Supporting Online Material for

Mechanism of RAD51-Dependent DNA Interstrand Cross-Link Repair

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Methods

Xenopus egg extracts, DNA replication and repair assay

The preparation of Xenopus egg extracts (HSS and NPE) was as described (1). For DNA replication, plasmids were first incubated in a high-speed supernatant (HSS) of egg cytoplasm (7.5 ng/μL) for 20 minutes at RT (21°C), leading to the formation of pre-replication complexes (pre-RCs). Next, two volumes of nucleoplasmic egg extract (NPE) was added, triggering Cdk2-dependent replication initiation at pre-RCs. For all figures, the 0 minute time point corresponds to the addition of NPE. Where indicated, BRC peptides were added to NPE (21 μM) prior to mixing with HSS. Reactions were supplemented with [α-32P]dATP for DNA labeling and 0.125 ng/μL of an undamaged plasmid that serves as an internal standard for quantification (pQuant). Reactions were stopped with Stop Solution A (0.5% SDS, 25 mM EDTA, 50 mM Tris-HCl pH 7.5) and replication products were purified as described (2). Replication products were separated by 0.8% native agarose gel and visualized using a phosphorimager to determine replication and repair efficiencies (described below). Preparation of pICL was as described (3).

Calculation of ICL repair efficiency

Repair efficiency was calculated essentially as in (2). pICL contains a single, site-specific cisplatin ICL that interrupts a SapI recognition site. ICL repair is assayed by SapI cleavage, which requires error-free removal of the crosslink. To quantify the amount of SapI cleavage, DNA samples were digested with either HincII alone, or
HincII and SapI, then separated by a native agarose gel and visualized using a phosphorimager. SapI cleavage of HincII-linearized molecules produces two fragments that are 2.3 and 3.3 kb in size. Fragments of similar size are also generated when ICL-stalled fork arms are broken or cleaved. Since these fragments do not represent ICL repair products, they were quantified in the HincII-digested samples and subtracted from the HincII/SapI-generated fragments. This yields the amount of fragments produced exclusively by SapI cleavage. To determine the efficiency of repair as a percentage of the total DNA replicated, radioactivity in each sample is normalized to correct for variation introduced during sample preparation. To this end, a small amount of an unrelated, undamaged plasmid (pQuant) was included in the reaction to serve as an internal standard for quantification. Note that addition of pQuant did not affect replication or repair efficiency. The percentage of SapI-cleavable products is then calculated by comparing the normalized value of SapI fragments to the radioactivity present in the known amount of pQuant (which is 1/20th the amount of pICL added to the reaction).

2D gel electrophoresis

Replicated pControl or pICL products were digested with HincII, then analyzed by 2DGE. The first-dimension gel consisted of 0.4% agarose run in 1xTBE buffer at 0.75 V/cm for 26 hours at RT. The desired lane was then cast across the top of the second-dimension gel, which consisted of 1% agarose with 0.3 μg/mL ethidium bromide, and run in 1xTBE containing 0.3 μg/mL ethidium bromide at 4.5 V/cm for
14 hours at 4°C. DNA from the resulting gel was transferred to a 0.45 μm positively charged nylon transfer membrane (GE Healthcare, Piscataway, NJ, USA), cross-linked with a 120 mJ/cm² UV exposure, and visualized using a phosphorimager. To promote branch migration, the first-dimension gel slice was soaked in branch migration buffer (10 mM Tris-HCl pH 8, 0.1 mM EDTA, and 100 mM NaCl) +/- 10 mM MgCl₂, where indicated, at 65°C for 4 hours. The second-dimension gel was cast and run normally.

**RuvC reactions**

HincII-digested replication products were incubated with the Holliday junction resolvase RuvC (Abcam, Cambridge, MA, USA) in reaction buffer (50 mM Tris-HCl pH 8, 5 mM MgCl₂, 1 mM DTT, and 100 μg/mL BSA) at 37°C for 1 hour. Reactions were stopped with Stop Solution A, incubated with 0.5 μg/μL Proteinase K at 37°C for 10 minutes, and analyzed by 2DGE.

**Peptide construction and purification**

The original BRC construct (pDEST15+GST-BRC4; containing residues 1511-1579 of human BRCA2) was a kind gift from V. Costanzo (London Research Institute, UK). Recombinant BRC peptides were expressed and purified as described (4), using BL21-AI cells induced by 0.2% (w/v) L-arabinose and Glutathione Sepharose 4B (GE Healthcare, Piscataway, NJ, USA). Where indicated, BRC peptides were added to a final concentration of 14 μM in egg extracts. BRC mutations were introduced using
the QuikChange Site-Directed Mutagenesis Kit (Qiagen, Valencia, CA, USA) with the following primers and their complements:

\[
\text{ACCTACTCTGTGGTTTTTCATGCAGCTAGCGGGAA} \quad \text{(BRC*)}
\]

\[
\text{GAATCTTTGGACAAAGTGAAAAACCTTGATGATGAAAAAGCAAGGTACTA} \quad \text{(BRC***)}
\]

\[
\text{GATCAAAGAACCTACTCTATTGGGTGATCATGCAGCTAGCGGGAAAAAGTTAAAAT} \quad \text{(BRC***)}
\]

**Sperm-chromatin binding assay**

Chromatin-bound proteins from 1.2 μL extract were isolated during replication of cross-linked *Xenopus* sperm chromatin (2). Freshly isolated chromatin was cross-linked with 0.5 μM trioxsalen (Sigma-Aldrich, St. Louis, MO, USA) then replicated as described above (with 4,500 sperm/μL in HSS/NPE). At the indicated times, a 15 μL reaction sample was mixed with 90 μL of ELB (10 mM HEPES-KOH pH 7.7, 2.5 mM MgCl₂, 50 mM KCl, 250 mM sucrose, and 1 mM DTT) containing 0.2% Triton X-100 (4°C), layered over 180 μL of ELB containing 500 mM sucrose (4°C) in 5x44 mm microcentrifuge tubes (Beckman, Fullerton, CA, USA). Chromatin was centrifuged at 16,000 x g for 45 seconds at 4°C in a horizontal microcentrifuge and the supernatant was aspirated, leaving behind 10 μL of the sucrose cushion. The chromatin pellet was washed with 200 μL ELB (4°C), resuspended in SDS-PAGE loading buffer, and analyzed by Western blot (0.12 μL extract loaded for INPUT).
**Nascent strand analysis**

Nascent strand analysis was performed as described (3). Briefly, purified DNA replication samples were digested with AflIII, followed by addition of 0.5 volumes Stop Solution B (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). Restriction fragments were separated on a 7% polyacrylamide sequencing gel, transferred to filter paper, dried, and visualized using a phosphorimager. Sequencing ladders were generated with primer S (5’-CATGGTTTACTAGCCAGATTTTTCCTCTCCTCCTG-3’; see Figure 3A) using the Cycle Sequencing kit (USB, Cleveland, OH, USA).

**ChIP and quantitative real-time PCR**

ChIP was modified from our existing procedure (5). 3 μL of reaction samples were cross-linked by adding 47 μL of 1% formaldehyde in ELB and incubated for 10 minutes at RT. Cross-linking was stopped by adding 5 μL of 1.25 M glycine for 10 minutes at RT, followed by passage through a Micro Bio-Spin 6 Chromatography column (Bio-Rad, Hercules, CA, USA) to remove excess formaldeyde. The flowthrough was diluted with sonication buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, 5 μg/mL Aprotinin+Leupeptin, and 2 mM PMSF) to 500 μL and subjected to sonication, yielding DNA fragments ~300-500 bp in size. Following immunoprecipitation with the indicated antibodies, formaldehyde cross-links were reversed and DNA was purified for analysis by quantitative real-time PCR as described (5) with the following primers: Primer pair I (5’-
AGCCAGATTTTTTCTCCTCCTC-3' and 5'-CATGCATTGGTCTGCACTT-3'), Primer pair II (5'-ACCTGGTTCTTTTCCAAC-3' and 5'-CTTTCATCTGAGCGCTCT-3'), and Primer pair III (5'-AACCCAATAGGGACTTTCC-3' and 5'-GGGCTACTGGAATATG-3'). Both RPA (6) and RAD51 antibodies were purified on Protein A Sepharose (GE Healthcare, Piscataway, NJ, USA) before use.

**Immunoprecipitation of pICL intermediates**

pICL was replicated in extract for 40 minutes and a 6.5 μL reaction sample was immunoprecipitated with RAD51 antibodies as described for the ChIP protocol, only without sonication. Recovered DNA was digested with HincII and analyzed by agarose gel electrophoresis (1 μL reaction sample loaded for INPUT).

**Antibodies and immunodepletion**

FANCD2, Mcm7, and RPA antibodies were previously described (3, 6, 7). A cDNA fragment encoding amino acids 3-101 of *X. laevis* RAD51 was amplified from a *Xenopus laevis* stage 18 cDNA library (8) using primers 5'-TCGTGGGATCCCCCAAGCGCACTATGAAGCCGAAG-3' and 5'-TCCGCTCGAGTGTACTGATCTGTATTATTTCAGAGCG-3', then subcloned in-frame into the BamHI and XhoI sites in the pGex5X-1 vector. The recombinant GST-tagged protein was expressed in BL21 Star (DE3) bacterial cells (Invitrogen, Carlsbad, CA, USA) and purified using Glutathione Sepharose (GE Healthcare, Piscataway, NJ, USA). Protein was dialyzed against PBS and used for immunization of rabbits. Characterization of
RAD51 antibodies is shown in Figure S9. Depletion of FANCD2 from *Xenopus* egg extracts was as described (2).
Supplemental Figure Legends

Figure S1. (A) Schematic model depicting how hemicatenanes may migrate off the ends of HincII-linearized replication intermediates under conditions that promote branch migration. (B) A 240-minute pICL replication sample was digested with HincII and analyzed by 2DGE (Mock) or a modified version of the 2DGE protocol (see Methods) where the 1st dimension gel slice is incubated under branch migration conditions either in the absence or presence of Magnesium prior to running the 2nd dimension (+BM, +BM and Mg). The X-arc region is shown at greater magnification in (C). (D) A 240-minute pICL replication sample was digested with HincII, then treated with buffer (Mock) or RuvC (50 or 500 nM) for 1 hour and analyzed by 2DGE. RuvC activity was tested separately (data not shown). (E and F) Raw data from which ICL repair efficiency was calculated for Figure 1E (described in Methods). pICL or pControl was replicated in extract for the indicated time. DNA samples were left uncut or digested with either HincII alone, or HincII and SapI. Samples were then separated by native agarose gel electrophoresis and visualized using a phosphorimager. Where indicated (+BM), digested samples were treated under conditions that promote branch migration prior to gel electrophoresis. Digested intermediates are indicated at right.

Figure S2. (A) BRC peptide sequences. A region of human BRCA2 containing BRC repeat #4 (BRC4) was fused to GST by a small linker region. Asterisk (*) denotes
amino acid mutations in BRC* and BRC*** sequences. (B) Replication efficiency calculated for Figure 2C and D as described (2).

**Figure S3.** DNA samples from Figure 3B were digested with HincII and separated by native 1D (A) or 2D (B) gel electrophoresis followed by analysis with a phosphorimager. Examples of dual-stalled fork position indicated with blue arrowhead. The intensity of dual-stalled fork intermediates from (A) and (B) was quantified and graphed in (C) and (D), respectively.

**Figure S4.** RAD51 completes its repair function just prior to SapI site regeneration. (A) To address the timing of RAD51 function during ICL repair, BRC peptides were added to egg extract at different times during pICL replication, and in all cases, SapI site restoration was measured at the end of the reaction (240 minutes) to assay repair efficiency. While addition of BRC*** at any time did not significantly affect repair (gray bars), BRCWT added at or before 60 minutes completely inhibited repair (black bars), indicating that RAD51 had not completed its function by this time. When BRCWT was added after 60 minutes, the final extent of repair was only marginally greater than the amount of repair achieved at the moment of addition (compare black and white bars). Since BRCWT displaces pre-loaded RAD51 within 30 minutes of its addition to extract [see (B)], this indicates that RAD51 completes its repair function just prior to SapI site regeneration. (B) Cross-linked sperm chromatin was replicated in extract for 60 min to allow RAD51 loading and then
supplemented with buffer (Mock), or the indicated BRC peptide. After an additional time (shown at top), chromatin-bound proteins (from 1.2 μL extract) were isolated through a sucrose cushion and analyzed by Western blotting with the indicated antibodies (0.12 μL extract loaded for INPUT). Four irrelevant lanes were removed between lanes 11 and 12. Note that pre-loaded RAD51 dissociates rapidly upon addition of BRCWT (lanes 8, 9).

**Figure S5.** Strand invasion occurs primarily between sister chromatids. (A) Schematics of pICL and an undamaged competitor plasmid (pComp) that was identical to pICL, except that it lacked the ICL and contained a mutated SapI site (converting SapI to an NheI site; sequence differences shown in red). (B) If recombination involves no preference for the sister chromatid, then addition of pComp should produce a linear decrease in SapI-cleavable repair products (black bars). pICL was replicated (2.5 ng/μL) with increasing amounts of pComp (0:1 – no pComp; 0.33:1 – 0.83 ng/μL pComp; 1:1 – 2.5 ng/μL pComp; 3:1 – 7.5 ng/μL pComp). Replication with up to a 3-fold excess of pComp caused no significant decrease in pICL repair (gray bars, measured at 240 minutes). This indicates that strand invasion occurs primarily between replicated sister chromatids. Error bars represent standard deviation from three separate experiments. (C) Representative raw data from which ICL repair was calculated in (B).
**Figure S6.** (A) ChIP results from Figure 4B presented with additional experimental controls. Binding of RAD51 and RPA was analyzed by ChIP using primer pairs I, II, and III during replication of pICL, pICL in the presence of BRC4 WT, pControl, or pICL in the presence of the replication inhibitor geminin. (B) Primary data from which dual-stalled fork accumulation was calculated for Figure 4C. DNA samples from Figure 4B and (A) were digested with HincII and analyzed by 2DGE. (C) Primary data from which -1 product accumulation was calculated for Figure 4C. DNA samples from Figure 4B and (A) were digested with AflIII and separated on a sequencing gel alongside a ladder generated with primer S. (D) Primary data from which ICL repair (above background) was calculated for Figure 4C. DNA samples from Figure 4B and (A) were digested with HincII alone, or HincII and SapI, then separated by native agarose gel and visualized using a phosphorimager.

**Figure S7.** (A) ChIP results from Figure 4F (primer pair I) presented with primer pairs II and III. (B) Western blot analysis of FANCD2 levels present in extracts used for Figure 4E and F (HSS and NPE combined). Mock-depleted (Mock), FANCD2-depleted (FANCD2Δ), FANCD2-depleted plus recombinant FANCI-FANCD2 (FANCD2Δ+ID). (C) Repair efficiency calculated for the reaction presented in Figure 4E and F. (D) Protein samples from Figure 3B were analyzed by Western blotting for FANCD2 modification and graphed. (E) Complete 2DGE time courses from Figure 4E.
**Figure S8.** Schematic model of ICL repair in *Xenopus laevis*. Parental DNA (black lines), Nascent strands (gray lines), RPA (orange circles), RAD51 (green circles). Strand extension steps shown in red for emphasis. Double Holliday junction (DHJ), DNA double-strand break (DSB), single-stranded DNA (ssDNA), double-stranded DNA (dsDNA).

**Figure S9.** Characterization of *Xenopus laevis* RAD51 antibody. The indicated samples were analyzed by Western blotting, first with pre-immune serum, then re-blotted with RAD51 antibodies (αRAD51). Position of the antigen (GST-RAD513-101) and endogenous RAD51 from extract (NPE) is indicated.
References

Figure S1

A

B

C

D

E

F

Figure descriptions:

A: Diagram showing HindIII digestion followed by branch migration.

B: Gel images showing pCRII (60 min) with lanes for Mock, +BM, +BM and Mg.

C: Gel images showing X-arc intermediates and 1x lines.

D: Gel images showing pCRII (60 min) with lanes for Mock, +RuxC (x), +RuxC (x).

E: Table showing HindIII, HindIII/SapI, pControl, and pControl + BM digestion analysis.

F: Table showing HindIII, HindIII/SapI, pCL, and pCL + BM digestion analysis.
Figure S2

A

<table>
<thead>
<tr>
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<th>Linker Region</th>
<th>BRC Motif</th>
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<tr>
<td>BRC$^+$</td>
<td>GST-P999MQLYKARCreD1KKEIIIPLGPTAGGDKVIAKESLDVGKKNLFDEEEQ737EITSPRQ14WATL2YFAECK2LELA</td>
<td></td>
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<tr>
<td>BRC$^{++}$</td>
<td>GST-P999MQLYKARCreD1KKEIIIPLGPTAGGDKVIAKESLDVGKKNLFDEEEQ737EITSPRQ14WATL2YFAECK2LELA</td>
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<tr>
<td>BRC$^{+++}$</td>
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B

![Graph showing replication in different conditions](image)
Figure S3

A

B

C

D

Mock  +BRC\textsuperscript{WT}  +BRC\textsuperscript{***}

10  20  60  120  180  240

0  50  100

0  80  160  240

Time (min)

Time (min)

Dual-stranded Forks (%)
Figure S6

A

Primer Pair I

Primer Pair II

Primer Pair III

RPA

RAD51

B

0 20 40 60 120 240 (min)

pCL

C

G A T C 0 20 40 60 120 240 G A

Extension Product

Insertion Product

D

pCL

HindIII HindIII/SapI

0 20 40 60 120 240 7 20 40 60 120 240
Figure S8

(i) Fork Pausing at -20 to -40
RPA binds to ssDNA gaps exposed on the lagging strand DNA template.

(ii) Approach to -1
RAD51 is loaded onto ssDNA.

(iii) Incisions and Insertion at 0
5'-3' resection of DSB generates additional ssDNA.

(iv) Extension
RAD51 binds to newly resected end, and RAD51 is displaced from the other sister chromatid.

(v) Strand Invasion / End Capture
RAD51-mediated strand invasion takes place between sister chromatids.

(vi) Extension
RPA is displaced during strand extension and RAD51 is removed from dsDNA.

(vii) DHJ Convergence

(viii) Hemicatenane Resolution

(ix) Nucleotide Excision Repair
Figure S9