Role of the RAD51-SWI5-SFR1 Ensemble in Homologous Recombination

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Running title: *Functional significance of SWI5-SFR1 and RAD51 interaction*

Supplementary Data

Supplementary Materials and Methods

DNA substrates

For DNA strand exchange assay, the 80-mer Oligo 1: 5'TTATGTTCATTTTTTATATCCTTTACTTTATTTTCTCTGTTTATTCATTTAC TTATTTTGTATTATCCTTATCTTATTTA was used for assembling the presynaptic filament. To prepare the target duplex 40-mer dsDNA, Oligo 2: 5'TAATACAAAATAAGTAAATGAATAAACAGAGAAAATAAAG was 5' end labeled with polynucleotide kinase (New England Biolabs) and [γ-^{32}P] ATP (PerkinElmer). Following the removal of the unincorporated nucleotide with a Spin 6 column (Bio-Rad), the radiolabeled oligonucleotide was annealed to its exact complement, by heating the mixture of the two oligonucleotides at 85°C for 10 min and slow cooling to 23°C. The resulting duplex was purified from a 10% polyacrylamide gel by electro-elution and filter-dialyzed in a Centricon-10 concentrator (Millipore) at 4°C into TE buffer (10 mM Tris-HCl, pH 8.0, and 0.5 mM EDTA).

Plasmids

***RAD51, RAD51 F86E, RAD51 A190L/A192L, and RAD51 S208E/A209D expression plasmids*:**

The mouse RAD51 cDNA was inserted into a pRSFDuet and pET51b vectors (Novagen) to add $(His)_6$ tag and Strep tags to the amino-terminal end of the protein, respectively. RAD51 F86E, RAD51 A190L/A192L, and RAD51 S208E/A209D expression plasmids were generated from pET51b-RAD51 by site-direct mutagenesis.

***SWI5-SFR1, SWI5^{dC9}-SFR1, SWI5^{dC20}-SFR1, SWI5 F83A-SFR1, SWI5 L85A-SFR1, SWI5 F83A/L85A-SFR1 and SWI5-SFR1^{dN202} expression plasmids*:**

For the co-expression of SWI5 and SFR1, the SWI5, SWI5^{dC9}, SWI5^{dC20}, SWI5 F83A, SWI5 L85A, or SWI5 F83A/L85A cDNA was introduced into the pRSFDuet vector that harbors $(His)_6$-tagged SFR1 or SFR1^{dN202}.

GST-BRC4 expression plasmid:

The cDNA that codes for mouse BRC4 peptide (KEPTLLSFHTASGKKVKIMQESLDKVKNLFDETQ) was inserted into the BamHI and NotI site of pGEX-6P-1 (GE Healthcare).

Protein expression and purification

Expression and purification of RAD51 and mutant variants

Amino-terminal $(His)_6$- or Strep-tagged mouse RAD51 was overexpressed and purified to near homogeneity from RecA-deficient *E. coli* strain BLR using our published procedure (27). For Strep-tagged mutant variants: RAD51 F86E, A190L/A192L and S208E/A209D were expressed and purified in the same way. Briefly, clarified cell lysate was subjected to Streptactin affinity step, followed by chromatographic fractionation in columns of Macrohydroxyapatite and Source Q. We note that $(His)_6$-tagged RAD51 was used in all the biochemical assays except the affinity pulldown experiments. Strep-tagged RAD51 was used only in affinity pulldowns with $(His)_6$-tagged SWI5-SFR1.

Expression and purification of SWI5-SFR1 and mutant variants

The wild type and mutant protein complexes with a $(His)_6$ tag at the amino-terminal end of SFR1 were expressed in *E. coli* Rosetta cells. SWI5-SFR1 and SWI5 F83A/L85A-SFR1 were purified with the same purification protocol as published (27). For the purification of SWI5^{dC9}-SFR1, SWI5 F83A-SFR1 and SWI5 L85A-SFR1 complexes, after the Talon affinity step, chromatographic fractionations in Source Q was conducted. The SWI5-SFR1^{dN202} complex was eluted from Talon affinity step, followed by chromatographic fractionation in Source Q and gel filtration in HiLoad 16/60 superdex 75. SWI5^{dC20}-SFR1 complex was fractionated in columns of Q Sepharose, Ni^{2+}-NTA agarose, Mono Q and Macrohydroxyapatite. Peak fractions were pooled and concentrated to 5-10 mg/ml in a Centricon-10 concentrator.

Expression and purification of GST-BRC4

Amino-terminally GST-tagged BRC4 from the mouse BRCA2 protein was overexpressed in *E. coli* BL21 Star. For protein purification, 12 g of cell pellet was suspended in 60 mL cell breakage buffer (25 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 150 mM KCl, and 1 mM 2-mercaptoethanol) containing the following protease inhibitors: aprotinin, chymostatin, leupeptin and pepstatin A at 3 μg/ml each, and 1 mM PMSF and Benzamidine. After centrifugation (100,000 X g for 60 min), the cell lysate was subjected to an affinity step by GST Sepharose^{TM} 4 Fast flow (GE Healthcare). The matrix was poured into a column and then washed with cell breakage buffer containing 300 mM KCl. GST-BRC4 was eluted with 10 mM of reduced glutathione in cell breakage buffer. The eluate was filter-dialyzed to remove the glutathione and concentrated to 20 mg/ml in a Centricon-10 concentrator.

Peptides

Mouse BRC4 peptide (KEPTLLSFHTASGKKVKIMQESLDKVKNLFDETQ) was purchased from Kelowna International Scientific Inc. and purified by HPLC, and their sequences were confirmed by mass spectroscopy.

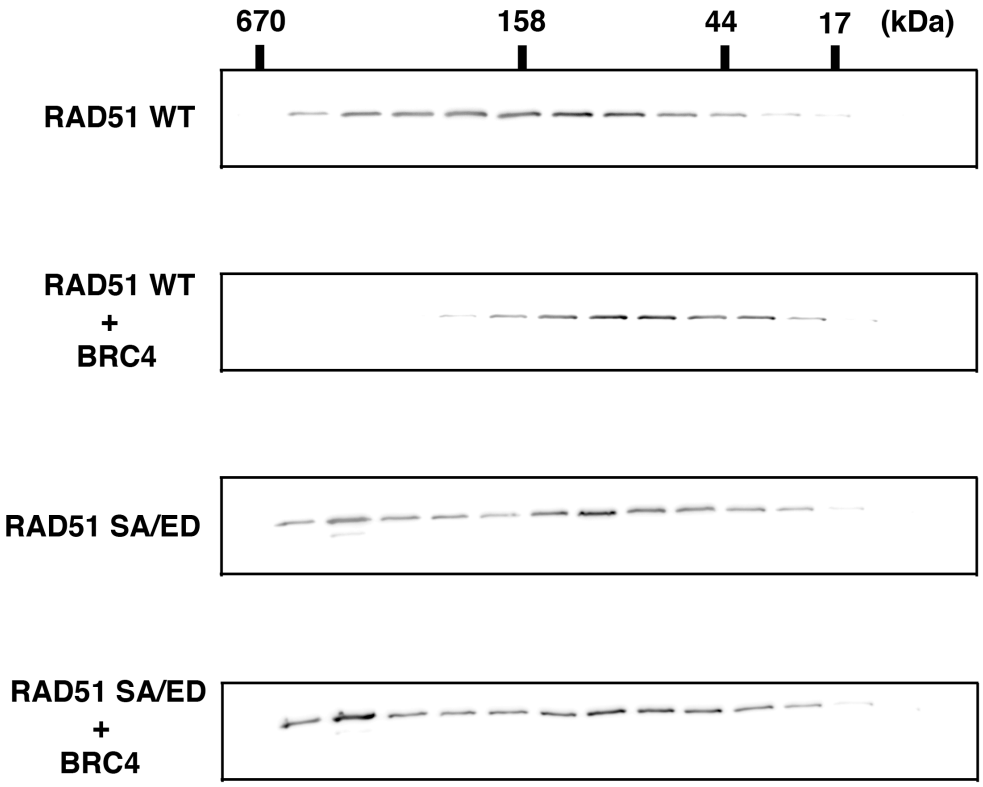
Gel filtration analysis

RAD51 or RAD51 S208E/A209D (10 μg each) with or without BRC4 (5 μg) was diluted to 0.5 ml with buffer C (25 mM Tris-HCl, pH 7.5, 10% glycerol, 0.01% Igepal, 300 mM KCl, 1 mM 2-mercaptoethanol) and analyzed by gel-filtration chromatography through a 24-ml Superdex 200 10/300 column (Amersham) equilibrated with the same buffer. Fractions (0.5 ml each, 24 ml total volume) were analyzed by SDS-PAGE and Western blotting. The method of determining the oligomeric status of mouse RAD51 F86E or A190L/A192L was described by Esashi et al. for human counterparts (31).

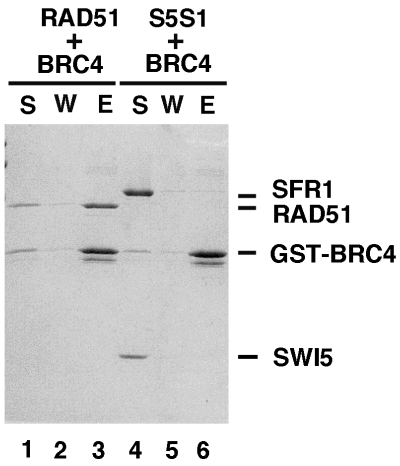
Electron microscopy

Reaction mixtures were assembled with combinations of 1 μM RAD51 with 1 μM SWI5-SFR1 or SWI5F83A/L85A-SFR1 and the 80-mer ssDNA (6 μM nucleotides) as described for the DNA strand exchange assay, except that BSA was omitted from the reaction buffer. After 30 min incubation, 3 μl of reaction was applied onto 400-mesh grids coated with fresh carbon film that had been glow-discharged. Samples were stained with 2% uranyl acetate for 1 min and examined in a FEI Tecnai 12 transmission electron microscope and operated at 120 keV in conjunction with a FEI charge coupled device camera at a nominal magnification 67,000X.

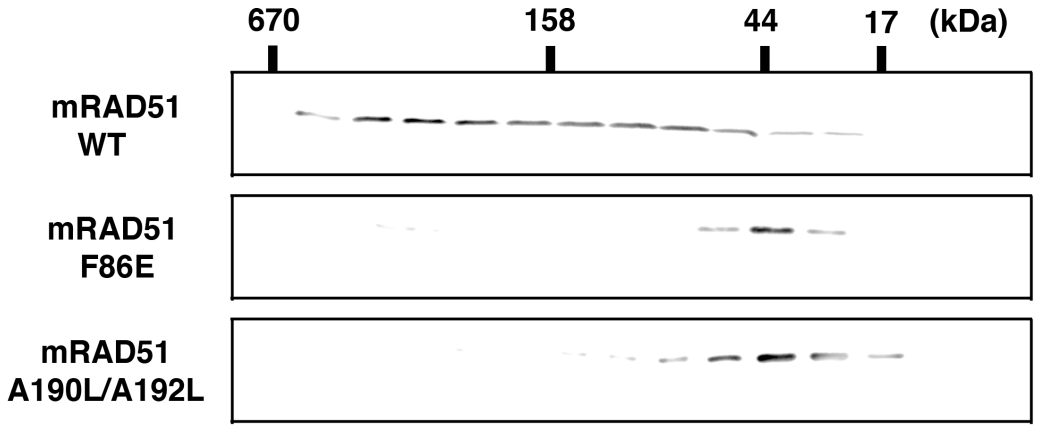
Supplementary Figures



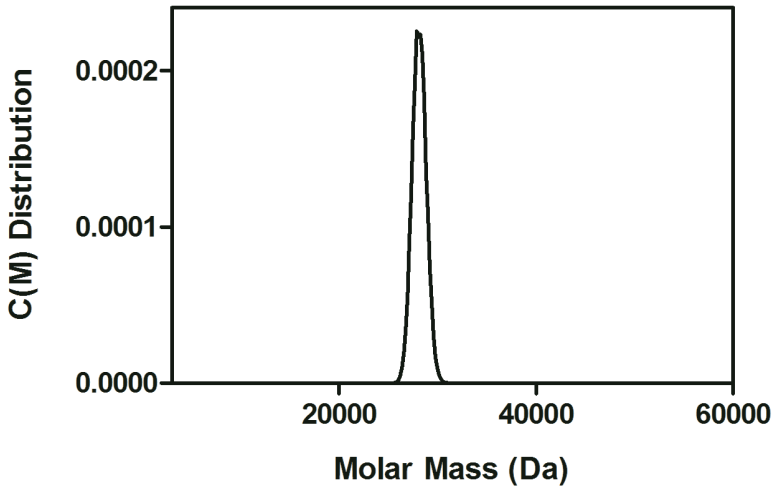
Supplementary Figure S1. Oligomeric status of RAD51 wild-type (WT) or RAD51 S208E/A209D (RAD51 SA/ED) with or without the presence of BRC4 was analyzed by gel filtration analyses.



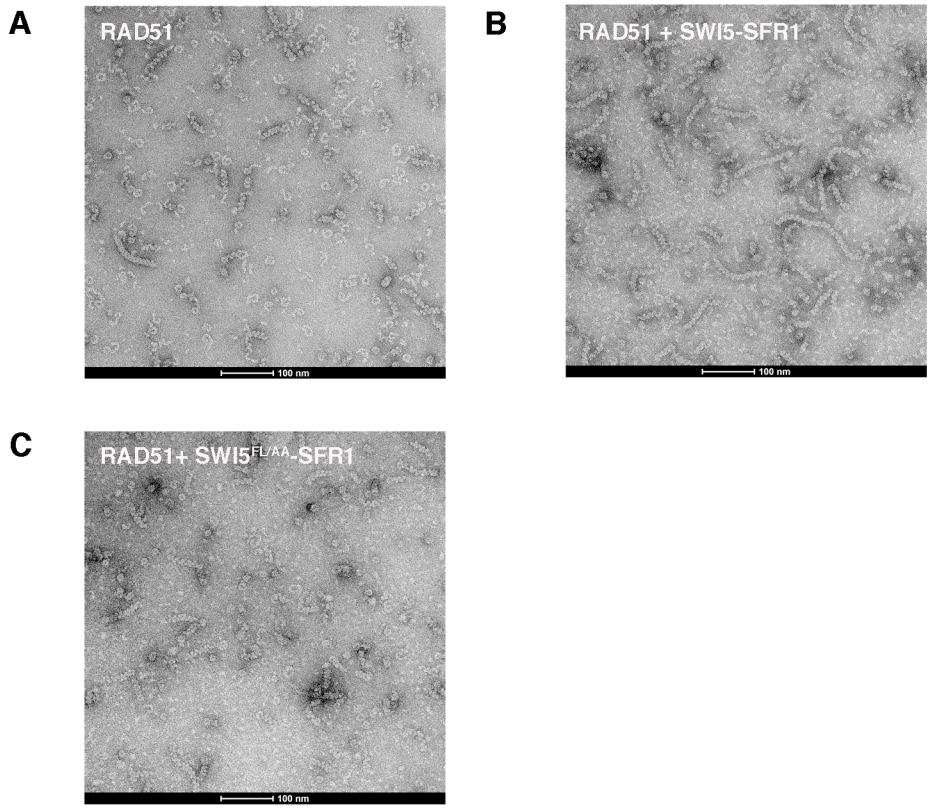
Supplementary Figure S2. GST-BRC4 physically interacts with RAD51 but not SWI5-SFR1 complex.
For affinity pulldown, purified GST-BRC4 was incubated with RAD51 or SWI5-SFR1 (S5S1) and then the mixtures were treated with GST beads to capture GST-BRC4. The supernatant (S), wash (W) and SDS eluate (E) were analyzed by 15% SDS-PAGE with Coomassie Blue staining.



Supplementary Figure S3. Oligomeric status of mouse RAD51 (mRAD51) wild-type (WT), F86E, or A190L/A192L was analyzed by gel filtration.



Supplementary Figure S4. Sedimentation velocity analysis of the SWI5-SFR1^{dN202} complex.
The experimental data from analytical ultracentrifugation were analyzed by the Sedfit program, which yielded an estimated molecular mass of 28.2 kDa.



Supplementary Figure S5. Representative images of negatively stained RAD51 presynaptic filaments as observed by electron microscopy.
RAD51 alone **(A)**, RAD51 with SWI5-SFR1 wild-type **(B)** or with the $SWI5^{FL/AA}$-SFR1 mutant variant **(C)** were examined by electron microscopy.

Supplementary References

27. Tsai, S.P., Su, G.C., Lin, S.W., Chung, C.I., Xue, X., Dunlop, M.H., Akamatsu, Y., Jasin, M., Sung, P. and Chi, P. (2012) Rad51 presynaptic filament stabilization function of the mouse Swi5-Sfr1 heterodimeric complex. *Nucleic Acids Res.*, **40**, 6558-6569.

31. Esashi, F., Galkin, V.E., Yu, X., Egelman, E.H. and West, S.C. (2007) Stabilization of RAD51 nucleoprotein filaments by the C-terminal region of BRCA2. *Nat. Struct. Mol. Biol.*, **14**, 468-474.