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Coordination of BRCA1/BARD1- and MRE11/RAD50/NBS1-dependent DNA transactions in breast tumor suppression

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14. ABSTRACT

The main objective of the proposal is to understand how the Mre11-Rad50-Nbs1 complex and the BRCA1-BARD1 complex interact on DNA and coordinate DNA transactions that are critical for the maintenance of genomic stability and to prevent breast tumor development. We have characterized the behavior of BRAC1/BARD1 on DNA, using a single molecule approach. We have developed new single molecule technologies to study the behavior of BRCA1/BARD1 and MRN complexes. Finally, we have established that MRN-BRAC1/BARD1 interactions are dispensable for MRN/CtIP dependent DNA end resection, the first step of homology-dependent repair.

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INTRODUCTION

BRCA1/BARD1 and MRE11/RAD50/NBS1 (MRN) play critical roles in preventing the onset of breast tumorigenesis. This is underscored by the fact that mutations in BRCA1 are associated with the most frequent form of hereditary breast cancer (*1*) and women who inherit mutations in the *BRCA1* gene have an estimated lifetime risk of breast and/or ovarian carcinoma as high as 85% (*2*). In addition, mutations in NBS1, RAD50 and Mre11 are associated with increased risk for breast cancer (*3, 4*) or with sporadic breast tumors (*5*). Finally, BRCA1/BARD1 and MRN associate in to form DNA damage-specific complexes, critical for damage checkpoint signaling (*6*). To better understand the functional significances of these interactions, we propose to determine how BRCA1/BARD1 and the MRN complex cooperate in the recognition, signaling and repair of DNA damage during DNA replication and at double-stranded breaks (DSBs) induced by DNA damaging agents. We hypothesize that each protein complex influences the behavior of the other on DNA and that the roles of these proteins in the maintenance of genomic stability are ultimately dictated by their dynamic interactions on DNA. The overall objective of this collaborative effort is to understand precisely how BRCA1/BARD1 breast tumor suppressor complex orchestrates DNA transactions critical for genome stability and how this process interfaces with MRN's diverse roles.

BODY

Significant progress has been made towards Task 1 and Task 2, as outlined in the Statement of Work. However, the project has not been completed as it was originally anticipated. The project involves the use of highly purified recombinant protein complexes (MRE11-RAD50-NBS1 and BRCA1-BARD1). These complexes are purified by chromatography following expression in baculovirus-infected cells. Our FPLC (to perform chromatography) apparatus broke in the fall of 2009. We made several attempts to fix it but we had to replace it eventually. This is a critical piece of equipment for Dr. Gautier's laboratory that recently acquired a new apparatus (AKTA purifier 10, $60,000). Given that it took some time to secure the funding to purchase the new equipment, our progress have been slower than anticipated. Accordingly, we have requested a No-Cost Extension for this proposal, which has been granted by the DOD.

In the First Task we proposed to start characterizing BRCA1/BARD1 interactions with DNA by Total Internal Reflection Fluorescent Microscopy (TIRFM).

We have previously reported (2009) our progress in purifying BRCA1/BARD1 protein complexes from baculovirus-infected cells. We purified enough protein complexes to perform the single molecule analysis proposed before our FPLC needed replacement.

We have completed initial characterization of quantum dot(QD)-tagged Brac1/Bard1 interactions with dsDNA using our single molecule DNA curtain imaging technology. We have shown that the QD tagged protein complexes work in our single molecule assays, that the labeling is specific, and that the labeled proteins are well-behaved in our single molecule assays. We have also demonstrated that BRCA1/BARD1 binds double stranded DNA at apparently random locations, and that the protein appears to undergo one-dimensional (1D) diffusion along the DNA in a direction that is biased by buffer flow (see below) before finally stopping at a fixed location (unpublished). We do not yet know the significance of the 1D sliding behavior nor do we know what DNA features influence the binding distributions, but it seems reasonable to expect that these properties reflect mechanistic attributes of BRCA1/BARD1 as it searches for appropriate binding targets. We have also continued to characterize quantum dot(QD)-tagged QD-tagged MRN interactions with dsDNA using our single molecule DNA curtain imaging technology. We have shown that the QD labeling strategy works for this protein complex in our single molecule assays and that the labeling is specific. We have also shown that MRN also slides rapidly along DNA, and that it can bring together two molecules of DNA. This MRN-mediated reaction appears to occur by a zippering mechanism where the molecules first join at

their free end, and then are brought together over regions spanning at 20-30 kilobases (unpublished). We are currently analyzing the data that we have obtained from both the BRCA1/BARD1 experiments and the MRN experiments (see below) before proceeding to the analysis of BRCA1/BARD1 and MRN with the larger complex made up of BRAC1/BARD1/MRN, and we also anticipate beginning work with the mutant proteins as described in the original proposal.

Furthermore, we have also perfected our new approach to making DNA curtains using nanofabricated barriers to lipid diffusion that are now made by electron-beam lithography.(*7*) This process now allows to make highly precise barriers for aligning thousands of molecules of DNA, and we can align them in a variety of configurations, including molecules with defined lateral dispersion,(*8*) molecules anchored by either end (referred to as double-tethered DNA curtains),(*9, 10*) and molecules organized into crisscrossing patterns (unpublished). The double-tethered curtains will be especially relevant to our studies of BRCA1/BARD1 and MRN, as initial characterization of these protein complexes has revealed 1D diffusive motion that is biased in one direction by buffer flow. Continuous buffer flow is an absolute requirement for all of our previous DNA curtain configurations, but our new double-tethered curtains eliminate the need for continuous buffer flow, which will now allow us to probe the exact mechanisms by which BCRA1/BARD1 and MRN travel along DNA.

Finally, in addition to the new DNA Curtain methods, we have established procedures for looking at DNA substrates bound by nucleosomes,(*10, 11*) and for making new dsDNA substrates that have long ssDNA overhangs resembling a processed DSB. These new procedures will be especially poignant for our work on BRCA1/BARD1 and MRN, as we can now study the proteins within the context of DNA substrates engineered to resemble naturally occurring DSBs within the context of chromain.

In the Second Task, we proposed to investigate the interactions between BRCA1/BARD1 and MRN complexes. Analysis by TIRFM and in cell-free extracts.

Progress towards the completion of second task have been impaired by our inability to generate highly purified MRN complexes required for the single molecule studies (see above). Therefore, we have focused our effort on the characterization of the MRN-CtIP interactions with BRCA1/BARD1 in cell-free extracts derived from Xenopus eggs, using the system described in the previous report.

The experimental system takes advantage of the ability to monitor synchronously the loading of signaling and repair proteins at DNA double-strand breaks (DSBs), including the MRN complex, ATM, CtIP, BRCA1 and RPA, the latter being used as a marker for processing of DSBs. The first event in DSB repair is the generation of single strand DNA (ssDNA). First, we assessed the role of Mre11-Rad50-Nbs1 complex (MRN) in resection using this new assay. We find that depletion of the MRN complex completely abrogates DNA end resection in M-phase extract (Figure 1, middle panel). Moreover, we show that CtiP depletion likewise abrogates resection in M-phase (Figure 1, right panel).

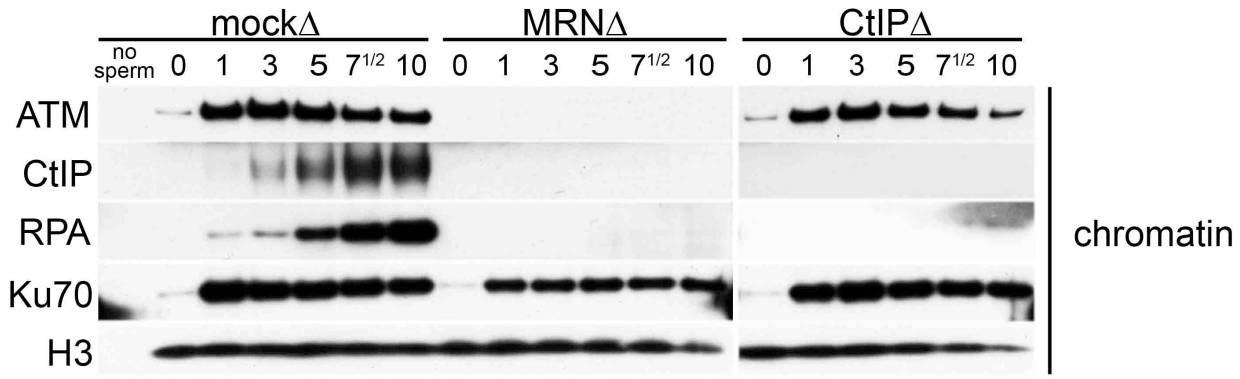
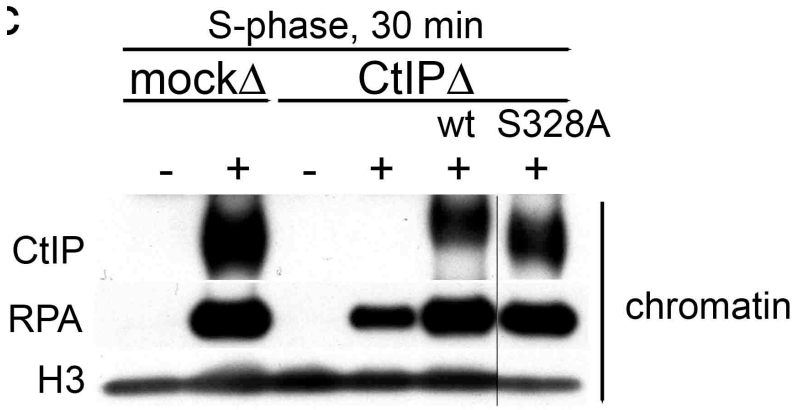


Figure 1. MRN and CtIP are absolutely required for DNA end resection in M-phase.
Kinetics of recruitment of proteins to DSB-containing chromatin in in Mock-depleted, Mre11-depleted, and CtIP-depleted M-phase extracts. M-phase extract (meiotic, CSF-arrested) was pre-incubated with sperm chromatin (5,000 sperm/μL). Aliquots of the sample (15 μL) were taken prior to (0 min) and at the indicated time (minutes) after addition of PflMI restriction endonuclease (0.05 U/ μL) and processed for chromatin-isolation followed by Western blotting with the indicated antibodies. Ku70 is a marker of DSBs generation following restriction digest. RPA is a ssDNA binding protein and is a marker for DNA end resection. Histone H3 (H3) is used as loading control.

It has been proposed that BRCA1 played a critical role in this early step of DSB repair (*12*). However, this point has been controversial and different results have been reported (*13, 14*). Our work shows unambiguously that MRN is required for this first step of DNA resection together with CtIP (Figure 1). CtIP is one of the few bona fide BRCA1-interacting protein with BACH1 and Abraxas. Phosphorylated CtIP interacts specifically with the BRCT tandem repeat of BRCA1 (*15*). Mutation of the phosphorylated serine to a non-phosphorylatable alanine, abrogates BRCA1-CtIP interaction in both chicken and mammalian cells. To evaluate the impact of BRCA1-CtIP interactions on CtIP-dependent resection, we generated and purified a non-phosphorylatable version of Xenopus CtIP (xCtIP-S328A). We then monitored resection in cell-free extracts depleted from endogenous xCtIP and supplemented with either wild type or mutant

recombinant xCtIP.

Figure 2. BRCA1-CtIP/MRN interactions are dispensable for MRN-dependent DNA end resection. Sperm chromatin was incubated in S-phase extract that was mock-depleted, CtIP-depleted, or CtIP-depleted supplemented with 75 nM wildtype or S328A xCtIP, and was treated with buffer (-) or PflMI (+) for 30 minutes. Samples were processed for chromatin-isolation and Western blotting as indicated. A. Control extract



As anticipated, depletion of CtIP from M-phase extracts abolished resection as seen by the lack of RPA bond to chromatin (Figure 2). Addition of wild type recombiannt xCtIP protein restored resection to control levels. Notably, addition of mutant CtIP (xCtIP-S328A) unable to bind to BRCA1 also restored CtIP-dependent resection to control levels (Figure 2). This established that BRCA1-CtIP interactions are not required for the initial step of DSB processing generated by restriction endonucleases.

KEY RESEARCH ACCOMPLISHMENTS

- Characterized the behavior of BRAC1/BARD1 on DNA, using a single molecule approach.
- Developed new single molecule technologies to study the behavior of BRCA1/BARD1 and MRN complexes.
- Established that MRN-BRAC1/BARD1 interactions are dispensable for MRN/CtIP dependent DNA end resection, the first step of homology-dependent repair.

REPORTABLE OUTCOME

S. Peterson, R. Baer, M. Gottesman and J. Gautier. Cdk1 uncouples CtIP-dependent resection and Rad51 filament formation in M-phase. (Submitted)

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CONCLUSION

Our experiments in cell-free extracts have allowed us to separate the functions of the BRCA1/BARD1 and MRN complex. Because both complex have been associated with the development of breast tumors, we hypothesize that their common role in checkpoint signaling might be critical to prevent breast tumor development.

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