental conditions. Data were analyzed using Origin 7.0 (OriginLab, Northampton, MA).

Light scattering analysis

Measurements were made with a Protein Solutions DynaPro temperature-controlled microsampler. The RAD52 1-209 protein was prepared in the absence of detergent and dialyzed samples were adjusted to the desired concentration (0.6 mg/ml), and particulates were removed by centrifugation for 20 min at 14,000 x g at room temp. The protein concentration was then determined directly using absorbance at 280 nm and a calculated molar extinction coefficient, taking the average of three separate readings. All samples were analyzed under identical conditions of 20° C in a buffer of 20 mM TrisHCl 8.0, 1 mM DTT, 10% glycerol, and either 1 M NaCl (high salt), or 0.15 M NaCl (low salt). Identical samples were treated with an 80-fold molar excess of 6-OH-dopa for 30 min on ice prior to light scattering analysis. The hydrodynamic radius and apparent molecular mass (MW-R) were calculated from dynamic light scattering measurements (at least 30 acquisitions per protein sample) using the DynaPro software version 5. The regularization graphs, calculated using an isotropic spheres model, portray multiple forms in solution as a histogram and percent mass on the Y-axis. A monodisperse sample was generally reflected in one or two histogram bars, while more polydispersed forms have several bars in the peak. The calculated MW-R for each peak was labeled above the histogram peak.

Cell lines and cell culture

U2OS cell lines with homologous recombination (DR-GFP), non-homologous end joining (EJ2-GFP), single strand annealing reporters (SA-GFP) were a kind gift from J. Stark(Gunn et al., 2011; Gunn and Stark, 2012). U2OS, MDA-MB236 and VC8 cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM). HCC1937 cells were grown in RPMI 1640 medium (Corning, USA). All media were supplemented with 20% fetal bovine serum (FBS), 2mM L-glutamine, 100 μg ml^{-1} streptomycin and 100 U ml^{-1} penicillin. BCR-ABL1-positive 32Dcl3 cell line was cultured in IMDM plus 10% FBS supplemented with 15% WEHI medium as a source of IL-3. Capan-1 cell line was cultured in IMDM plus 10% FBS. AML and CML cells were cultured in StemSpan H3000 media supplemented with a cocktail of growth factors (100 ng/ml stem cell factor, 20 ng/ml interleukin3 [IL-3], 100 ng/ml fms-related tyrosine kinase 3 ligand, 20 ng/ml granulocyte colony-stimulating factor, 20 ng/ml IL-6). All cell lines were cultured at a constant temperature of 37° C in a 5% carbon dioxide (CO_2) humidified atmosphere.

DNA repair reporter assays

U2OS cells (1×10^5) were plated in triplicates on 24 well plate, treated with indicated doses of compounds or DMSO (vehicle control) and transfected 24 hours later with 0.8 μg pCMV-3xNLS-I-SceI or 0.8 μg control vector pCMV-3xNLS using Lipofectamine 2000 (Invitrogen). GFP^+ frequencies were measured 3 days post transfection by FACS using GUAVA flow cytometer (Millipore) in triplicates and corrected for transfection efficiency and background events. Transfection efficiency was measured simultaneously by parallel transfection with 0.05 μg wt GFP expression vector (pCMV-3xNLS-GFP). For siRNA experiments, cells were transfected with 20 pmol siRNA + 0.5 μg of pCMV-3xNLS-I-SceI (or control vector, GFP expression vector) per well.

Western blotting

For western blotting analysis, a portion of cells from the reporter or survival assays performed with siRNA was used. Whole-cell lysates were prepared by lysing the cells in RIPA buffer (25 mM TrisHCl, 125 mM NaCl, 1% Nonidet-P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and a Protease Inhibitor Cocktail (Pierce)). Equal amounts (20 μg) of cell lysates were separated by SDS–polyacrylamide gel electrophoresis and then transferred to membrane overnight at 4° C, immunoblotted with antibodies against

RAD52 (Santacruz) and Actin (Abcam). Blots were stained with an enhanced chemi luminescence detection kit (ECL-Plus, Amersham Biosciences). In the case of western blot from native gel, purified protein incubated with or without 6-OH-dopa was separated by native gel electrophoresis then transferred to membrane overnight at 4° C, immunoblotted with antibody against His-tag (Abcam).

Survival assays

For the growth assays, cells were plated in 384-well plate at 400 or 800 cells/well and treated with 6-OH-dopa at the indicated concentration 6 hours later. On day 3, cells were lysed with CellTiter-Glo® Luminescent Cell Viability Assay reagent (Promega) and luminescence was read using EnVision plate reader. Percent cell growth was calculated relative to DMSO treated cells. For clonogenic assays, cells were seeded at 1,000 cells per well on 6 well plates in triplicates. Twenty four hours later, 0 μM, 2.5 μM, 5 μM, or 10 μM of 6-OH-dopa was added in the case of MDA-MB-436 cells and 0 μM, 5 μM, 10 μM, or 20 μM in VC8/V79 and CAPAN-1 cells, followed by treatment with the compound every 48 hours. Colonies were fixed with Acetic acid/methanol (1:7) and stained with 0.5% crystal violet solution. For the HCC1937 viability assay, both BRCA proficient and deficient cells were plated on day 0 in triplicate at 10,000 cells/well in 24 well plates. On days 1 and 3, cells were treated with 0 μM, 2.5 μM, 5 μM, or 10 μM 6-OH-dopa. Cells were counted on day 4 with a hemocytometer using Trypan Blue exclusion, and immediately were plated for clonogenic assay in 6 well plates at a density of 1,000 living cells/well. After two weeks, colonies were fixed/stained with 0.05% of 10mg/ml ethidium bromide in 50% ethanol. AML patient cells were previously selected based on having either high (BRCA proficient) or low (BRCA deficient) BRCA1/2 expression levels(Cramer-Morales et al., 2013)(Supplementary Fig. 6). A pool of 3 patient cells, either BRCA proficient or deficient, were plated at 10,000 cells per well in 96 well plates on day 0, treated with 6-OH-dopa (0 μM, 2.5 μM, 5 μM, or 10 μM) or olaparib (0 μM, 1.25 μM, 2.5 μM, or 5 μM) on days 0 and 2, and living cells were counted on day 4 with Trypan Blue. Lin-CD34+ primary CML and normal cells were obtained by magnetic sorting using the EasySep negative selection human progenitor cell enrichment cocktail followed by human CD34 positive selection cocktail (StemCell Technologies). For clonogenic assay in HCC1937, AML and CML cells, colonies were visualized with Alphaimager gel imager (Alpha Innotech). For the viability assay, CD34+ CML and normal cells were plated at 10,000 cells per well in 96 well plates. On days 0 and 2, they were treated with 0 μM, 2.5 μM, 5

μM, or 10 μM 6-OH-dopa, and living cells were counted on day 4 with Trypan Blue. Immediately following counting, the colony forming cell (CFC) assay was performed. In brief, all cells from each subpopulation were plated in duplicate in Methocult H4230 (StemCell Technologies). Colonies were counted after 7 days.

Microscopy and immunofluorescence

For detection of GFP-RAD52 foci, we used a previously published murine hematopoietic BCR-ABL1-positive 32Dcl3 cell line that stably expresses GFP-RAD52(Cramer-Morales et al., 2013) and MDA-BRCA1, 293T cells transiently transfected with GFP-RAD52. BCR-ABL1-positive 32Dcl3 cells were plated in 12 well plates at 500,000 cells/ml and pretreated for 4 hours with 0, 2.5, 5, or 10 μM 6-OH-dopa. After 4 hours, the cells were treated with 3ug/ml cisplatin for 16 hours. Following cisplatin treatment, cytospins were prepared using polylysine coated slides (Thermo Scientific). DNA was counterstained with DAPI. Foci were visualized with an inverted Olympus IX70 fluorescence microscope equipped with a Cooke Sensicam QE camera (The Cooke Co., Auburn Hills,MI, USA). Staining and images from 30-40 cells/group were processed using SlideBook 3.0 (Intelligent Imaging Innovation). MDA-BRCA1 and 293T cells expressing GFP-RAD52 were treated with 0, 5 or 10 μM 6-OH-dopa for 72 hours, irradiated with 5 Gy, fixed in 10% formaldehyde solution at room temp for 30 min, followed by 10 min permeabilization in PBS containing 0.5% Triton X-100. Cells were washed in PBS, followed by PBS with 10% FBS to block non-specific binding by antibody. Cells containing GFP-RAD52 were counter stained by DAPI and visualized directly after fixation and permeabilization with confocal microscope (Leica microsystems). Nuclei containing > 5 RAD52 foci were scored as positive. For γH2AX staining, MDA-MB 436 cells were treated with 0, 5 or 10 μM 6-OH-dopa for 72 hours. Cells were fixed in 4% formaldehyde solution for 1 hr at room temp, followed by permeabilization with 0.1% Triton X-100. Cells were stained for γH2AX with rabbit polyclonal antibody (Milipore) overnight at 4° C, followed by incubation with goat anti rabbit FITC conjugated secondary antibody (Thermoscientific) and counterstaining with DAPI. Cells were visualized by confocal microscopy (Leica microsystems). Nuclei containing > 5 γH2AX foci were scored as positive.

RAD52 knockdown

U20S/MDA-436 cells (1×10^5) were plated on 6 well plate and 100 pmol of siRNA for RAD52 (Dharmacon) was transfected using Lipofectamine (Life Technologies) according to manufacturer's instructions. siRNA treated cells were used for assays 24 hours after transfection.

Apoptosis assay

VC8 and V79 cells were treated with 0, 5, or 10 μM 6-OH-dopa for six days and stained for Annexin V according to manufacturer's instructions (Affymetrix ebioscience). Cells were counterstained with propidium iodide and quantified for Annexin V staining by FACS using GUAVA flow cytometer (Millipore). HCC1937 and HCC1937 BRCA1 complemented cells were treated with 0, 5, 10 or 20 μM 6-OH-dopa for six days and stained for Annexin V according to manufacturer's instructions (Affymetrix ebioscience). Cells were counterstained with propidium iodide and quantified for Annexin V staining by FACS using GUAVA flow cytometer (Millipore).

DNA

RP40 was 5'-^{32}P labeled using T4 polynucleotide kinase (NEB) and [γ-^{32}P] ATP (PerkinElmer). DNA oligonucleotides (Integrated DNA Technologies) are as follows (5'-3'):

RP316, FAM-TTTTTTTTTTTTTTTTTTTTTTTTTTTT;

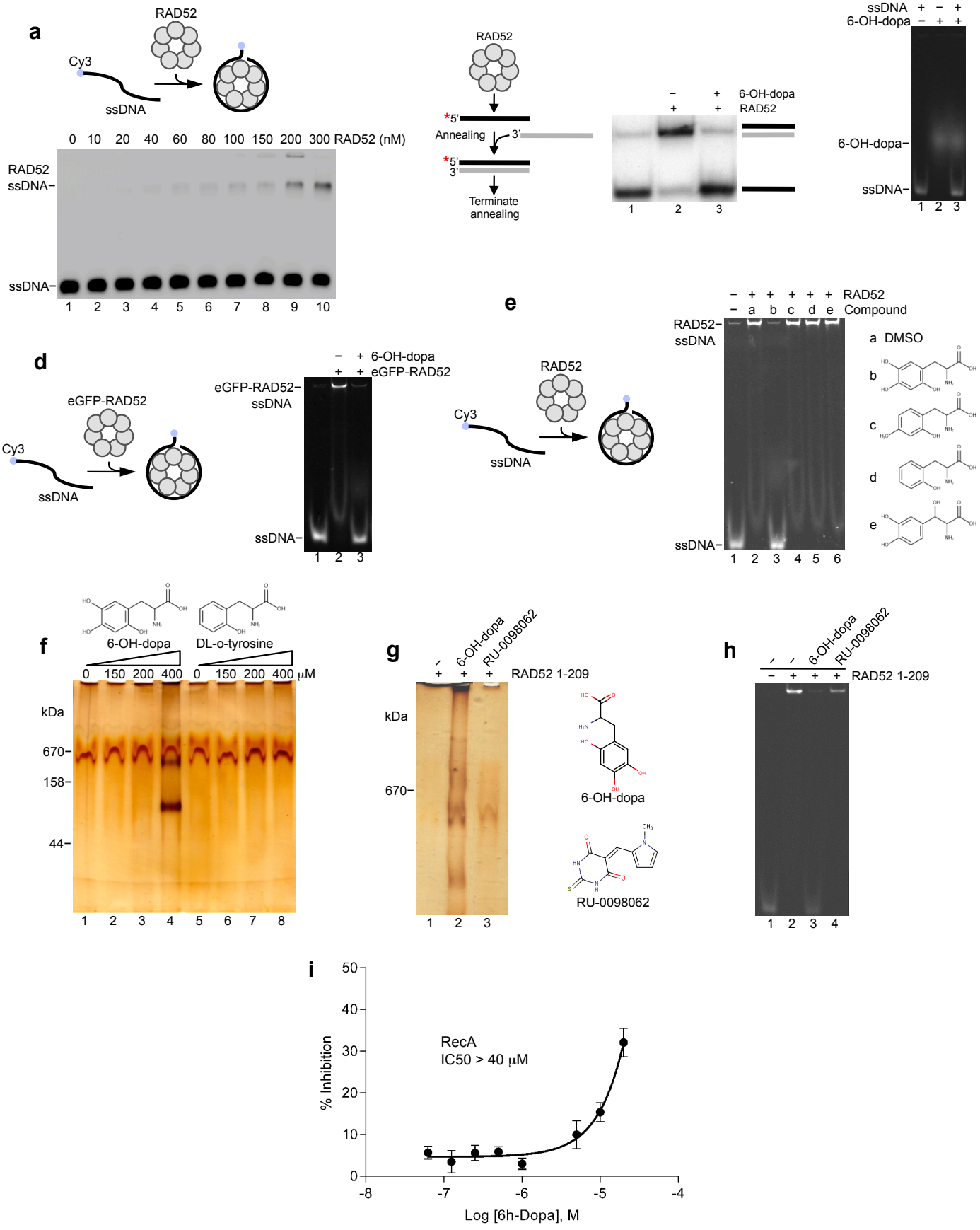
RP334, Cy3-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT;

RP40, TTATCATCGATATTAATACGACTCACTATAGGGAGGAGGGAGGGATGAGAATATT;

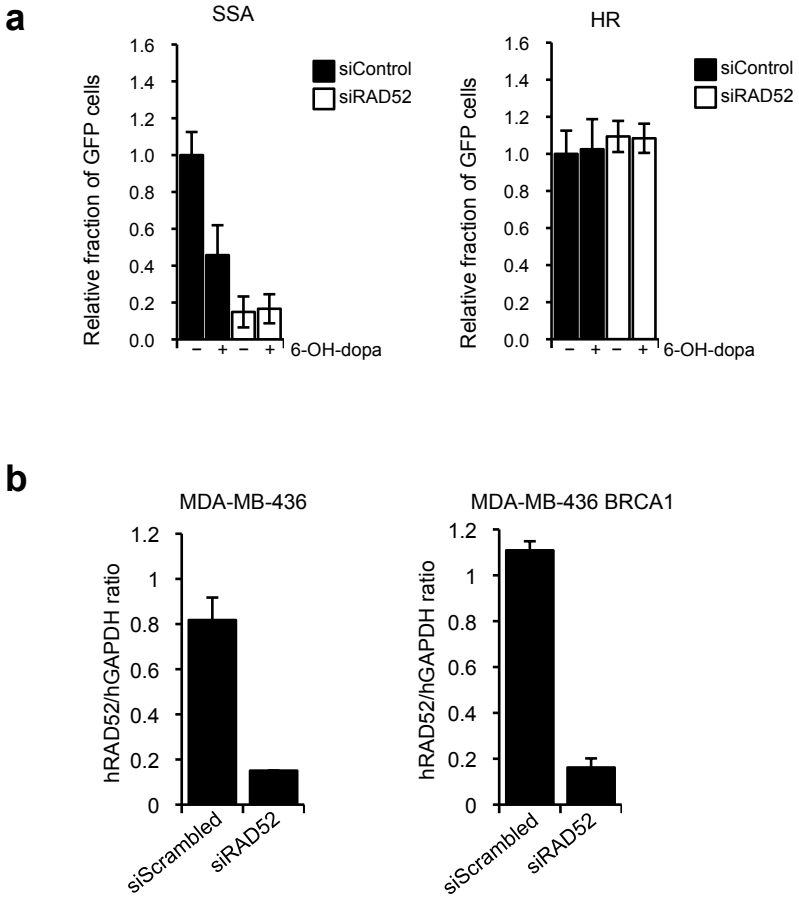
RP40C, AATATTCTCATCCCTCCCTCCTCCCTATAGTGAGTCGTATTAATATCGATGATAA

Supplementary Figures

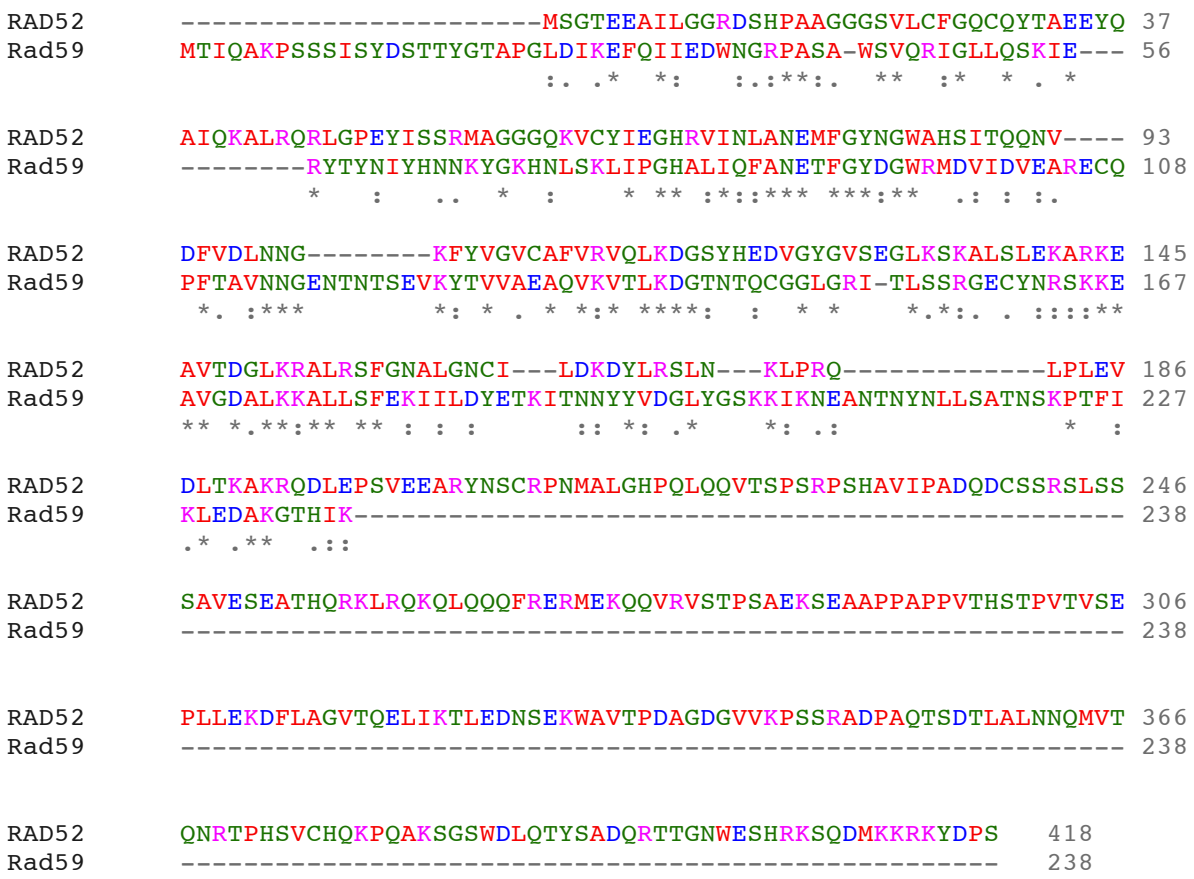
Supplementary Fig. 1



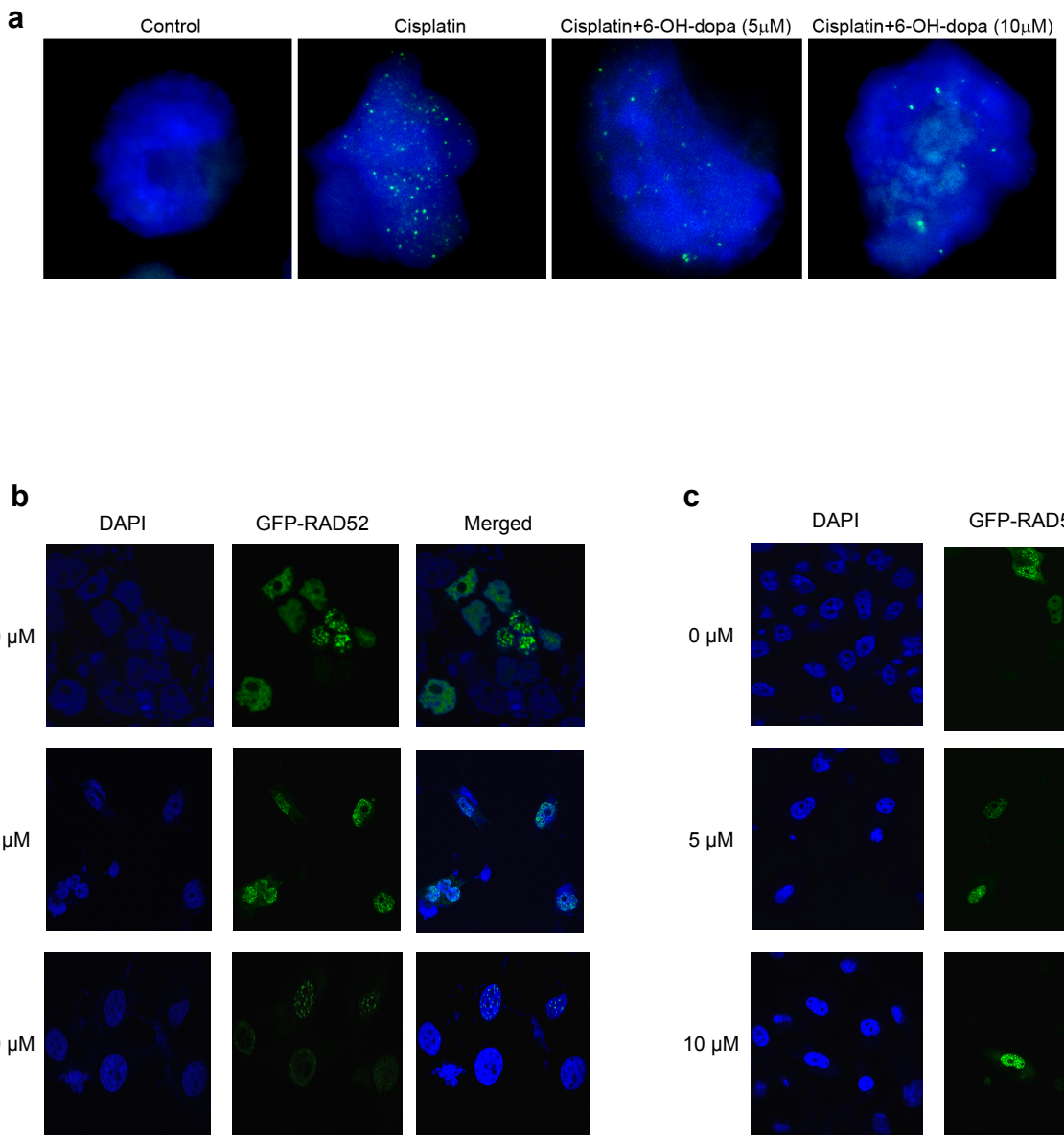
Supplementary Fig. 2



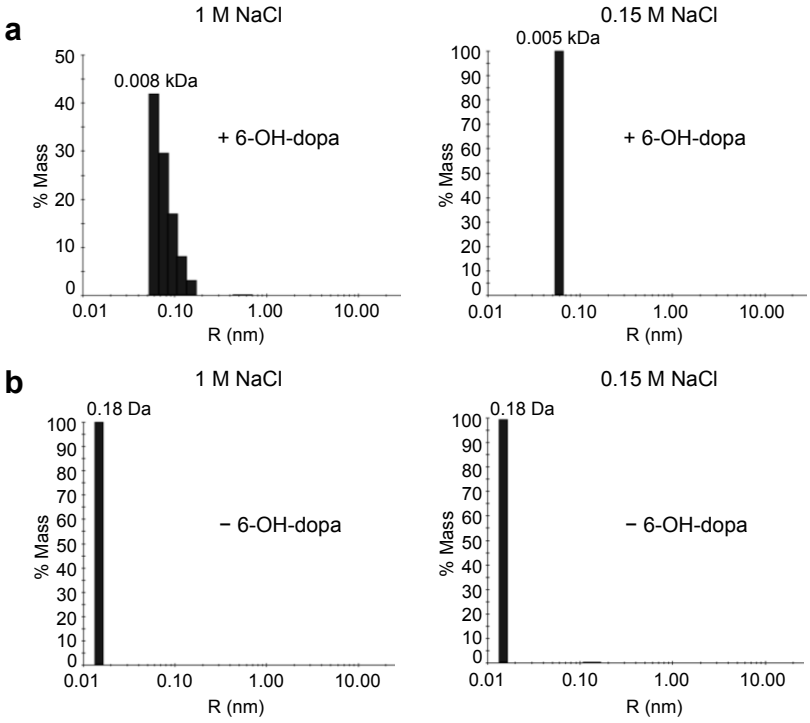
Supplementary Fig. 3



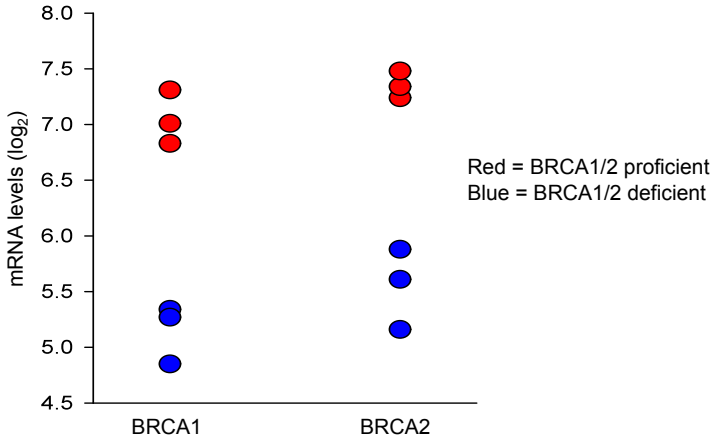
Supplementary Fig. 4



Supplementary Fig. 5



Supplementary Fig. 6



Supplementary Figure Legends

Supplementary Figure 1 RAD52 and 6-OH-Dopa Biochemical Controls. Figure S1 related to Fig. 1,2, and 5.

a, Resolution of RAD52-ssDNA complexes in an agarose gel. Schematic of assay (top). Electrophoresis mobility shift assay (EMSA) showing RAD52-ssDNA complex formation. RAD52-ssDNA complexes were resolved in a non-denaturing agarose gel (bottom). **b,** 6-OH-dopa inhibits RAD52 single-strand annealing (SSA). Schematic of assay (left). Non-denaturing gel showing RAD52 mediated SSA in the presence (lane 3) and absence (lane 2) of 60 μM 6-OH-dopa (right). * = ^{32}P. **c,** 6-OH-dopa has no effect on the mobility of ssDNA in the EMSA. Non-denaturing gel showing the mobility of Cy3 conjugated ssDNA (lane 1), 6-OH-dopa (lane 2), and Cy3 conjugated ssDNA and 6-OH-dopa combined (lane 3). **d,** Control showing ssDNA binding activity of eGFP-RAD52. Schematic of EMSA (left). Non-denaturing gel of EMSA in the presence (lane 3) and absence (lane 2) of 60 μM 6-OH-dopa. **e,** Small molecules similar to 6-OH-dopa do not inhibit RAD52. Schematic of EMSA (left). Non-denaturing gel of EMSA in the presence of 60 μM of the indicated small molecules. b = 6-OH-dopa; c = Beta-(2-Hydroxy-4-methylphenyl) alanine, d = DL-*o*-tyrosine; e = L-DOPS. **f-h,** Small molecule effects on RAD52 composition. **f,** Silver stained non-denaturing gel of RAD52 1-209 following incubation with indicated amounts of 6-OH-dopa and DL-*o*-tyrosine. Lower molecular weight product in lane 4 demonstrates 6-OH-dopa dissociation of RAD52 1-209 undecamer rings. **g,** Silver stained non-denaturing gel of RAD52 1-209 following incubation with buffer (lane 1), 400 μM of 6-OH-dopa (lane 2) or RU-0098062 (lane 3). Lower molecular weight product in lane 2 demonstrates 6-OH-dopa dissociation of RAD52 1-209 undecamer rings. **h,** Non-denaturing gel of EMSA showing small molecule inhibition of RAD52 1-209 ssDNA binding. EMSA was performed in the presence of RAD52 1-209, Cy3-conjugated ssDNA and the indicated small molecules. **i,** 6-OH-dopa has a small effect on RecA. Plot showing percent inhibition of RecA as a function of 6-OH-dopa concentration. Data shown as average ± s.d. from 3 independent experiments.

Supplementary Figure 2 RAD52 and 6-OH-Dopa Cellular Controls. Figure S2 related to Fig. 2 and 6.

a, 6-OH-dopa has no effect on single-strand annealing or homologous recombination in RAD52 depleted cells. U2OS cells harboring the single-strand annealing (left) or homologous recombination (right) GFP reporter were treated with DMSO or 5 μm of 6-OH-dopa followed by co-transfection with I-Sce I expressing vector and siRAD52 or scrambled siRNA as a control. The percentage of GFP positive cells was assessed 72 hours post transfection. Data shown as average ± s.e.m. from 3 independent experiments. **b,** Demonstration of RAD52 knockdown in MDA-MB-436 cells via siRNA. Plots showing the ratio of mRNA levels for RAD52 and GAPDH following transfection of MDA-MB-436 cells (left) and MDA-MB-436 cells complemented with BRCA1 (right) with the indicated siRNAs. mRNA levels were determined by real-time quantitative PCR.

Supplementary Figure 3 Sequence alignment of *Homo sapiens* RAD52 and *Saccharomyces cerevisiae* Rad59. Figure S3 related to Fig. 2.

The indicated amino-acid sequences were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/; European Bioinformatics Institute) default settings. * = identical residues, : = residues sharing very similar properties, . = residues sharing some properties. Red, small and hydrophobic; Blue, acidic; Magenta, basic; Green, hydroxyl, sulfhydryl, amine. RAD52 and Rad59 share 31.5% sequence identity.

Supplementary Figure 4 Representative fluorescent microscopy images showing 6-OH-Dopa suppression of GFP-RAD52 foci. Figure S4 related to Fig. 3.

a, IR induced GFP-RAD52 foci in BCR-ABL1-positive 32Dcl3 cells. Cells were transfected with GFP-RAD52, pre-treated with indicated amounts of 6-OH-dopa for 4 hours, followed by treatment with 3ug/mL cisplatin for 16 hours. DAPI (blue); RAD52 (green). **b,c** IR induced GFP-RAD52 foci formation in the presence of indicated amounts of 6-OH-dopa in 293T (b) and MDA-MB-436 BRCA1 complemented (c) cells. Representative images showing GFP-RAD52 foci at 6h. Cells containing at least 5 distinct foci were denoted and counted. Left (DAPI-Blue); Middle (GFP-RAD52-green); Right (Merged).

Supplementary Figure 5 Controls for light scattering analysis. Figure S5 related to Fig. 5.

a,b, Plots showing light scattering data of buffer with (a) and without (b) 6-OH-dopa in the presence of 1 M (left) and 0.15 M (right) NaCl.

Supplementary Figure 6 Levels of BRCA1 and BRCA2 mRNA in acute myeloid leukemia (AML) patient cells. Figure S6 related to Fig. 6. Plots showing microarray detection of mRNA for BRCA1 and BRCA2. Each circle represents a different patient. Red circles = BRCA1/2 proficient, blue circles = BRCA1/2 deficient. Data adapted from previous studies(Cramer-Morales et al., 2013).