AWARD NUMBER: W81XWH-16-1-0599

TITLE: Dissecting the Mechanisms of Drug Resistance in BRCA1/2-Mutant Breast Cancers

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CONTRACTING ORGANIZATION:
The Geneva Foundation

Tacoma, WA 98402

REPORT DATE: October 2018

TYPE OF REPORT: Annual Report

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2018	Annual	30 Sept 2017-29 Sept 2018

4. TITLE AND SUBTITLE

Dissecting the Mechanisms of Drug Resistance in BRCA1/2-Mutant Breast Cancers

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-1-0599

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

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

Poly(ADP-ribose) polymerase (PARP) inhibition provides a promising therapeutic modality for targeting homologous recombination (HR) deficient tumors such as BRCA1 and BRCA2-mutated triple negative breast cancers (TNBCs). Although PARP inhibitors have shown activity in the BRCA-associated TNBCs, several of these tumors develop *de novo* as well as acquired PARP inhibitor (PARPi) resistance. Besides attenuation in intracellular uptake of drugs, the only known mechanism that drives chemotherapy resistance of BRCA1/2-deficient cancers is through the restoration of HR. Recent studies from our laboratories (Nussenzweig and D'Andrea) indicate that deregulation of pathways that promote extensive degradation of nascent DNA strands and alternative end-joining (Alt-EJ) can render BRCA1/2-deficient cells resistant to PARPi in a HR-independent manner. The objective of our project is to collaboratively test the hypothesis that complex processes involving Alt-EJ or replication fork stability promote survival and drive resistance to chemotherapy. A detailed assessment of the critical mediators that regulate the balance between HR, Alt-EJ and replication fork degradation should identify novel means to overcome acquired chemoresistance in BRCA1/2-mutated breast cancers. During the second year of the DOD funding, we have made significant progress in clarifying mechanisms and identifying proteins which could contribute to replication fork stability and chemoresistance in BRCA2-deficient tumors.

15. SUBJECT TERMS Breast cancer, BRCA1, BRCA2, PARP inhibitors, chemotherapy, resistance, HR, replication fork stability, EZH2, PAR, FK866, NMNAT-1/2/3

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

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1. INTRODUCTION:

The inactivation of the tumor suppressor genes *BRCA1* and *BRCA2* by mutations or epigenetic silencing is a critical event in breast and ovarian carcinogenesis. *BRCA1* and *BRCA2* encode proteins that are essential for accurate double strand break (DSB) repair by homologous recombination (HR). BRCA1 functions early during DSB resection, BRCA2 functions later in HR by catalyzing RAD51 nucleo-filaments at processed DSBs. Accordingly, HR deficient breast and ovarian tumors are highly sensitive to poly(ADP-ribose) polymerase (PARP) inhibitors, since PARP inhibitors exhibit synthetic lethality in tumors with defective HR DNA repair. PARP inhibitors are currently in development for BRCA- or otherwise HR repair-deficient cancers, with FDA approval of Olaparib, Rucaparib and Niraparib. Of these drugs, Olaparib has been the most widely studied thus far, and it has been approved by the FDA as a monotherapy for treatment of ovarian cancer patients with germline *BRCA1* or *BRCA2* mutations. Nonetheless, *de novo* and acquired PARP inhibitor (PARPi) resistance, is a pressing clinical problem in patients with BRCA-deficient cancers treated with PARP inhibitors. Therefore, identification of the mechanisms underlying PARPi resistance is crucial for improving treatment and predicting tumor responses. Besides attenuation in intracellular uptake and increased efflux of drugs, the other known mechanism of PARPi resistance in BRCA-deficient tumors include restoration of HR due to somatic reversion of *BRCA1/BRCA2* or loss of other genes such as 53BP1, RIF1 or REV7. Recently, it was shown that BRCA1 and BRCA2 protect stalled replication forks from Mre11-mediated degradation, independent of their roles in HR. Accordingly, restoration of either HR capacity or replication fork stability is also associated with PARPi resistance in BRCA-deficient tumors. Indeed, a recent study from the Nussenzweig laboratory indicated that loss of *PTIP* protects replication forks from degradation in both *BRCA1*- and *BRCA2*- deficient cells and confers PARPi resistance. The D'Andrea laboratory has recently identified a novel DNA repair pathway, the so-called PARP/POLQ end-joining pathway, which, when upregulated, provides the HR-deficient breast tumor cell with an alternative mechanism of DNA repair. Collectively, recent studies from both laboratories indicate that deregulation of pathways that promote extensive degradation of nascent DNA strands and alternative end-joining (Alt-EJ) can render BRCA1/2-deficient cells resistant to PARPi in a HR independent manner. *We had therefore hypothesized that replication fork protection and PARP mediated Alt-EJ are novel and potentially interlinked mechanisms by which BRCA1/2-deficient breast cancers acquire resistance to chemotherapy.* Accordingly, the objective of our project is to provide a more detailed assessment of the factors that contribute to replication fork protection and Alt-EJ. This could lead to therapeutic approaches to overcome acquired resistance by targeting new vulnerabilities in both *BRCA1/2*-mutant and *BRCA1/2*–wildtype breast cancer.

2. KEYWORDS:

Breast cancer, BRCA1, BRCA2, PARP inhibitors, chemotherapy, resistance, HR, replication fork stability, EZH2, PAR, FK866, NMNAT-1/2/3, iPOND, mass spectrometry, PTIP, MLL

3. ACCOMPLISHMENTS:

The major goal of the project is to identify the molecular mechanisms of PARPi resistance in BRAC1/BRCA2 mutated breast tumors in order to improve therapeutic options for breast cancer patients.
The following specific aims were proposed:

> **Specific Aim 1**. Understand how PTIP-MLL3/4 and PARP1 confers chemoresistance and replication fork (RF) degradation in BRCA1/2-deficient cells.
> **Specific Aim 2**. Determine the interactions of BRCA2, FANCD2, and POLQ in replication fork (RF) stability and Alt-EJ
> **Specific Aim 3**. Assess mechanisms of PARPi resistance in mouse models and patient derived xenografts

- What was accomplished under these goals?

We have described major activities, specific objectives, significant results or key outcomes, conclusions and other achievements related to each specific aim in the following section. Both Drs. Nussenzweig and D'Andrea have noted tasks for which they were responsible (**Site 1, NCI, NIH; Site 2, DFCI**).

Specific Aim 1: Understand how PTIP-MLL3/4 and PARP1 confers chemo-resistance in BRCA1/2-deficient cells

Major Task 1. Defining the functional domains of PTIP and the contribution of MLL3/4 to drug resistance (**Site 1, NCI, NIH, Dr. Nussenzweig**)

Preliminary results described in our Breakthrough Award proposal and in Arnab Ray Chaudhuri et. al., *Nature* 2016, led to the hypothesis that the loss of PTIP rescues *BRCA1*- and *BRCA2*-deficient cells from replication fork (RF) degradation by impairing Mre11 nuclease association with nascent DNA. PTIP has been shown to constitutively associate with PA1 and with MLL3/MLL4 histone methyltransferases which catalyze methylation of histone H3 at lysine 4. Since the recruitment of PTIP to stalled forks is independent of 53BP1, we speculated that its interaction with the MLL3/4 complex is relevant.

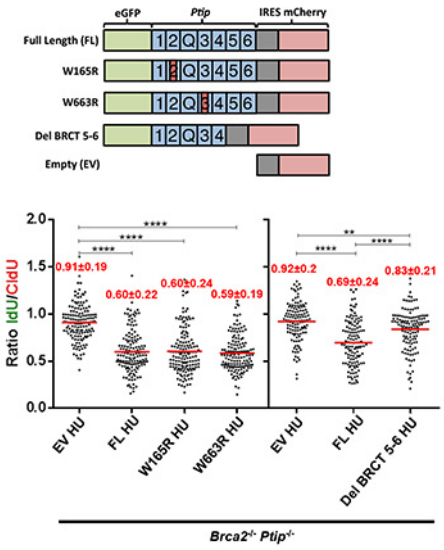

Fig. 1. MLL3/4 promotes replication fork degradation in *Brca2*- deficient cells
Upper panel shows the schematic of the retroviral PTIP mutant constructs used to identify the domain of PTIP involved in driving replication fork degradation. Different BRCT domains in PTIP are numbered and Q represents the glutamine rich region between the 2nd and the 3rd BRCT domains. **Lower panel** shows the ratio of IdU *vs.* CldU upon HU treatment of *Brca2*$^{-/-}$ *Ptip*$^{-/-}$ B lymphocytes retrovirally infected with either EV, FL, W165R, W663R and Del BRCT 6-5 PTIP-mutant constructs and sorted for GFP or mCherry expression. Numbers in red indicate the mean and standard deviation for each sample (**$P \leq 0.01$, ****$P \leq 0.0001$, Mann-Whitney test). 125 replication forks were analyzed for each condition.

To identify the region of PTIP that promotes RF degradation in Brca2-deficient cells, we expressed EV (empty vector), FL (full-length PTIP), W165R (disrupting interactions with PA1), W663R (disrupting interactions with 53BP1 at DSBs) or Del-BRCT5-6 (disrupting interaction with MLL3/4 independently of DSBs) in Brca2/Ptip doubly deficient cells (**Fig. 1, upper panel**). We observed that only reconstitution of Brca2/Ptip-deficient cells with PTIP-Del-BRCT5-6 maintained fork protection (**Fig. 1, lower panel**).

We next tested whether the recruitment of MRE11 at stalled forks was dependent on MLL3/4. We observed that MRE11 association at RFs was dependent on MLL3/4 as monitored by iPOND (isolation of proteins on nascent DNA) and immunofluorescence analysis. We also observed an enrichment of H3K4me1 and H3K4me3 at nascent forks upon HU treatment that was PTIP- and MLL3/4-dependent. Thus, deposition of MRE11 on newly synthesized or stalled chromatin correlates with the establishment of H3K4me1 and H3K4me3 at RFs (**Fig. 2, upper right panels**).

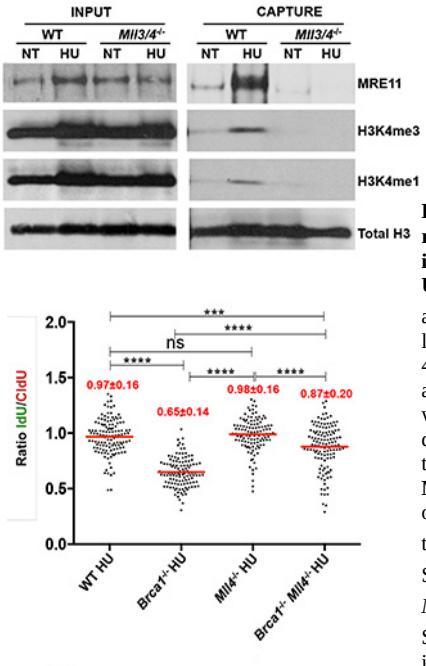

Fig. 2. MLL3/4 promotes replication fork degradation in *Brca2*- deficient cells
Upper Panel: WT and *Mll3/4*$^{-/-}$ MEFs were EdU-labeled for 15 min and treated with 4 mM HU for 4 hr. Proteins associated with replication forks were isolated by iPOND and detected by Western blotting with the indicated antibodies.
Middle and Lower Panels: Ratio of IdU *vs.* CldU upon HU treatment in WT, *Brca1*$^{-/-}$, *Mll4-SET*$^{-/-}$, *Brca1*$^{-/-}$ *Mll4*$^{-/-}$, *Brca2*$^{-/-}$ *Mll4*$^{-/-}$ and *Brca1*$^{-/-}$ *Mll4-SET*$^{-/-}$ B cells. Numbers in red indicate the mean and standard deviation for each sample (ns, not significant, ****$P \leq 0.0001$, Mann-Whitney test). At least 125 replication forks were analyzed for each genotype.

To determine whether MLL4 contributes to degradation of stalled forks in Brca-deficient cells, we examined RF degradation in Brca1$^{-/-}$ Mll4$^{-/-}$ and Brca2−/−Mll4−/− B cells. Brca1$^{-/-}$Mll4$^{-/-}$ and Brca2$^{-/-}$ Mll4$^{-/-}$ cells displayed a significant rescue of fork degradation (**Fig. 2, middle panel**). To test whether MLL4 methyltransferase activity is critical for this rescue, we targeted the catalytic SET domain of MLL4 in Brca1-deficient B cells. We observed a significant rescue of fork degradation in Brca1$^{-/-}$ Mll4-SET$^{-/-}$ cells, suggesting that the methyltransferase activity is important for promoting fork degradation (**Fig. 2, lower panel**).

Major Task 2: Determine the mechanisms by which PARP1 modulates chemosensitivity in BRCA2 deficient cells (**Site 1, NCI, NIH, Dr. Nussenzweig**)

Our preliminary results and data published in Ding et. al., *Nat. Commun* 2016 indicated that PARP1 is required for the recruitment of MRE11 to stalled replication forks (as measured by iPOND experiments). We, therefore, speculated that PARP1 deficiency protects replication forks from degradation in the absence of BRCA2. We have tested this hypothesis directly by monitoring the shortening of the IdU fiber track upon HU treatment in conditional knockout B cells. We generated B lymphocytes from WT, Parp1$^{ko/ko}$, Brca2$^{cko/cko}$ and Parp1$^{ko/ko}$;Brca2$^{cko/cko}$ mice. The cko allele was deleted by transducing the cells with CRE-expressing retrovirus after stimulating B cells with lipopolysaccharide+interleukin-4 and RP105. On HU treatment, the average IdU to CldU ratios were determined to be 0.957, 0.976, 0.624 and 0.979 for WT, Parp1$^{ko/ko}$ and Brca2$^{cko/cko}$ and Parp1$^{ko/ko}$;Brca2$^{ko/ko}$ cells, respectively (**Fig. 3**). Thus, consistent with our observation in mESCs (Ding et. al., *Nat. Commun* 2016), loss of PARP1 resulted in protection of the nascent strand in Parp1$^{ko/ko}$; Brca2$^{ko/ko}$ B cells.

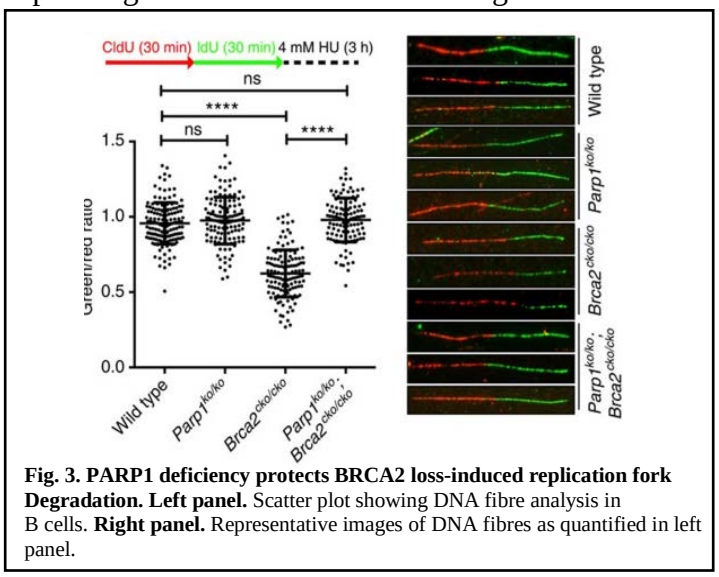

Fig. 3. PARP1 deficiency protects BRCA2 loss-induced replication fork Degradation. Left panel. Scatter plot showing DNA fibre analysis in B cells. **Right panel.** Representative images of DNA fibres as quantified in left panel.

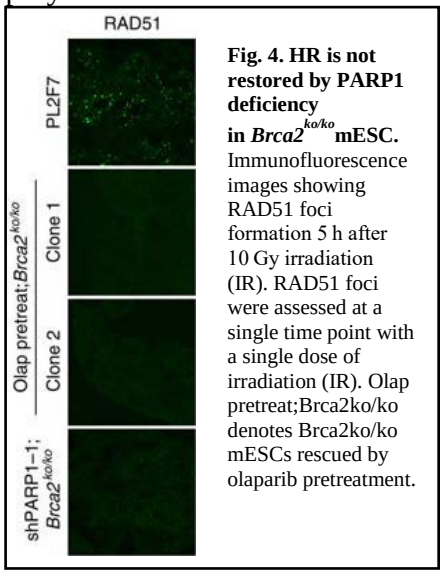

Fig. 4. HR is not restored by PARP1 deficiency in *Brca2$^{ko/ko}$* mESC. Immunofluorescence images showing RAD51 foci formation 5 h after 10 Gy irradiation (IR). RAD51 foci were assessed at a single time point with a single dose of irradiation (IR). Olap pretreat;Brca2ko/ko denotes Brca2ko/ko mESCs rescued by olaparib pretreatment.

Since BRCA2 is essential for the recruitment of RAD51 at DSBs to mediate HR, we examined whether RAD51 recruitment in response to DSB induction was restored in Brca2$^{ko/ko}$ cells rescued by PARP1 knockdown by shRNAs or by olaparib treatment (**Fig. 4**). We did not observe any RAD51 foci in these cells in response to irradiation (**Fig. 4**), although RAD51 was expressed at levels similar to the control cells (data not shown). Similarly, the loss of PARP1 in Brca1-deficient B cells failed to rescue RAD51 foci formation (**Fig. 5**). Taken together,

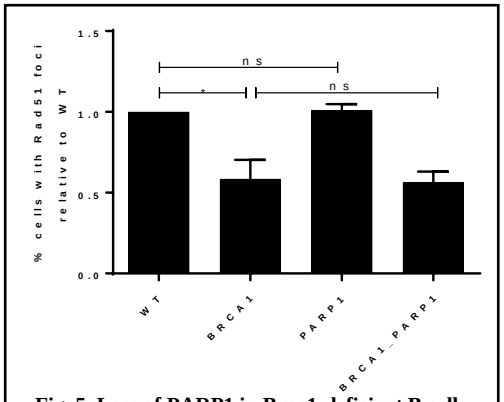

Fig. 5. Loss of PARP1 in Brca1-deficient B cells protects nascent DNA from degradation without restoring HR. RAD51 foci formation 5 h after 10 Gy irradiation (IR). RAD51 foci were assessed at a single time point with a single dose of irradiation (IR).

these results demonstrate that DSB-induced HR is not restored in rescued Brca1 or Brca2-deficient cells when PARP1 (or PTIP) is depleted.

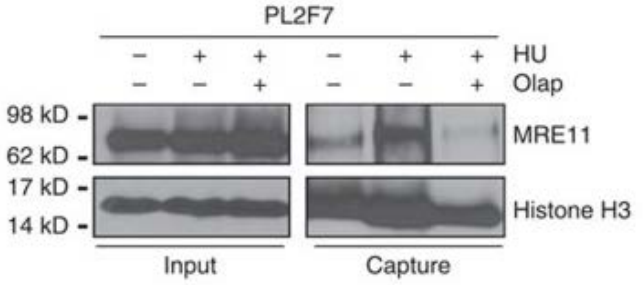

Fig. 6. Impaired MRE11 recruitment contributes to fork protection and cell survival after BRCA2 loss. Western blot of iPOND samples in PL2F7 (mESC) cells showing MRE11 and Histone H3 levels at the replication fork. HU, 4 mM, 4 h. Olaparib (Olap), 1 μM, 2 h pretreatment, with HU for 4 h.

We proceeded to examine the molecular mechanism of fork protection that may contribute to the rescue of $Brca2^{ko/ko}$ mESCs. Since MRE11 nucleolytic activity is responsible for BRCA2 loss-induced fork degradation and MRE11 has been reported to interact with PARP1, we investigated whether the effect of PARP1 deficiency was mediated via MRE11 recruitment. We, therefore, examined the presence of MRE11 at the fork by iPOND in PL2F7 cells (mESCs) treated with HU (4 mM, 4 h) or HU with olaparib (1 μM, 2 h pretreatment followed by HU for another 4 h). HU treatment induced marked increase in MRE11 recruitment to stalled replication forks. However, olaparib reduced the association of MRE11 to stalled replication forks (**Fig. 6, top right panel**). This led us to conclude that the impaired recruitment of MRE11 to stalled replication forks by PARP inhibition or PARP1 deficiency contributes to fork protection in $Brca2^{ko/ko}$ mESC.

In our previous annual report and described in Ray Chaudhuri et. al., *Nature* 2016 and Ding et. al., *Nat. Commun* 2016, we used a variety of distinct and complementary approaches to demonstrate that cellular PARylation levels affect genomic instability in Brca2-deficient cells. Specifically, we had shown the presence of significantly elevated levels of PAR polymer proteins in Brca2-deficient cells. Since PARPs are NAD+-dependent enzymes and require a source of NAD+, we observed that supplementation with the NAD+ precursors further increased cellular levels of PARylation and chromosomal aberrations in Brca2-deficient cells. In contrast, the knock-down of Nmnat-1, which is a central enzyme in NAD+ biosynthesis in the nucleus, resulted in significantly decreased chromosomal aberrations in Brca2-deficient cells. Strikingly, we also showed that a deficiency in NMNAT-1 rescued the lethality of Brca2-deficient ESCs. In addition, down regulation of cellular PAR levels by overexpressing PAR hydrolases, such as PARG (an enzyme which cleaves the linkage between ADP-ribose and acceptor proteins), in Brca2-deficient B cells resulted in decreased genomic instability upon cisplatin treatment. These results indicated that cellular PARylation levels affect genomic instability in Brca2-deficient cells.

Since cells lacking Brca2 are HR-deficient, we sought to identify HR-independent mechanisms that might explain the effects of PARylation on genomic instability. Work described in the Year 1 annual report and this report (above) show that replication fork stabilization can mediate chemoresistance and rescue genome instability in Brca2-deficient cells. We, therefore, focused on identifying proteins associated at replication forks of Brca2-defective cells which could play a role in replication fork stabilization and concurrently

inform our understanding of PAR metabolism. We utilized iPOND (isolation of proteins on nascent DNA) coupled with quantitative SILAC (stable isotype labeling of amino acids in cell culture) mass spectrometry (technical details in **Major Task 3**, below) to investigate protein enrichment at replication forks in Brca2-defective cells. Replication fork proteomes of Brca2^{Y3308X} mutant mESCs (hypomorphic ESCs with a C-terminal truncation in Brca2) were compared with wild-type cells (**Fig. 7**). Interestingly, we identified NMNAT-1 as a reproducibly enriched protein at replication forks of Brca2^{Y3308X} mutant cells (**Fig. 7**). CHEK1 is known to be activated at replication forks that encounter damaged DNA and stabilize replication forks and/or restart stalled replication forks. The enrichment of CHEK1 at replication forks of Brca2^{Y3308X} mutant cells is consistent with the fact that Brca2^{Y3308X} mutant cells have a defect in HR. In these analyses, the abundance of replication proteins, like the PCNA or MCM complex were unchanged between wild-type and Brca2^{Y3308X} mutant cells. Previous reports have shown that PARP1 and NMNAT-1 physically and functionally interact at target gene promoters where it produces NAD+ to support PARP1 catalytic activity. These results indicate that the hyperactivation of PARP1 observed in BRCA2-defective cells could be meditated by NMNAT-1 enrichment at replication forks leading to defective fork protection.

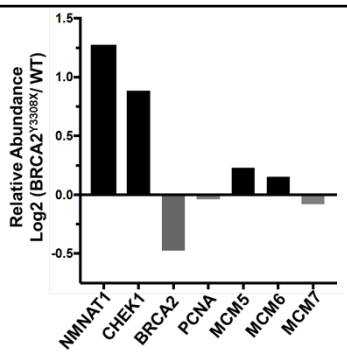

Fig 7. NMNAT1 is recruited at forks in BRCA2^{Y3308X} hypomorphic mutant mESCs. iPOND-SILAC-MS identified proteins enriched at replication forks in *Brca2*^{Y3308X} hypomorphic mutant mESCs. Log_2 abundance ratios for selected proteins is depicted.

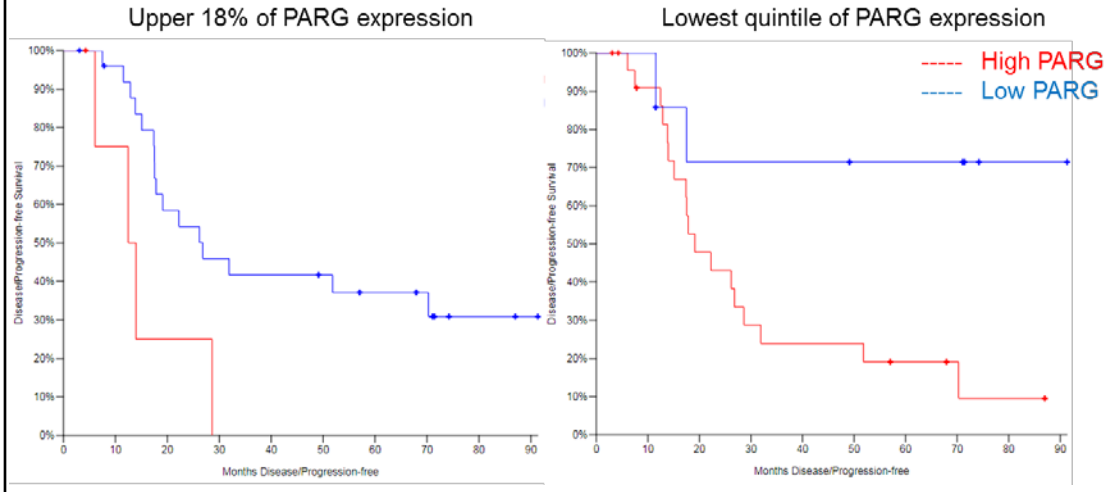

Fig. 8 Difference in progression-free survival (PFS) of *BRCA2*-mutated ovarian serious adenocarcinoma patients with standard platinum-based regimens. (L, R panels) Data were obtained from the TCGA project. Patients were separated into PARG low or high-expression on the basis of the upper 18% (**L**) or lower quintile (**R**) of PARG expression z-scores. The difference between the PFS of PARG-low versus PARG-high was assessed by univariate log-rank *P* value (*P*=0.018 and *P* =0.025 in **L** and **R**, respectively). PFS curves for PARG-low and PARG-high expressing tumors were generated by the Kaplan-Meier method. All reported *P* values are two-sided.

Next, we wanted to know whether PARylation of Brca2-defective cells have any clinical associations. To test whether differential levels of the PAR hydrolase PARG expression could

be an indicator of patient responses to platinum chemotherapy, we queried clinical information from The Cancer Genome Atlas (TCGA) of patients with BRCA2-mutated ovarian serous adenocarcinomas treated with platinum chemotherapy. Survival analysis demonstrated that platinum-treated BRCA2 mutants with high PARG expression were correlated with a shorter progression-free survival (PFS) (**Fig. 8**). Lower expression of PARG also predicted a longer PFS in BRCA2-associated ovarian cancers (**Fig. 8**). Taken together, these data suggest that PARG levels could be used as a biomarker for acquired resistance to platinum-based chemotherapy in BRCA2-mutated ovarian cancers.

In the future, we aim to focus on the molecular mechanisms regarding how PARylation modulates fork protection in Brca2-defective cells. We are generating Nmnat-1 stable knock-downs and Parg overexpressed clones in Brca2^{Y3308X} mutant mESCs to investigate whether defective fork protection is rescued in this context. PARP1 is required for the recruitment of the MRE11 nuclease to stalled replication forks and its loss rescues the embryonic lethality and replication fork stability in Brca2-null ESCs. We will, therefore, also investigate whether MRE11 recruitment to stalled replication forks is attenuated in Nmnat-1 knock-downs or Parg overexpressed BRCA2^{Y3308X} mutant mESC clones. These experiments will clarify mechanistic details of synthetic viability and drug resistance in Brca2-deficient cells.

<u>Major Task 3</u>: Determine the changes in replisome composition associated with replication fork degradation and protection (**Site 1, NCI, NIH, Dr. Nussenzweig**)

Loading of the central recombinase RAD51 at DSBs by BRCA2 is essential for mediating error free HR repair and is considered to be essential for cellular viability. However, it is unclear how tumor cells are viable in the absence of BRCA2. Our data suggests that Brca2^{Y3308X} mES cells deficient for HR dependent DSB repair can perform recombination at normal rates as measured by sister chromatid exchanges (SCEs) (**Fig. 9**). Spontaneous and damage induced SCEs have been previously shown to be dependent on RAD51. This led us to analyze the effect of RAD51 loading at unchallenged and HU stalled replication forks in WT and Brca2^{Y3308X} hypomorphic mES cells. Our data showed that RAD51 was fully competent to be loaded at stalled replication forks in BRCA2 defective cells, although at reduced levels (**Fig. 10, top right panel**) when compared with stalled forks in WT cells.

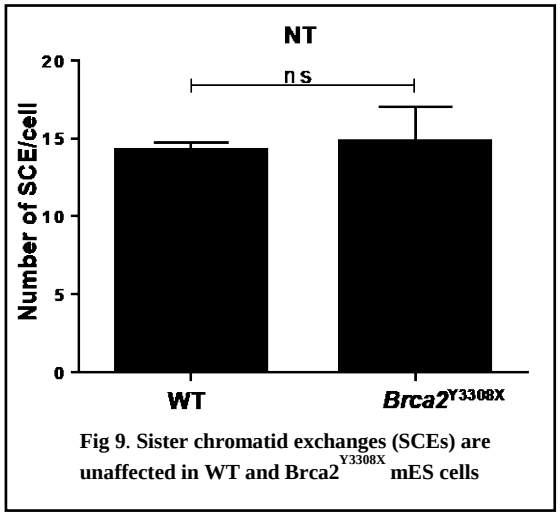

Fig 9. Sister chromatid exchanges (SCEs) are unaffected in WT and Brca2^{Y3308X} mES cells

Further systematic analysis of the composition of proteins at active and stalled replication forks in WT and Brca2^{Y3308X} mESCs necessitated an unbiased approach. We, therefore, coupled iPOND with mass spectrometry as shown in the schema in **Fig. 11**.

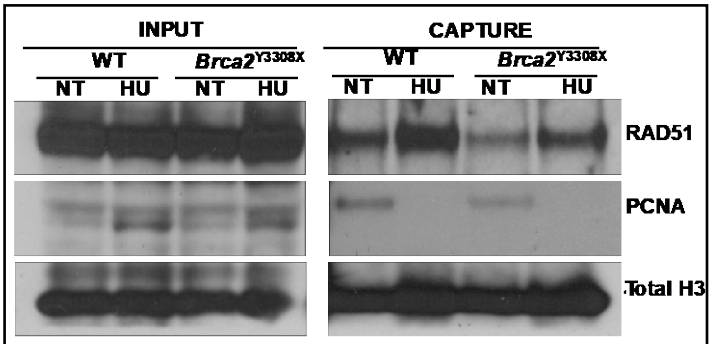

Fig. 10. iPOND analysis in WT and Brca2^{Y3308X} mES cells. Western Blot for RAD51 and PCNA at untreated (NT) and hydroxyurea (HU) stalled forks. Results indicate that there is a substantial increase of RAD51 loading at stalled forks even in BRCA2 deficient cells.

To accomplish this, we used wild type murine ESCs and established a feeder free cell culture system. With the help of the lab of David Cortez (Vanderbilt University and a collaborator on this proposal) who developed this technology originally in human lines, we labeled these murine cells with heavy and light amino acids (**Fig. 11**). We performed proof of principle experiments in wild type (WT) ESCs by analyzing protein recruitment to replication forks in the presence and absence of the replication stalling agent hydroxyurea (HU). Our experiments showed a high degree of concordance and reproducibility between biological replicates - resulting in 85% overlap between the two WT samples. Analysis of highly enriched proteins at stalled replication forks in WT cells revealed proteins of the checkpoint kinases, ATR pathway, homologous recombination (HR) proteins like BRCA1, BRCA2 and Rad51, Fanconi Anemia (FA) proteins and ssDNA binding proteins like RPA (data not shown).

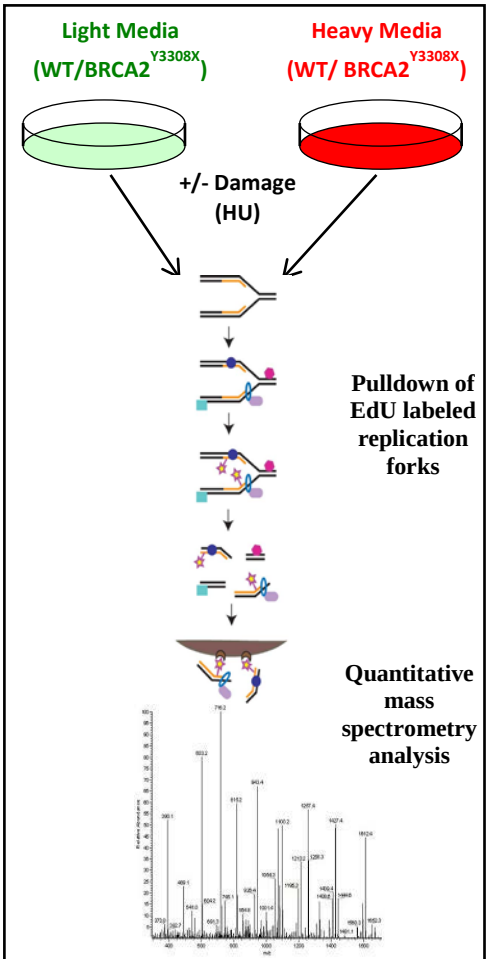

Fig 11. Methodology for iPOND coupled to SILAC-mass spectrometry analysis. WT mES or BRCA2^{Y3308X} cells were differentially labeled with light or heavy amino acids. These cells are then labeled with EdU for 15 mins and the heavy labeled cells are treated with HU for 2 hrs and crosslinked. Equal numbers of heavy and light labeled cells are mixed. EdU labeled replication forks with associated proteins are pulled down after click reaction chemistry, de-crosslinked and analyzed by mass spectrometry.

We next labeled WT and BRCA2^{Y3308X} cells with light and heavy amino acids. These cells were then treated with the replication stalling agent hydroxyurea and analyzed for differential protein recruitment at stalled replication forks. The experimental sets included comparisons between WT (heavy vs light) and BRCA2^{Y3308X} (heavy vs light) cells. Each experiment was repeated twice and the overlap of proteins detected between them was further analyzed. A comparison between the WT and the BRCA2^{Y3308X} sets resulted in an overlap of approximately 900 proteins - 85% of detected proteins were found in both WT and BRCA2^{Y3308X} datasets.

We then triaged this list by only examining proteins enriched at stalled replication forks upon HU treatment that were common to both WT and Brca2^{Y3308X} cells. Our analysis revealed that a significant subset (~ 100 proteins) were enriched by 1.5 fold at stalled replication forks - independent of BRCA2 status. As expected, quantitative analysis by mass spectrometry detected RAD51 in BRCA2 defective cells, albeit at reduced levels than WT cells, confirming our previous observation (**Fig. 12 and Fig. 10 above**).

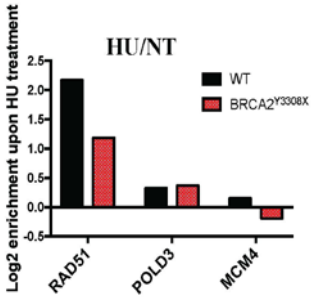

Fig. 12. Enrichment of RAD51 at stalled replication forks as analyzed by iPOND-mass spectrometry upon HU treatment when compared to untreated cells in WT and Brca2^{Y3308X}. MCM4 and POLD3 serve as controls where these proteins are not enriched at significant levels at stalled replication forks. Results indicate that there is a substantial increase of RAD51 loading at stalled forks even in BRCA2-deficient cells

A more comprehensive pathway analysis of the proteins recruited at stalled replication forks in WT and Brca2^{Y3308X} cells revealed that, independent of BRCA2 status, the majority of proteins could be classified as belonging to DNA repair, checkpoint, sister chromatid cohesion, chromatin modification, transcription and origin firing pathways (**Fig. 13**). Further categorization of the proteins associated with DNA repair showed an enrichment of activities involved in the HR pathway. These HR proteins include MMS22L, TONSL, RAD51 paralogs and the BRCA1-A complex (**Fig. 14**). We speculate that some of these proteins could mediate RAD51 loading at stalled replication forks. Our mass spectrometry data also showed enrichment of chromatin associated proteins like ATRX, DAXX, H2AY, independent of BRCA2. Interestingly, reports have shown that loss of these latter proteins also display replication stress phenotypes and impaired homologous recombination. It is feasible to assume that these proteins are involved in the loading of RAD51 through the modulation of the chromatin environment at stalled replication forks.

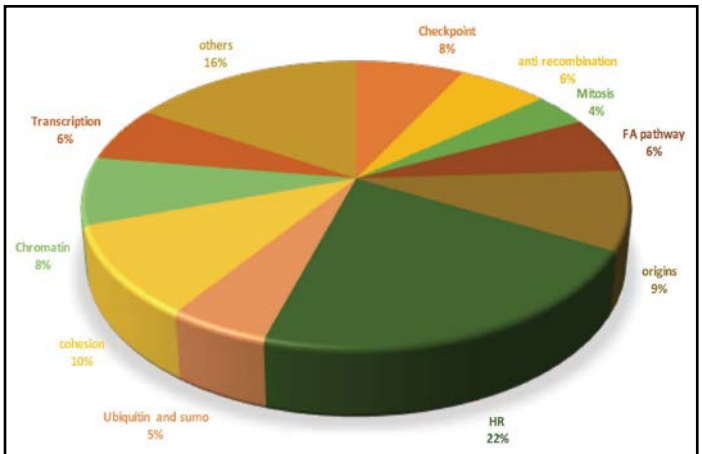

Fig. 13. Pie chart depicting the enrichment of proteins for specific pathways upon HU treatment that are common to both WT and *Brca2*^{Y3308X} mES cells at stalled replication forks analyzed by iPOND-mass spectrometry

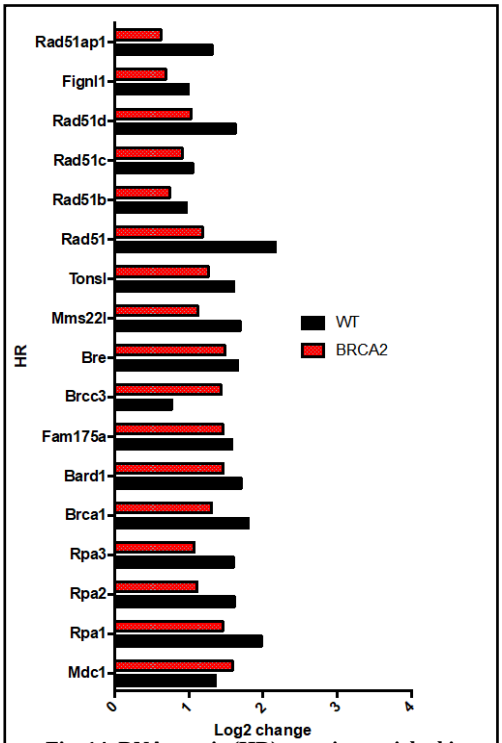

Fig. 14. DNA repair (HR) proteins enriched in both WT and Brca2^{Y3308X} mES cells upon replication stress

Specific Aim 2: Determine the interactions of BRCA2, FANCD2, and POLQ in Replication Fork stability and Alt-EJ

Major Task 4: To determine whether the concurrent deletion of BRCA2 and POLQ results in synthetic lethality and reduced breast tumorigenesis.

We have recently published a report indicating that BRCA1/2 deficient breast tumors have a hyperdependency on POLQ expression and activity (Ceccaldi et al, Nature, 2016). More recently, we have determined that BRCA2-deficient breast tumor cells can become resistant to PARP1 inhibitors, by another novel mechanism. Specifically, during the second year of DOD support, Dr. D'Andrea's laboratory has made considerable progress in the understanding of mechanisms of PARP inhibitor resistance. At the beginning of the year, Dr. D'Andrea's laboratory published a critical paper (Rondinelli B, Gogola E, Yücel H, Duarte AA, van de Ven M, van der Sluijs R, Konstantinopoulos PA, Jonkers J, Ceccaldi R, Rottenberg S, D'Andrea AD. EZH2 promotes degradation of stalled replication forks by recruiting MUS81 through histone H3 trimethylation. Nat Cell Biol. 2017 Nov;19(11):1371-1378). This paper, which cites support from this DOD grant, demonstrated that BRCA2-deficient breast cancers, but not BRCA1-deficient breast cancers, can develop resistance to PARP inhibitors (PARPi) by downregulating a novel pathway – namely, the EZH2/MUS81 pathway. Our laboratory demonstrated that, during normal replication fork progression, EZH2 methylates H3K27, which contributes to the recruitment of the nuclease, MUS81, to the RF. While MUS81 plays a normal role in maintaining RF progression, in the setting of BRCA2 deficiency, MUS81 is toxic and causes degradation of reversed forks. Through our ovarian and breast murine studies, supported by this DOD grant, and through our collaborations with the Rottenberg lab, we have shown that EZH2 and MUS81 are indeed downregulated in PARPi resistant tumors (Figure 15).

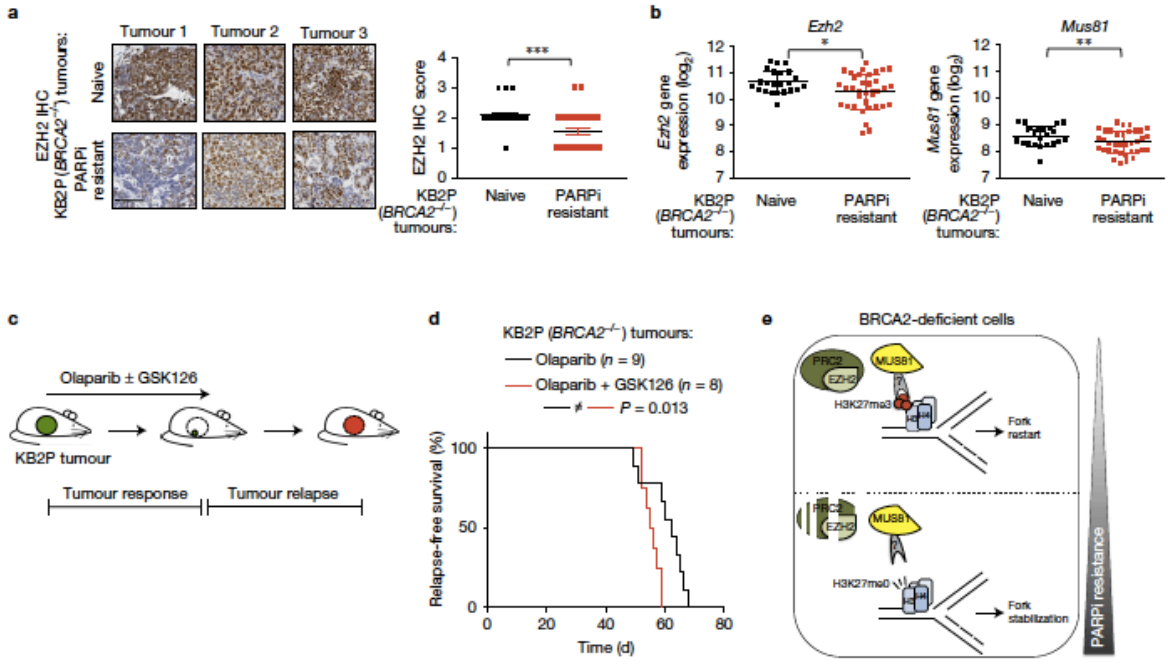

Figure 15. Ezh2 inhibition drives relapse f KB2P-derived breast tumors in mice following PARPi treatment. (**a**) Representative staining (left) and quantification (right) of EZH2 expression by immunohistochemistry (IHC) in naive (n = 24) versus PARPi (olaparib)-resistant (n = 37) KB2P-derived tumours. Scale bar, 50 µm. $^{***}P$ = 0.00051. (**b**) Normalized gene expression of *Ezh2* and *Mus81* using RNA-seq of a panel of naive (n = 24) and resistant (n = 37) KB2P-derived tumours. $^{*}P$ = 0.028 for Ezh2 and $^{**}P$ = 0.0059 for Mus81. (**c**) Outline of the PARPi treatment in mice. Following tumour outgrowth to approximately 200 mm^3, mice were treated with either olaparib alone (n = 9) or olaparib + GSK126 (n = 8). Relapse-free survival was defined as the time needed for the tumours to grow back to the initial volume (200 mm^3) after the administered treatment. (**d**) Relapse-free survival of mice carrying orthotopically injected KB2P-derived tumours that were treated as indicated. (**e**) Model for EZH2-mediated PARPi resistance in BRCA2-deficient cells. EZH2 methylates Lys27 on histone 3 (H3K27me2/3) at stalled forks. Methylated H3K27 allows the binding of the endonuclease MUS81, ultimately promoting fork degradation and restart. Following EZH2 downregulation, Lys27 on histone 3 remains unmethylated (H3K27me0). As a consequence, MUS81 recruitment to stalled forks is reduced, resulting in increased fork stabilization. In agreement with this, low EZH2 or MUS81 levels at stalled forks cause PARPi resistance. Data in **a** and **b** represent mean ± s.d. and significance was determined by the two-sided Mann–Whitney test. Significance in **d** was determined by the two-sided log-rank (Mantel–Cox) test, for which the P value is indicated and the confidence interval is 95%. In **a** and **b**, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, with exact P values (with a confidence interval of 95%) noted in the legends for the relevant panels.

<u>Major Task 5</u>: To determine the mechanism of replication fork instability in FANCD2-deficient cells

To study fork stability in more detail, we have performed the following experiments. Specifically, during the second year of support, we have also identified new mechanisms of PARPi resistance. The D'Andrea laboratory has generated a BRCA1-null cell line (RPE p53 mutant, BRCA1 mutant) and selected these cells for PARPi resistance. Ten novel resistant clones were isolated. These clones become PARPi resistant, either by restoring HR repair, or by restoring replication fork stability. Functional analysis of these clones is shown in Figure 16.

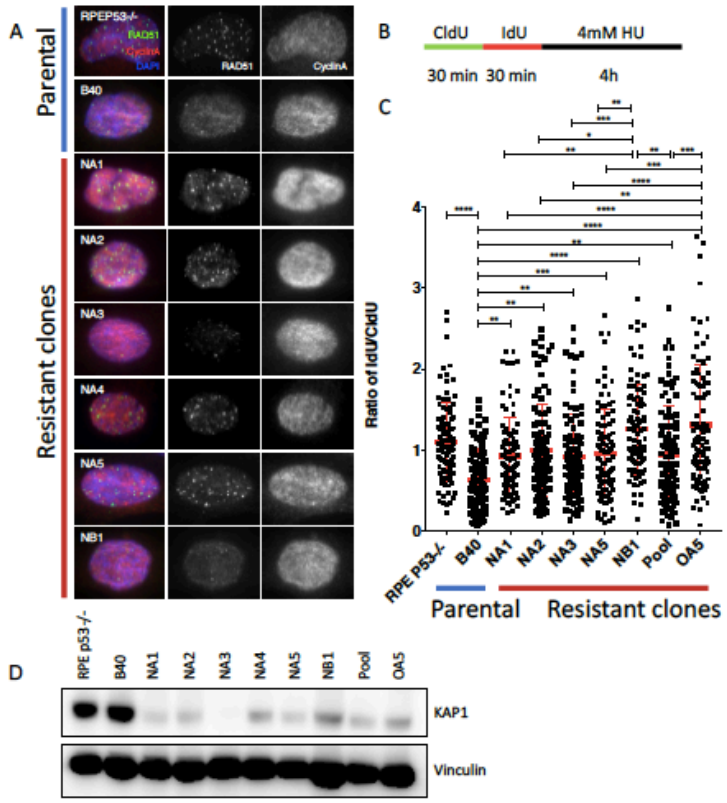

Figure 16. We characterized HR restoration with RAD51 foci (**A**), replication fork protection with the DNA fiber assay (**B, C**), and DDR activation of the clones with western blotting (**D**). We found that 2 out of 6 (33%) of the clones (NB1 and NA3) did restore HR. Using the fiber assay (**B**), all clones had significantly more stable replication forks when compared to the parental B40 cell line (**C**). All subclones had decreased levels of KAP1 (**D**). * p<0.05, ** p<0.005, *** p<0.001, **** p<0.0001

We are now analyzing these clones in more detail, by RNA-seq, to determine the mechanism of PARPi resistance.

Major Task 6: To identify and characterize proteins which cooperate with FANCD2 and POLQ in replication fork stability and Alt-EJ

We have not completed Major Task 6. We plan to complete this task within the next 6 months. Specifically, we plan to identify and characterize additional proteins which cooperate with FANCD2 and POLQ.

Specific Aim 3: Assess mechanisms of PARPi resistance in mouse models and patient derived xenografts

Major Task 7: Evaluate replication fork stability relative to PARPi/cisplatin response in genetically engineered mouse models

We have established additional studies to evaluate synthetic lethal relationships, using mouse models. Specifically, during the second year of DOD support, the D'Andrea laboratory also determined another critical component of replication fork stability in BRCA1 deficient tumor cells. The lab demonstrated that BRCA1-deficient tumor cells are hyperdependent on the USP1/UAF1 complex. This DUB complex deubiquitinates FANCD2-Ub and PCNA-Ub at the fork. Importantly, loss or inhibition of the DUB results in increased PCNA-Ub at the fork, leading to prolonged recruitment of TLS polymerases. The persistent activation of these TLS polymerases is toxic in the BRCA1-deficient setting. The synthetic lethal relationship of BRCA1 knockdown with USP1/UAF1 knockdown was confirmed through multiple experiments. First, BRCA1/USP1 synthetic lethality was confirmed by analysis of the Broad Institute Dependency Database. Second, using a xenograft (mouse) model, as proposed in the DOD grant, we determined that simultaneous knockdown of BRCA1 and USP1 results in breast tumor cytotoxicity (Figure 17). This

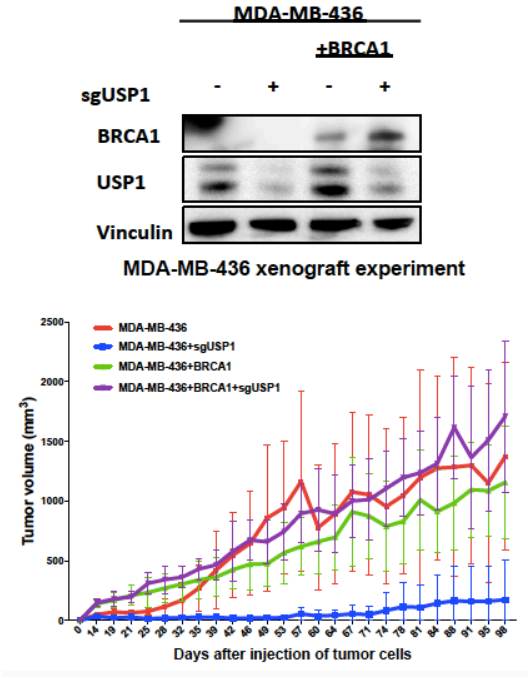

Figure 17. Reduced tumor growth of BRCA1-deficient MDA-MB-436 breast cancer cells with CRISPR-mediated knockout of USP1. Upper panel: Western blot of the lysates from MDA-MB-436 cells and MDA-MB-436+BRCA1 cells following USP1 knockout. Lower panel: Tumor growth of MDA-MB-436 cells, MDA-MB-436+sgUSP1 cells, MDA-MB-436+BRCA1 cells and MDA-MB-436+BRCA1+sgUSP1 cells in athymic nude mice. 5 mice/group were used and the tumor cells were implanted on both the flanks of mice. (P-values of MDA-MB-436+sgUSP1 group vs every other group <0.0001).

important result suggests that the USP1 inhibitor, ML323, might be efficacious for treatment of some BRCA1 mutant tumors. Milestone: This work is currently under review in the journal Molecular Cell (Lim KS, Li H, Roberts EA, Gaudiano EF, Clairmont C, Ponnienselvan K, Liu JC, Sambel L, Yang C, Parmar K, Yusufzai T, Zheng N, D'Andrea AD. USP1 is Required for Replication Fork Protection in BRCA1-Deficient Tumors. 2018, Mol Cell, Submitted).

Major Task 8: Evaluate Replication Fork Stability and HR competence in PARP Inhibitor Sensitive and Resistant TNBC-PDX models

In our attempts to complete Major Task 8, we made an unexpected discovery which has now become an important new direction for the D'Andrea laboratory. Specifically, during the second year of DOD support, the D'Andrea laboratory has identified another novel mechanism of PARPi resistance – namely, the amplification and overexpression of the TRIP13 gene (Clairmont C, Sarangi P, Ponnienselvan K, Galli L, Csete I, Moreau L, Adelmant G, Chowdhury D, Marto J, D'Andrea, AD. TRIP13 Inactivates REV7 and Enhances Homologous Recombination. 2018, Nature, Submitted). Recent studies indicate that the active (closed) isoform of the protein REV7 binds to SHLD1, 2, 3 proteins and forms a functional SHLD complex, capable of blocking DSB resection. Importantly, knockdown of components of the SHLD complex (i.e., knockdown of REV7 or SHLD1, 2, 3) results in enhanced dsb resection and the acquisition of PARPi resistance of BRCA1-deficient cells. Importantly, our laboratory has determined that the ATPase, TRIP13, can open and inactivate REV7, thereby disassembling the SHLD complex and promoting dsb end resection. Moreover, breast tumors are known to have amplified copies of the TRIP13 gene, resulting in PARPi resistance.

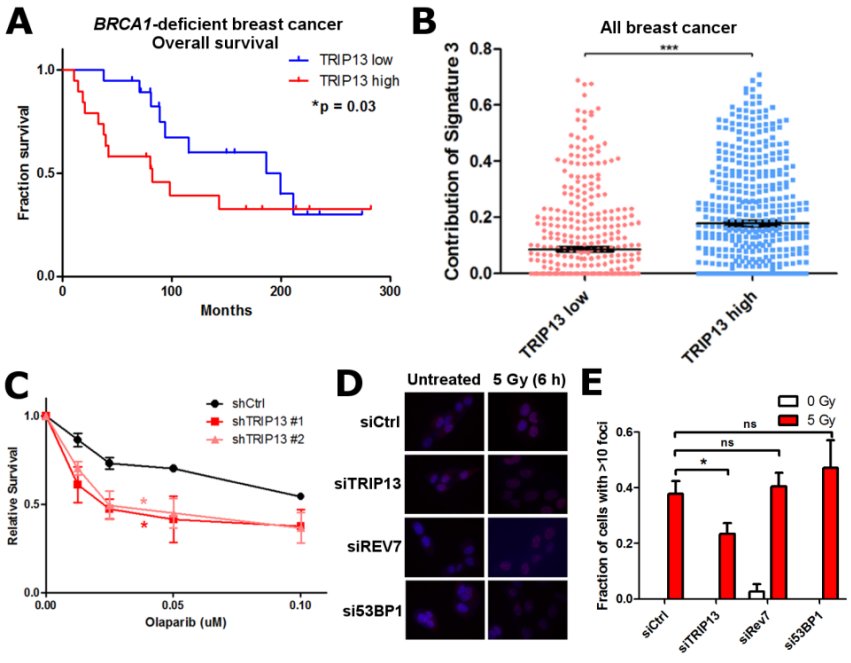

Figure 18: TRIP13 overexpression correlates with poor prognosis in *BRCA1*-deficient breast cancer and enhances homologous recombination in a cell line model (A) Kaplan-Meier overall survival plot of *BRCA1*-deficient breast cancer patients stratified by median TRIP13 expression level, Gehan-Breslow-Wilcoxon test. (B) Relative contribution of HR deficiency mutational signature (Signature 3) in breast cancer patients from TCGA cohort stratified by TRIP13 expression levels. ***p<0.001 (C) 14-day clonogenic survival assay of SUM149PT cells with nontargeting or TRIP13-targeted shRNAs treated with varying Olaparib doses. *p<0.05 (Student's paired t-test, two-tailed) (D) Representative images of IR-induced RAD51 foci in SUM149PT cells. (E) Proportion of SUM149PT cells with greater than 10 RAD51 foci 6 hours following IR treatment. *p<0.05 (Student's paired t-test, two-tailed). Error bars indicate SEM.

To confirm these studies, we have analyzed a BRCA1-mutant breast cancer line, SUM149 (Figure 18). This cell line has biallelic BRCA1 mutations but is PARPi resistant, presumably due to TRIP13 upregulation. Interestingly, knockdown of TRIP13 in these cells results in reduction of HR, and enhanced PARPi sensitivity. A critical mouse experiment planned for the last year of the DOD grant is to generate a xenograft model with the SUM149 cell line, with and without dox-inducible expression of the TRIP13 shRNA. An important predictor of these studies is that knockdown of TRIP13 in this tumor in vivo will result in tumor regression. Again, these studies are directly supported by our DOD grant.

Summary of the key research accomplishments:

During the second year of DOD funding, we have made significant progress in understanding the mechanisms by which proteins such as PTIP and PARP1 contribute to replication fork stability and chemoresistance in BRCA2-deficient tumors. Using a mass spectrometry approach to identify proteins associated with stalled replicating forks, we have identified numerous candidate proteins that could contribute to replication fork protection or destabilization.

We have made progress in identifying the proteins which contribute to the replication fork stability and we have identified a new mechanism of chemoresistance in BRCA2-deficient tumors. We have determined that loss of function of EZH2 in BRCA2-deficient cancer cells promotes PARPi resistance. Importantly, our results identify EZH2 expression as a biomarker of BRCA2-deficient tumor response to chemotherapy. We have published a manuscript describing our EZH2 results. We have also identified USP1 and TRIP13 as proteins that regulate fork stability and HR repair.

What opportunities for training and professional development did the project provide?

Nothing to report

- How were the results disseminated to communities of interest?

Results were shared with the scientific community via informal discussions, posters and presentations at scientific meetings and through publications in peer-reviewed journals

- What do you plan to do during the next reporting period to accomplish the goals and objectives?

4. IMPACT:

Nothing to Report

What was the impact on the development of the principal discipline(s) of the project?

We have identified replication fork protection as a new non-homologous repair independent pathway to achieve chemoresistance in BRCA2-deficient tumors. Besides PTIP and PARP1, we have identified additional proteins that are likely involved in fork protection or destabilization using an unbiased mass spectrometry approach. Characterization of these proteins will give us a better understanding of how other (DNA repair) factors, not involved in the HR pathway, contribute to the prevention of replicative stress and prevent the onset of cancers by maintaining genome stability through the protection of replication forks

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:
Nothing to Report

Significant changes in use or care of human subjects:
Not applicable

Significant changes in use or care of vertebrate animals:

Nothing to Report

Significant changes in use of biohazards and/or select agents:

Nothing to Report

6. PRODUCTS

Publication: Journal publications.

Nothing to Report

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers, and presentations.

Dr. Andre Nussenzweig (Site 1, NCI, NIH)

Invited Speaker, workshop on Chromosomal Instability: From Molecular Mechanisms to Disease, Baeza, Spain, November 2017
Invited Speaker, 46th Autumn Immunology Conference, Chicago, Illinois, USA
Invited Speaker, Seminar Boston Children's Hospital, Boston, MA, February 2018
Keynote Speaker, Annual Gynecologic Malignancies Retreat Dana-Farber Cancer Institute, Boston, MA, March 2018
Invited Speaker, 9th. Journee Roger Monier, Cellular Stress and Cancer, Paris, France, April 2018
Invited Speaker, Seminar Princess Margaret Cancer Centre, University Health Network, Toronto, Canada, April 2018
Invited Speaker, ESHG Conference, Milan, Italy, 2018
Invited Speaker, Gordon Conference, Topoisomerases in Biology & Medicine, South Hadley, MA, 2018
Keynote Speaker, Cancer and Aging Meeting, Pittsburgh, PA, June 2018
Keynote Speaker, 2018 Mutagenesis Gordon Research Conference, Newry, ME, June 2018
Keynote Speaker, Cancer Genome Dynamics Meeting- NYU Perlmutter Cancer Center, New York, September 2018
Invited Speaker, Understanding Sequence-Specific Mutations in Cancer, Memorial Sloan Kettering Cancer Center, New York, October 2018
Invited Speaker, Oncology Programme of the Institute for Research in Biodedicine (IRB), Barcelona, Spain, October 2018

Invited Speaker, workshop on Chromosome Architecture and Topological Stress, Baeza, Spain, October 2018

Website(s) or other Internet site(s)

Nothing to Report

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Andre Nussenzweig
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to Project:	Dr. Nussenzweig was responsible for the project management and collaboration with Dr. D'Andrea.
Funding Support:	Dr. Nussenzweig is supported by the NIH Intramural Research Program

Name:	Elsa Callen
Project Role:	Staff Scientist
Researcher Identifier (e.g. ORCID ID):	N/A

Nearest person month worked:	3
Contribution to Project:	Dr. Callen has been instrumental in developing the replication fork protection assay and in assessing fork stability in a variety of genetic contexts
Funding Support:	Dr. Callen is supported by the NIH Intramural Research Program

Name:	Kenta Shinoda
Project Role:	Post-doctoral fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6
Contribution to Project:	Dr. Shinoda has performed work in the area of PARP1 biology and how affecting co-factor (NAD+) levels influences enzymatic activity and genome stability in BRCA-deficient cells
Funding Support:	Dr. Shinoda is supported by the NIH Intramural Research Program

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS:

COLLABORATIVE AWARDS: We have marked the tasks assigned to us and accordingly we have provided a progress made for each task.

QUAD CHARTS: Not applicable

9. **APPENDICES:** Nothing to report