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Award Number: W81XWH-13-1-0063

TITLE: A New Cell-Free System to Study BRCA1 Function

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CONTRACTING ORGANIZATION:
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REPORT DATE: May 2014

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
May 2014	Annual	1 May 2013 – 30 April 2014

4. TITLE AND SUBTITLE
A New Cell-Free System to Study BRCA1 Function
A New Cell-Free System to Study BRCA1 Function A New Cell-Free System Stu dy BRCA1 Function

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0063

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

8. PERFORMING ORGANIZATION REPORT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Harvard Medical School

Boston, MA 02115

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This proposal is based on our finding that in a cell-free system based on Xenopus egg extracts, the tumor suppressor BRCA1 is required for a novel step in the repair of DNA interstrand cross-links (ICL). Specifically, prior to our application of funding, we had found that in the absence of BRCA1, when replication forks collide with an ICL, leading strands stall 20 nucleotides from the ICL and fail to be extended towards the ICL lesion. In the last year, we have shown that leading strand extension is critical for ICL repair (Aim 1). In addition, we found that in BRCA1-depleted egg extracts, the CMG helicase that unwinds DNA ahead of DNA polymerases, fails to be unloaded from the stalled fork (Aim 2). This explains the leading strand arrest at the -20 position and identifies a potentially new function for BRCA1 in ICL repair and tumor suppression. We have also developed new ways of inhibiting BRCA1 function in egg extracts and examined the role of potential BRCA1 effectors (FANCJ, FANCM, CTIP) in promoting the extension step. We conclude that BRCA1 does not perform its function by acting through FANCJ, FANCM, or CTIP.

15. SUBJECT TERMS
none provided

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	9	**19b. TELEPHONE NUMBER** *(include area code)*

Table of contents

Page

Introduction

Mutations in BRCA1 account for a significant fraction of familial breast and ovarian cancers. BRCA1 is thought to suppress genome instability by promoting homologous recombination, which it does in part by helping to recruit BRCA2 and the RAD51 recombinase to sites of DNA damage. However, more recently, it has been proposed that BRCA1 performs functions in DNA interstrand cross-link (ICL) repair that are not related to HR (Bunting et al., 2012). For example, BRCA1 appears to help recruit the ICL repair factor FANCD2 to sites of damage. To further explore the role of BRCA1 in ICL repair, we employed Xenopus egg extracts, which we previously showed support ICL repair (Raschle et al., 2008). In the grant proposal, we presented preliminary data that BRCA1 depletion from egg extracts inhibits ICL repair (see Figure 2B of the proposal) and that RAD51, BRCA2, and FANCD2 binding to the damage are inhibited, as expected from previous reports (see Figure 2E-G of the proposal). However, we also found that ICL repair was inhibited at an early step called "Approach" (see Figure 2C of the proposal). Thus, in the absence of BRCA1, the leading strands of replication forks that have stalled at an ICL are not efficiently extended from the -20 position to the -1 position. The grant proposes to understand how BRCA1 promotes the Approach step.

Body

The original Statement of Work is reproduced below in grey. Our progress on each Task is described in black. All experiments were performed at least twice, usually three times or more. Representative examples are shown.

Task 1: Determine whether blocking Approach inhibits FANCI-FANCD2 loading.

1a. Replicate pICL in egg extract and add aphidicolin 12 minutes after NPE addition; then perform ChIP with antibodies to FANCI, FANCD2, DNA pol e, FANCA, FANCM.

We first wanted to test whether Approach, which is inhibited in BRCA1-depleted egg extracts, is required for ICL repair. To this end, we inhibited this step by an orthogonal means. pICL was replicated for 12 minutes to allow the majority of forks to arrive at the -20 position (Figure 1A, lane 7). Reactions were then split and supplemented with buffer or the DNA polymerase inhibitor aphidicolin. Aphidicolin-treated samples exhibited little or no Approach (Figure 1A, compare lanes 15-19 with 9-13), as well as a ~25% decrease in total nucleotide incorporation due to degradation of some forks that had not yet stalled at the crosslink (Figure 1B). Chromatin immunoprecipitation (ChIP) showed that BRCA1, RAD51, and FANCD2 were still recruited to the ICL in aphidicolin-treated samples (Figure 1C-E), although total recovery was also decreased by ~25%. In contrast, DNA incisions were inhibited, as measured by persistence of the converged fork structure (Figure 1F and data not shown). In addition, ICL repair was completely absent (Figure 1G). Together, these results indicate that Approach is required for incisions and downstream repair events, but not for the recruitment of BRCA1, RAD51, or FANCD2. We infer that the defect in Approach seen in BRCA1-depleted egg extracts readily explains the inhibition of ICL repair in the absence of BRCA1. However, the defect in FANCD2, BRCA2, and RAD51

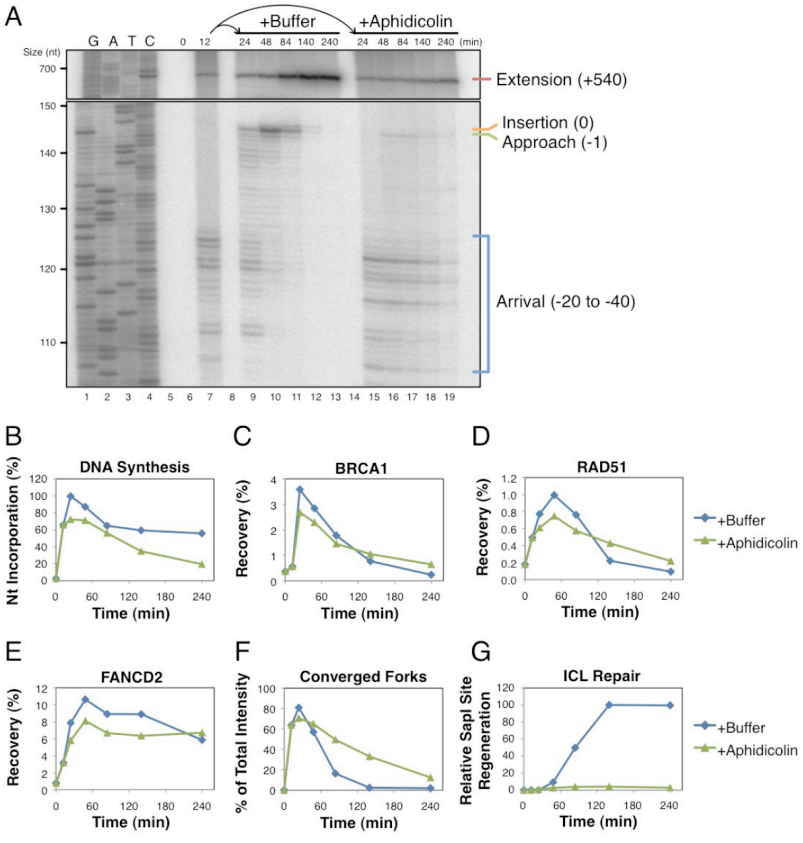

Figure 1: Approach is required for ICL repair. (A) pICL was replicated in egg extracts. After fork convergence (12 min), buffer or aphidicolin was added and leading strands were analyzed at the indicated time points. DNA synthesis (B), BRCA1 ChIP (C), RAD51 ChIP (D), FANCD2 ChIP (E), the abundance of converged forks (F), and the efficiency of ICL repair (G) were determined at the indicated time points.

loading in BRCA1-depleted extracts is not explained by defective Approach. In other words, the intermediate generated during Approach is not necessary for loading of FANCI-FANCD2, BRCA2, or RAD51.

1b. Clone, express, and purify ubiquitylated FANCI, FANCD2$^{1-561}$, and Ub-FANCD2$^{562-1443}$ in insect cells. If this approach does not work, reconstitute FANCI-FANCD2 ubiquitylation with UBE2T and FANCL.

This Task was proposed primarily as a follow up if Task 1a had shown that Approach is required for loading of FANCD2. This was not the case. Nevertheless, since we were interested in generating ubiquitylated FANCI-FANCD2 for other reasons, we proceeded with this task. We expressed and purified FANCI, FANCD2$^{1-561}$, and Ub-FANCD2$^{563-1443}$ in insect cells, but the two halves of FANCD2 did not interact efficiently, even in the presence of FANCI (data not shown). We have temporarily abandoned this approach.

1c. Perform gel shift experiments with the protein complex prepared under 1b and different DNA substrates to determine the complexes DNA binding specificity.

Given the outcome of Task 1a, this task is not applicable.

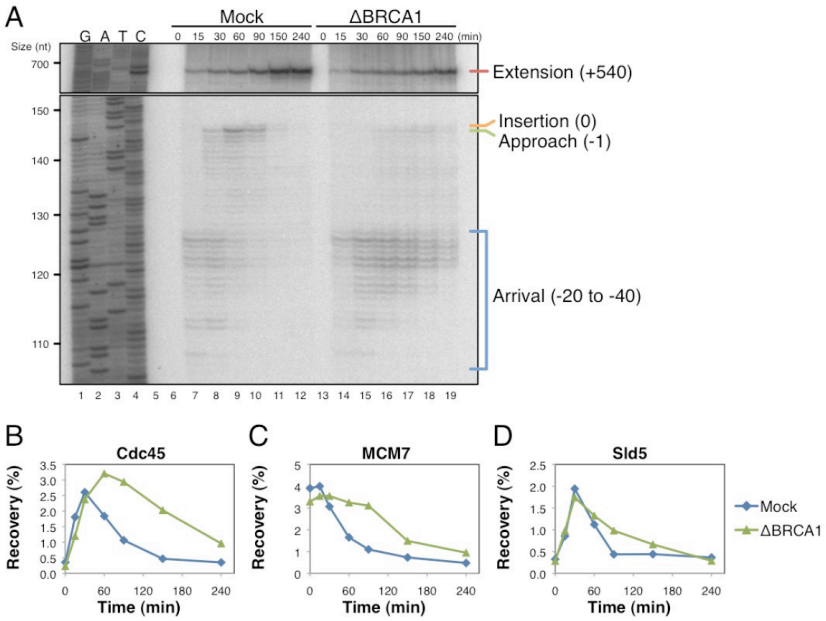

Figure 2: BRCA1 is required for CMG dissociation. (A) Leading strands were analyzed in Mock and BRCA1-depleted egg extracts. (B-D) In the same reactions, Cdc45, MCM7, and SLD5 were analyzed by ChIP at the ICL.

Task 2. Determine whether BRCA1 is required for MCM2-7 dissociation from ICLs.

2a. Replicate pICL in mock-depleted and BRCA1-depleted egg extracts and perform ChIP with antibodies to Mcm5, Mcm7, Cdc45, GINS, DNA pol e, Rad51, BRCA2, and RPA.

We had previously shown that Approach correlates with the dissociation of the CMG helicase from replication forks that have stalled at an ICL (Fu et al., 2011). We therefore postulated that the defect in Approach in BRCA1-depleted extracts is due to a defect in CMG dissociation. To test this, we performed ChIP in mock-depleted and BRCA1-depleted egg extracts. BRCA1-depletion not only inhibited Approach (Figure 2A) as we reported in the proposal, but also greatly slowed the dissociation of three CMG subunits, Cdc45, Mcm7, and Sld5, from the ICL (Figure 2B-D). Binding to a distal site on the plasmid was not affected (data not shown). This result shows that BRCA1 is required for CMG dissociation.

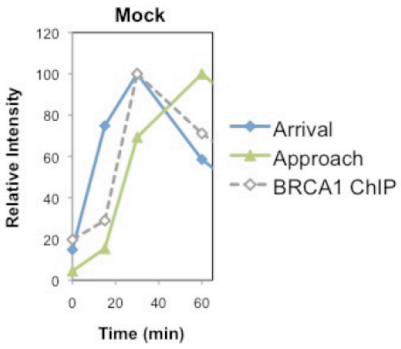

Figure 3: BRCA1 binds to ICLs after arrival of forks at the -20 position and before Approach of the leading strands at the -1 position. In the same experiment, BRCA1 ChIP was compared with the kinetics of arrival and Approach.

To determine whether BRCA1 functions directly in the unloading of the CMG complex, we examined the binding of BRCA1 to the plasmid using ChIP. Importantly, BRCA1 binding occurred shortly after leading strands arrived at the -20 position and shortly before they were extended to the -1 position (Figure 3). Thus, BRCA1 is present at the site of the ICL during the Approach step. We also showed that BRCA1's interaction partner BARD1 binds to the ICL with exactly the same kinetics as BRCA1 (data not shown). We conclude that BRCA1-BARD1 is present at ICLs during CMG dissociation, consistent with it promoting this process directly.

Task 3. Determine the functional interplay between BRCA1 and FANCJ /FANCM

3a. Replicate pICL in mock-depleted and BRCA1-depleted egg extracts and perform ChIP with antibodies to FANCJ and FANCM.

BRCA1-BARD1 might itself promote CMG unloading or act through an effector. We considered four possible effectors of BRCA1-BARD1. Two of these, FANCM and FANCJ, are DNA helicases. We speculated that their helicase activities might be required to displace CMG. FANCJ was particularly attractive since it binds to BRCA1. We considered two other potential effectors, ABRAXAS and CTIP, both of which also bind to BRCA1. So far, we have raised antibodies to FANCM, FANCJ, and CTIP and depleted the corresponding proteins. While all three proteins appear to be required for ICL repair, in all cases the defect occurs *downstream* of MCM2-7 unloading. We conclude that none of these proteins are BRCA1 effectors. We are currently raising antibodies to ABRAXAS. If ABRAXAS depletion inhibits CMG unloading, it will most likely be due to a failure to localize BRCA1 at sites of damage, and not necessarily due to a direct role for ABRAXAS in this process. Consistent with the above results, ChIP showed that FANCJ recruitment to ICLs is not reduced by BRCA1 depletion (data not shown). We did not extend the ChIP analysis to FANCM because it is not involved in CMG unloading.

3b. Perform co-immunoprecipitations between FANCJ and BRCA1.
Given that FANCJ is not involved in CMG unloading, we did not perform this task.

Task 4. Identification and characterization of new BRCA1 effectors using mass spectrometry

4a. Prepare chromatin from mock-depleted and BRCA1-depleted egg extracts in preparation for mass spectrometry analysis.
We have not initiated this task.

4b. Send chromatin samples to our collaborators in Germany for mass spectrometry analysis.
We have not initiated this task.

4c. For novel proteins whose binding to chromatin depends on BRCA1: clone the gene, express the protein, raise antibodies, immunodeplete the protein from egg extracts, and measure the effect on ICL repair, Approach, MCM2-7 dissociation, and FANCJ/FANCM loading.
We have not initiated this task.

Task 5. Identification of BRCA1 domains that are required for ICL repair

5a. Clone and express BRCA1-BARD1, BRCA1^{S1379F}-BARD1, BRCA1^{I26A}-BARD1, BRCA1$^{\Delta CC}$-BARD1, or other mutants in insect cells.
We expressed wild type BRCA1-BARD1 in insect cells and purified the protein. We have not expressed the mutant proteins.

5b. Supplement BRCA1-depleted egg extracts with recombinant BRCA1-BARD1, BRCA1^{S1379F}-BARD1, BRCA1^{I26A}-BARD1, or BRCA1$^{\Delta CC}$-BARD1 and measure the effects on ICL repair.
Re-addition of recombinant wild type BRCA1-BARD1 to BRCA1-depleted egg extract did not restore ICL repair (data not shown). To rule out that our BRCA1 antibody non-specifically depleted a protein required for CMG dissociation, we depleted BRCA1 with other antibodies. We found that depletion of egg extract with these antibodies also led to the stabilization of CMG on DNA (data not shown). We believe that most likely, BRCA1 depletion co-depletes an essential BRCA1 co-factor. Since we have ruled out the BRCA1-interacting proteins CTIP and FANCJ as BRCA1 effectors, we are focusing on ABRAXAS which binds to BRCA1 and helps it localize to sites of DNA damage. We are currently raising an antibody to ABRAXAS to determine whether it was co-depleted with BRCA1 from egg extracts. We will also purify ABRAXAS and add it back to BRCA1 depleted egg extracts, together with recombinant BRCA1-BARD1, to determine whether it rescues the effect of BRCA1 depletion.

To gain further evidence that the BRCA1-BARD1 complex participates in CMG unloading, we sought to disrupt the BRCA1-BARD1 complex, which forms through interactions between the RING domains of BRCA1 and

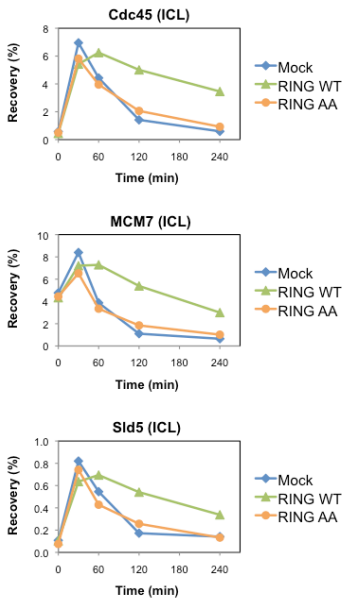

Figure 4: A BARD1 RING peptide stabilizes CMG at the ICL. pICL was replicated in the presence of buffer (blue), WT RING peptide (green), or mutant RING peptide (yellow), and CMG binding to ICLs was measured using Cdc45, MCM7, and Sld5 ChIP.

BARD1. Cell-based experiments had shown that expression of a BARD1 fragment corresponding to the BARD1 RING domain disrupts the BRCA1-BARD1 complex and phenocopies BRCA1 deficiency (Westermark et al., 2003). We purified this RING peptide and added it at high concentrations to Xenopus egg extracts. Importantly, this disrupted the endogenous BRCA1-BARD1 complex, and it caused a similar defect in CMG unloading as BRCA1 depletion (Figure 4, green traces). In contrast, a mutant RING peptide that binds poorly to BRCA1 did not cause CMG stabilization (Figure 4, yellow traces). These data support our model that the BRCA1-BARD1 complex is involved in CMG unloading.

To further investigate the role of BRCA1 in ICL repair and CMG unloading, we sought to inhibit BRCA1-BARD1 binding to ICLs. The BRCA1-BARD1 complex is recruited to sites of DNA damage by ubiquitin signaling (Yan and Jetten, 2008). To disrupt ubiquitin signaling, we employed ubiquitin vinyl-sulfone (UbVS), a highly specific, irreversible inhibitor of deubiquitylating enzymes. Incubation of *Xenopus* egg extract with UbVS blocks ubiquitin turnover, leading to the depletion of free ubiquitin (Dimova et al., 2012). Extracts were incubated with buffer, UbVS, or UbVS and excess free ubiquitin prior to addition of pICL. Although DNA synthesis was not significantly inhibited by the addition of UbVS (Figure 5A), ICL repair was abolished (Figure 5B). Repair was only partially rescued by the addition of free ubiquitin, suggesting that ubiquitin turnover is essential for efficient repair.

To examine the effect of UbVS on BRCA1 loading at ICLs, we used chromatin immunoprecipitation (ChIP). As shown in Figures 5C-E, Rap80, BRCA1, and FANCD2 were not recruited to ICLs when UbVS was present. Recruitment was rescued by the addition of free ubiquitin, indicating that recruitment defects were due to ubiquitin depletion.

To investigate how UbVS affects ICL repair, nascent strand products (Figure 5F) were analyzed by denaturing polyacrylamide gel electrophoresis. UbVS treatment had no effect on the arrival of leading strands at the ICL (Figure 5G, compare lanes 7, 13, and 19), consistent with replication proceeding normally (Figure 5A). In contrast, UbVS completely blocked the Approach of leading strands to the -1 position, as well as formation of all downstream nascent strand products (Figure 5G, compare lanes 7-11 with 13-17). Addition of free ubiquitin

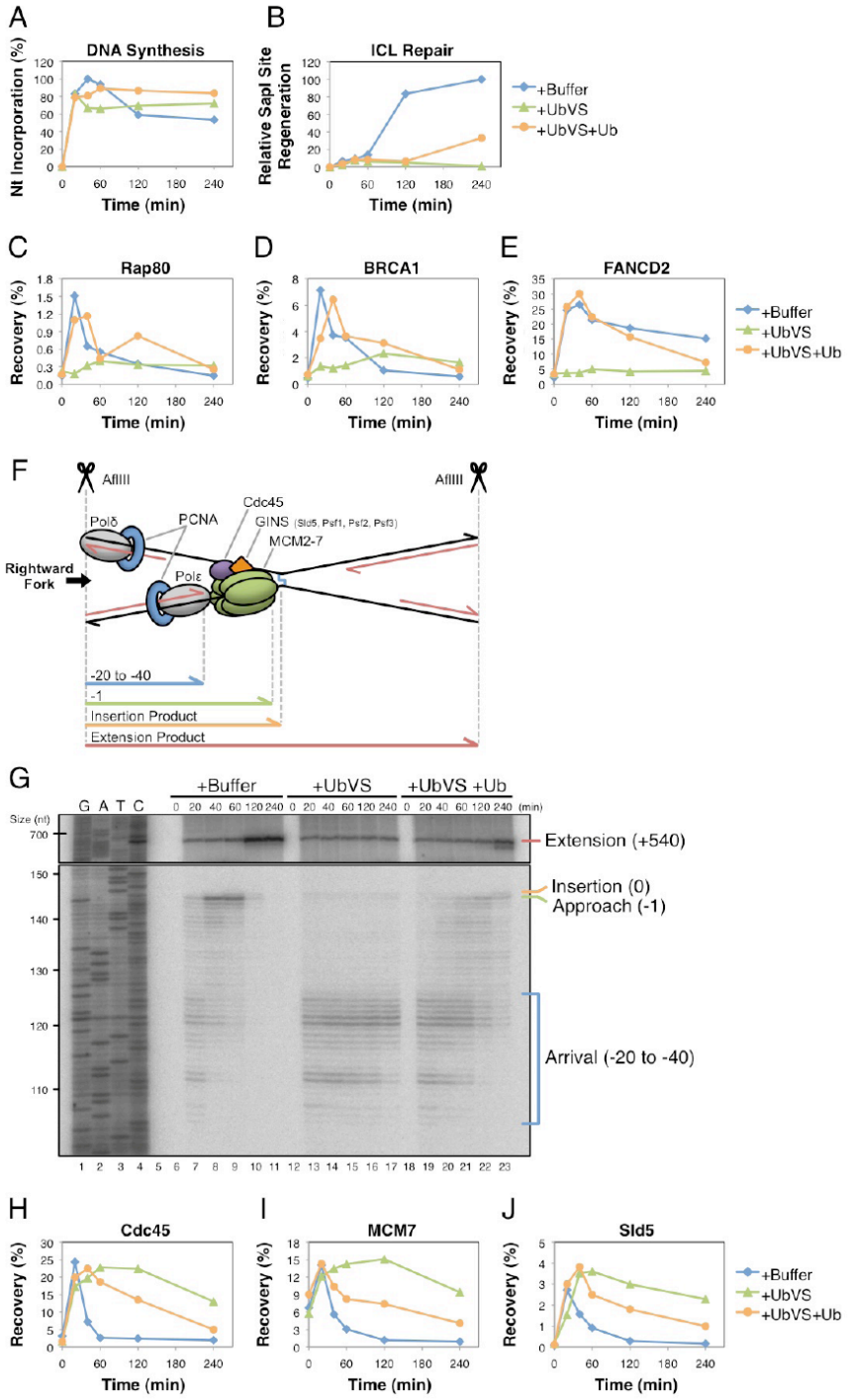

Figure 5: Ubiquitin signaling is required for CMG dissociation and BRCA1 localization to ICLs. Egg extracts were supplemented with buffer, UbVS, or UbVS + free ubiquitin, and we measured DNA synthesis (A), ICL repair (B), RAP80, BRCA1, and FANCD2 ChIP (C-E), leading strand stalling at ICLs (G), and CDC45, MCM7, and SLD5 ChIP (H-J). (F) Cartoon illustrating the intermediates detected when leading strands stall at an ICL.

with UbVS restored Approach, Insertion, and Extension, albeit with delayed kinetics (Figure 5G, lanes 19-23). Importantly, unloading of Cdc45, MCM7, and Sld5 was severely delayed in UbVS-treated reactions (Figure 5H-J). As for Approach, CMG unloading was partially restored by the addition of free ubiquitin. Together, these results demonstrate that ubiquitin signaling is required to both recruit BRCA1 and remove the CMG helicase from stalled replication forks, further supporting the idea that BRCA1 promotes CMG dissociation.

Notably, addition of free ubiquitin did not efficiently restore ICL repair. This suggests that ubiquitin turn-over is required for efficient ICL repair.

5c. Deplete CTIP or FANCJ to determine whether this mimics any defects in ICL repair observed for the BRCA1^{S1379F}-BARD1 mutant.

Depletion of neither CTIP nor FANCJ mimiced the CMG unloading defect seen in BRCA1-depleted extracts (see task 3a).

Task 6. Identification of BRCA1 ubiquitylation targets via mass spectrometry analysis

6a. Prepare chromatin from BRCA1-depleted egg extracts supplemented with rBRCA1-BARD1 or rBRCA1^{I26A}-BARD1 in preparation for mass spectrometry analysis.

We have not initiated this task.

6b. Send chromatin samples to our collaborators in Germany for mass spectrometry analysis.

We have not initiated this task.

6c. Ubiquitylation events that are BRCA1-dependent will be functionally characterized. For example, MCM2-7 with the ubiquitylation site(s) mutated to arginine will be expressed in insect cells and then added to MCM2-7-depleted egg extracts to determine the effects on ICL repair.

We have not initiated this task.

Key Research Accomplishments

- BRCA1-BARD1 binds to ICLs at the time of CMG unloading and Approach
- BRCA1 depletion with various different antibodies inhibits CMG unloading and Approach
- A BARD1 RING peptide that disrupts the BRCA1-BARD1 complex inhibits CMG unloading and Approach
- Ubiquitin depletion inhibits BRCA1 binding to sites of damage, CMG unloading, and Approach
- Approach is required for ICL repair

Reportable Outcomes

1. The work implicating BRCA1 in the unloading of the CMG complex was submitted to the journal *Molecular Cell* and received supportive reviews. We are currently preparing a revision.

2. Dr. David Long, a post-doctoral fellow, who showed that BRCA1 depletion prevents the unloading of the CMG helicase, has obtained an Assistant Professor position at the Medical University of South Carolina.

Conclusions

To date, it has been assumed that BRCA1's primary role in ICL repair is to support homologous recombination. However, our data strongly support the novel concept that BRCA1 also promotes the first step in ICL repair, the removal of the replicative DNA helicase from forks that have stalled at the lesion. This insight sheds new light on the mechanism by which BRCA1 might suppress genome instability and cancer. We are now trying to understand how BRCA1 performs this function. An obstacle has been our inability so far to rescue the BRCA1 depletion with purified BRCA1-BARD1 complex. In addition to the approaches listed in the proposal, we are raising an antibody to ABRAXS to determine whether this protein is co-depleted with BRCA1. If so, we will add not only BRCA1-BARD1 but also ABRAXAS to BRCA1-depleted egg extract. If co-addition of ABRAXAS with BRCA1-BARD1 rescues BRCA1 depletion, we will be able to perform the proposed structure-function analysis of BRCA1. If not, we will pursue the mass spectrometry-based approaches outlined in the proposal to identify other proteins that might have been co-depleted with BRCA1.

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