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: Oct 2019

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
Oct 2019	Annual Report	30 September 2018 – 29 September 2019

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)
The Geneva Foundation
917 Pacific Ave. # 600
Tacoma, WA 98402

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 drives 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 first year of the DOD funding, we have made progress in identifying the proteins which contribute to the replication fork stability and we have identified new mechanisms of chemoresistance in BRCA2-deficient tumors.

15. SUBJECT TERMS

Breast cancer, BRCA1, BRCA2, PARP inhibitors, chemotherapy, resistance, HR, replication fork stability, haploinsufficiency, RNF168, 53BP1, PTIP, Shieldin, end resection

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

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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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, haploinsufficiency, RNF168, 53BP1, PTIP, Shieldin, end resection

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

Subtask 5: To perform structure/function studies using PTIP mutants (**Site 1, NCI, NIH, Dr. Nussenzweig**)

We have previously shown that PTIP (as part of the MLL3/4 methyltransferase complex) promotes replication fork (RF) degradation in *Brca1/2*-deficient cells and that deletion of PTIP restored RF stability leading to chemoresistance (Ray Chaudhuri et al, Nature 2016). In addition to MLL3/4, PTIP interacts with 53BP1, a prominent anti-recombination protein that is known to suppress BRCA1-dependent HR but has no effect on RF stability. Given that loss of 53BP1 function is a recurrent and clinically relevant mechanism by which *Brca1*-deficient tumors acquire resistance to PARP inhibitors, we performed structure/function studies to clarify how PTIP-53BP1 binding impacts HR and chemoresistance. The PTIP W663R mutant, which disrupts interaction with 53BP1, has been described to cause embryonic lethality in the mouse, limiting its usefulness. Instead, we developed a new transgenic mouse model carrying a point mutation in 53BP1 (S25A) that abolishes its binding to PTIP (**Fig. 1**).

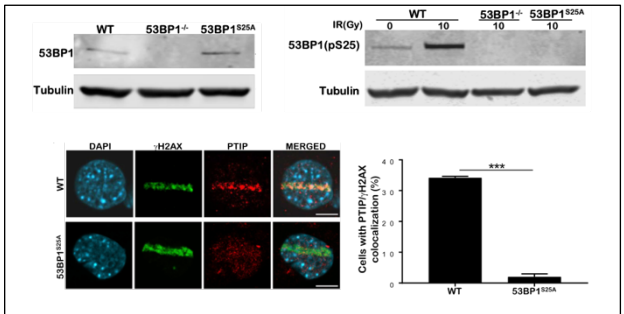

Fig.1. 53BP1(S25A) mutation disrupts PTIP-53BP1 binding. (A) Expression of 53BP1 protein in WT, *53BP1*$^{-/-}$ and *53BP1*^{S25A} B cells. (B) DNA damage-induced phosphorylation of 53BP1 on serine 25 in WT, *53BP1*$^{-/-}$ and *53BP1*^{S25A} B cells at baseline and after exposure to 10 Gy of ionizing radiation (IR). (C) 53BP1-dependent recruitment of PTIP to laser-induced DSB sites in WT, and *53BP1*^{S25A} MEFs.

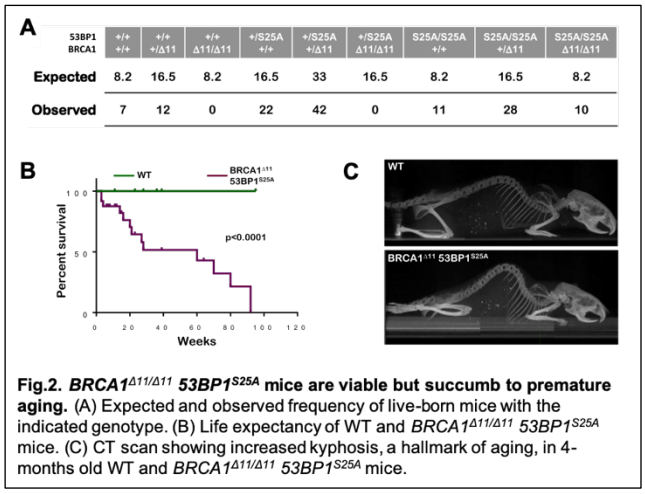

Fig.2. *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*^{S25A} mice are viable but succumb to premature aging. (A) Expected and observed frequency of live-born mice with the indicated genotype. (B) Life expectancy of WT and *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*^{S25A} mice. (C) CT scan showing increased kyphosis, a hallmark of aging, in 4-months old WT and *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*^{S25A} mice.

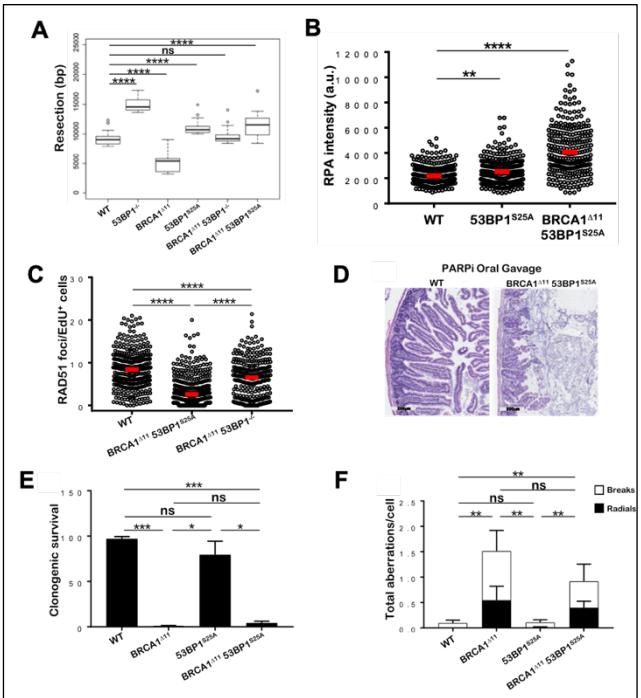

Fig.3. Disruption of PTIP-53BP1 interaction restores DSB processing but not RAD51 loading in *BRCA1*$^{\Delta11/\Delta11}$ cells. (A) Average lengths of resection tract at endonuclease (AsiSI)-induced DSBs as measured by END-seq. (B) Quantification of IR-induced RPA binding on damaged chromatin in WT, *53BP1*^{S25A} and *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*^{S25A} MEFs. (C) Quantification of IR-induced RAD51 foci formation in WT, *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*$^{-/-}$ and *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*^{S25A} MEFs. (D) Histological section of mouse small intestine showing massive destruction of tissues in *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*^{S25A} mice following PARPi treatment by daily oral gavage. (E) Colony formation capacity of MEFs with the indicated genotypes following continuous PARPi treatment. (F) Quantification of chromosomal aberrations in MEFs with the indicated genotypes after exposure to PARPi for 16 h.

Employing mouse genetics, we found that loss of PTIP-53BP1 binding (by S25A) rescued the embryonic lethal phenotype of *Brca1*-deficient (*Brca1* $^{\Delta11/\Delta11}$) mice to the same extent as complete *53BP1* ablation. Yet *Brca1* $^{\Delta11/\Delta11}$*/53BP1*^{S25A} mice succumbed to premature aging, unlike *Brca1* $^{\Delta11/\Delta11}$*/53BP1*$^{-/-}$ mice, indicating that not all aspects of 53BP1 function rely on its interaction with PTIP (**Fig. 2**). Indeed, our evidence suggested that loss of PTIP-53BP1 binding restored the initiation of HR in *Brca1*-deficient cells by alleviating the inhibition 53BP1 normally exerts on DSB end resection (**Fig. 3A, B**). Nonetheless, *Brca1* $^{\Delta11/\Delta11}$*/53BP1*^{S25A} cells were not capable of completing subsequent steps of HR due to an inability to load the RAD51 recombinase and consequently remained hypersensitive to PARP inhibition (**Fig. 3C-F**). Thus, the function of 53BP1 in HR can be functionally separated into its previously known role on inhibiting DSB processing and a hitherto unknown role whereby it can directly block RAD51 loading after end resection.

We have shown earlier that in both mouse and human cells lacking functional Brca1, RAD51 loading is accomplished by an alternative pathway initiated by the E3 ubiquitin ligase RNF168, which through chromatin modification recruits the RAD51 loaders PALB2 and Brca2 (Zong et al, Mol Cell 2019). Importantly, we found that PTIP binding to 53BP1 modulates the latter's ability to associate with another protein complex, composed of RIF1, REV7 and Shieldin (itself a complex consisting of SHLD1, SHLD2 and SHLD3) (**Fig. 4A**). In the absence of PTIP binding,

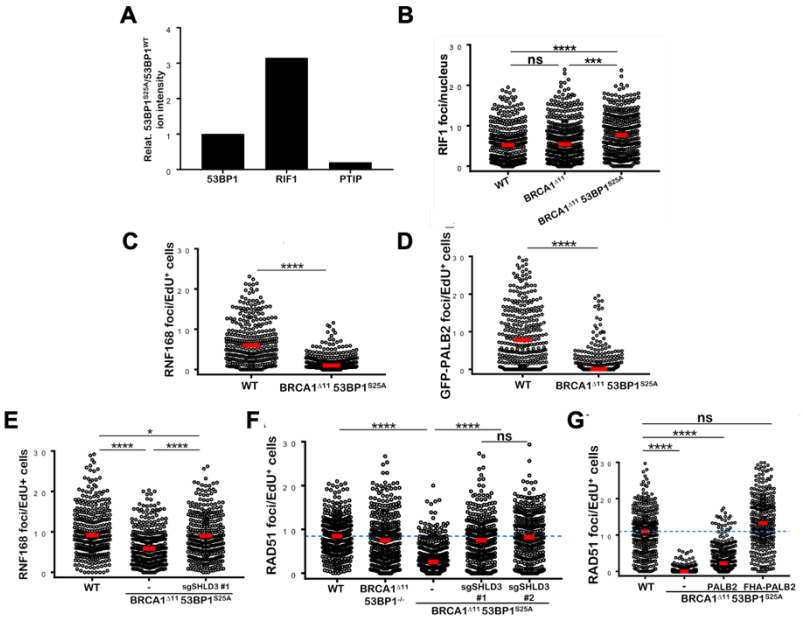

Fig.4. Loss of PTIP-53BP1 interaction enhances RIF1 and Shieldin binding at DSB sites and suppresses a RNF168-mediated alternative RAD51 loading pathway. (A) Relative ion intensities for 53BP1, RIF1, and PTIP peptides associated with the 53BP1 complex in HeLa-S cells expressing FLAG-HA-53BP1(S25A) or FLAG-HA-53BP1WT, as determined by mass spectrometry. (B) Quantification of IR-induced RIF1 foci formation in WT, *BRCA1*$^{\Delta11/\Delta11}$ and *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*^{S25A} MEFs. (C) Quantification of IR-induced RNF168 foci formation in WT and *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*^{S25A} MEFs. (D) Quantification of IR-induced PALB2 foci formation in WT and *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*^{S25A} MEFs. (E) Quantification of IR-induced RNF168 foci formation in WT, *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*^{S25A} and *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*^{S25A} MEFs where Shieldin (SHLD3) is deleted using CRISPR-Cas9. (F) Quantification of IR-induced RAD51 foci formation in WT, *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*$^{-/-}$, *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*^{S25A} and *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*^{S25A} MEFs where Shieldin (SHLD3) is deleted using CRISPR-Cas9. (G) Quantification of IR-induced RAD51 foci formation in WT, *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*^{S25A} and *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*^{S25A} MEFs overexpressing either WT PALB2 or FHA-PALB2.

RIF1/Shieldin is recruited to and associates with 53BP1 at DNA damage sites at supra-physiological levels (**Fig. 4B**). Shieldin (through SHLD2) can bind single stranded DNA (ssDNA) and excessive accumulation of Shieldin significantly impaired the ability of RNF168 to promote alternative RAD51 loading at resected DSBs (**Fig. 4C, D**). This would explain why *Brca1* $^{\Delta11/\Delta11}$*/53BP1*^{S25A} cells do not gain HR competency, even though they are proficient in end resection. Indeed, deletion of Shieldin (SHLD3) completely restored RNF168 recruitment and subsequent RAD51 loading in *Brca1* $^{\Delta11/\Delta11}$*/53BP1*^{S25A} cells (**Fig. 4E, F**). Likewise, efficient RAD51 loading could be achieved in *Brca1* $^{\Delta11/\Delta11}$*/53BP1*^{S25A} cells through forced targeting of PALB2 to DSB sites (FHA-PALB2) (**Fig. 4G**), bypassing the need for RNF168.

Finally, as Brca1 and 53BP1 have been shown to mutually antagonize one another, we examined which domains within the Brca1 protein are responsible for suppressing the distinct pre- and post-resection functions of 53BP1. We found that sequences encoded by *Brca1* exon 11 (frequently mutated in human breast cancers) are primarily involved in overcoming 53BP1's block on resection initiation, while its RING finger domain (frequently mutated in human breast cancers) is important for counteracting 53BP1's block on RAD51 loading post-resection (**Fig. 5**). Altogether, our study provides important new insights into the function of PTIP in HR regulation and demonstrate how 53BP1 utilizes distinct mechanisms to block two Brca1-dependent steps in HR – end resection and RAD51 loading – and their potential consequences for chemoresistance. This data was published recently in Callen et al (Molecular Cell, in press).

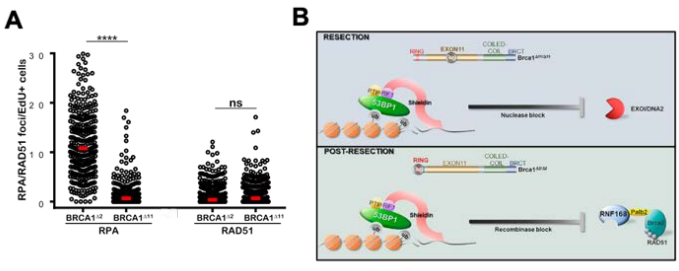

Fig.5. BRCA1 regulates end resection and RAD51 loading using distinct domains. (A) Quantification of IR-induced RPA and RAD51 foci formation in *BRCA1*$^{\Delta11/\Delta11}$ and *BRCA1*$^{\Delta2/\Delta2}$ MEFs. (B) Model depicting how 53BP1 differentially engage with PTIP and RIF1 to suppress the key steps of HR – end resection and RAD51 loading – and how BRCA1 utilizes distinct domains to relieve 53BP1 enforced pre- and post-resection blocks on HR.

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

Subtask 1: To determine whether BRCA2 and POLQ are also synthetic lethal in tumorigenesis by using the conditional BRCA2/p53 knockout mouse model (K14CRE;BRCA2f/f;p53f/f, KB2P) (proposed time line: 12-15 months) (**Site 2, DFCI, Dr. D'Andrea**).

Conditional BRCA2/p53 knockout mice develop breast cancer within 100-300 days after birth. We aim to breed these mice with POLQ knockout mice and evaluate breast cancer progression in triple knockout mice. We anticipate that concurrent knockout of BRCA2/p53 and POLQ will result in synthetic lethality and the triple knockout mice will be tumor free. We are still waiting for ACURO approval. Therefore, these animal experiments have not been yet initiated. However, we have already sufficient number of POLQ knockout mice through our breeding colony of POLQ mutant mice. POLQ mice are being bred using a non-DOD funding source. Of note, during the first year of the DOD funding, we have confirmed all the cellular and biochemical phenotypes of POLQ deficiency.

Milestone(s) Achieved: We have confirmed a role of POLQ protein in Alt-EJ and in replication fork stability.

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

Subtask 1: To test that PTIP/MRE11-mediated nucleolytic degradation is responsible for the replication fork instability in FANCD2-/- cells (proposed time line: 12-18 months).

We had proposed to generate double knockouts in B-cells by crossing PTIPf/f-FANCD2-/- mice with the CD19 CRE transgenic mice and use B-cells from the double knockout mice for their competence in HR and fork stability. As we are still waiting for an ACRURO approval, these animal studies have not been initiated (**Site 2, DFCI, Dr. D'Andrea**).

Milestone(s) Achieved: None

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

Subtask 1: To identify and characterize proteins which cooperate with FANCD2 and POLQ in replication fork stability and Alt-EJ using iPOND/mass spectrometry (time line:6-18 months) (**Site 2, DFCI, Dr. D'Andrea**).

We have not yet performed the experiments proposed for this subtask.

Subtask 2: Validation of FANCD2 and POLQ interacting proteins identified by mass spec using coimmunoprecipitation, shRNA knockdown, changes in POLQ nuclear foci and Alt-EJ template assays (proposed time line: 18-30 months) (**Site 2, DFCI, Dr. D'Andrea**).

We have not yet performed the experiments proposed for this subtask.

During the last year, we have further evaluated the role of POLQ in alternative end joining (Alt-EJ). We have demonstrated that inhibition of POLQ blocks Alt-EJ and selectively kills HR-deficient tumor cells. Specifically, we devised an *in vitro* screen to identify a small molecule inhibitor of Polymerase Q. The POLQ protein has an N-terminal ATPase domain and a C-terminal Polymerase domain. Since the N-terminal ATPase domain has strong ATPase activity *in vitro*, we were able to perform a high throughput screen. We identified Novobiocin (NVB) as a potent and specific inhibitor of the POLQ-ATPase. We have performed several mouse xenograft and GEMM models to confirm that NVB kills BRCA1-deficient tumor cells, as well as other HR-deficient tumor cells. See Specific Aim 3 below.

Also, as part of Subtask 2, we have identified a new mechanism by which BRCA1-deficient PARP inhibitor sensitive tumor cells became PARP inhibitor resistant. We have shown that BRCA1-deficient tumor cells can amplify the gene TRIP13 as a mechanism for restoring HR repair. Specifically, the TRIP13 gene encodes an ATPase which can inactivate REV7, leading to an opening and inactivation of the SHLD complex. The relevance of the SHLD complex results in increased dsb end resection, increased RAD51 loading, and increased HR, leading to acquired PARPi resistance (Figure 6). This work has recently been accepted for publication in the journal, Nature Cell Biology (Clairmont CS*, Sarangi P*, Ponnienselvan K, Galli LD, Csete I, Moreau L, Adelmant G, Chowdhury D, Marto JA, D'Andrea AD. TRIP13 Regulates DNA Repair Pathway Choice through REV7 Conformational Change. Nature Cell Biol, 2019, In Press).

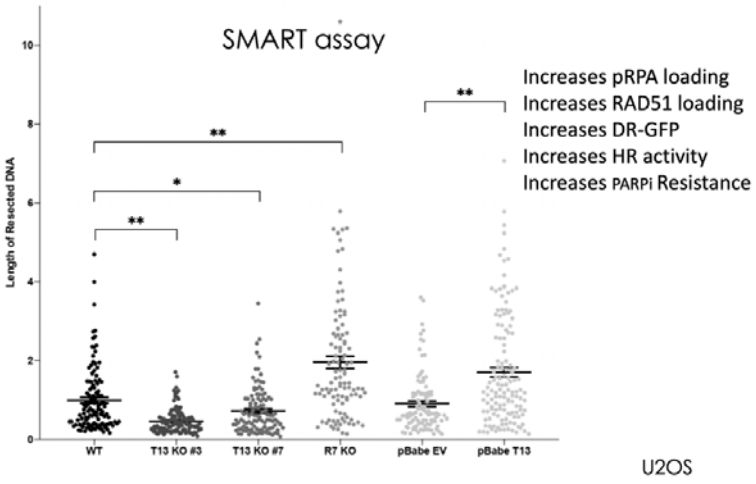

Fig. 6. TRIP13 overexpression increases double strand break end resection, promotes HR repair, and causes PARPi resistance. As shown, overexpression of TRIP13 causes an increase in dsb end resection while CRISPR knockout of TRIP13 reduces end resection. TRIP13 increases end resection by releasing the SHLD complex (Clairmont CS*, Sarangi P*, Ponnienselvan K, Galli LD, Csete I, Moreau L, Adelmant G, Chowdhury D, Marto JA, D'Andrea AD. TRIP13 Regulates DNA Repair Pathway Choice through REV7 Conformational Change. Nature Cell Biol, 2019, In Press).

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

<u>Major Task 7</u>: **Evaluate replication fork stability relative to PARPi/cisplatin response in genetically engineered mouse models (Site 1, NCI, NIH, Dr. Nussenzweig**)

A long-standing question in the field is why mutation of a single allele of *Brca1* in the germline predisposes patients to the development of breast/ovarian cancers that are characterized by genome instability, when heterozygous *Brca1* mutant cells are not overtly defective in either RF protection or HR. To reconcile these observations, we proposed that alternative pathways can compensate for the partial loss of Brca1 function, thus masking the effect of heterozygous *Brca1* mutations. If our hypothesis is proven correct, it would not only significantly enhance our understanding of *Brca*-associated tumorigenesis, but could potentially reveal a means to expand the utility of PARPi/cisplatin beyond the subset of *Brca*-deficient tumors.

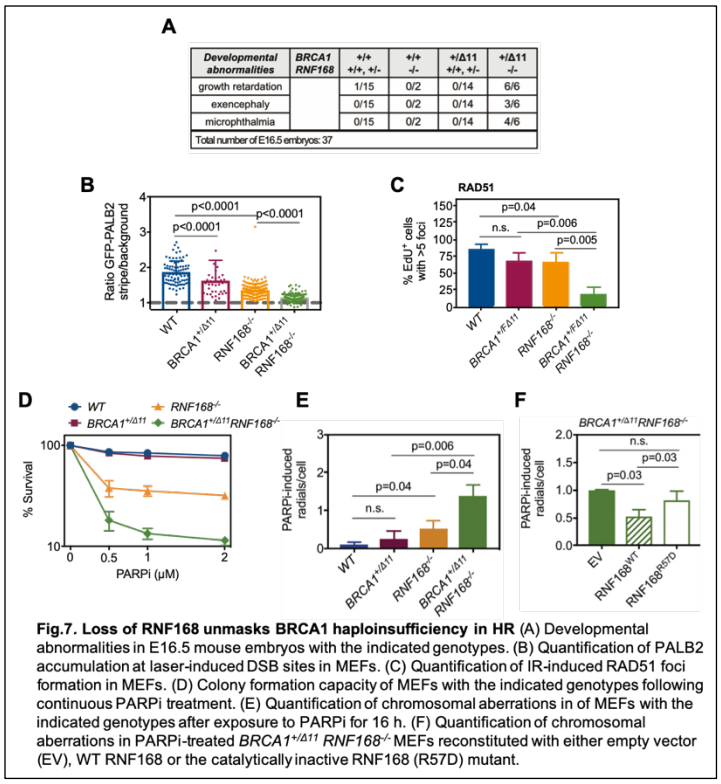

Fig.7. Loss of RNF168 unmasks BRCA1 haploinsufficiency in HR (A) Developmental abnormalities in E16.5 mouse embryos with the indicated genotypes. (B) Quantification of PALB2 accumulation at laser-induced DSB sites in MEFs. (C) Quantification of IR-induced RAD51 foci formation in MEFs. (D) Colony formation capacity of MEFs with the indicated genotypes following continuous PARPi treatment. (E) Quantification of chromosomal aberrations in of MEFs with the indicated genotypes after exposure to PARPi for 16 h. (F) Quantification of chromosomal aberrations in PARPi-treated *BRCA1*$^{+/\Delta11}$ *RNF168*$^{-/-}$ MEFs reconstituted with either empty vector (EV), WT RNF168 or the catalytically inactive RNF168 (R57D) mutant.

Using a series of genetically engineered mouse models, we have now obtained substantial amounts of experimental evidence that support the concept that certain functions of Brca1 are haploinsufficient, but these latent defects are normally masked by the activation of alternative pathways (Zong et al, Mol Cell 2019). Briefly, we found that loss of the E3 ubiquitin ligase RNF168 was highly deleterious in a mice harboring a heterozygous *Brca1* exon 11 mutation (*Brca1*$^{+/\Delta11}$) whereas loss of RNF168 in *Brca1*-proficient counterparts were relatively well tolerated (**Fig. 7A**). Mechanistically, the catalytic activity of RNF168 is capable of supporting an alternative mode of RAD51 loading in cells where HR is partially compromised by a heterozygous *Brca1* exon 11 mutation (**Fig. 7B, C**). As a result, *Brca1*$^{+/\Delta11}$*RNF168*$^{-/-}$ cells became hypersensitive to PARPi and cisplatin whereas *Brca1*$^{+/\Delta11}$ cells remained largely resistant to the same treatments (**Fig. 7D-F**). Interestingly, *Brca1*$^{+/\Delta11}$ cells did not show RF protection defects, even when RNF168 was deleted (**Fig. 8A**). Moreover, loss of RNF168 itself also did not seem to impact RF stability (**Fig. 8A**). Thus, RNF168 specifically masks Brca1 haploinsufficiency in HR, contributing to chemoresistance.

Interestingly, we have previously reported that loss of PTIP conferred chemoresistance to PARPi and cisplatin in *Brca1/2*-deficient cells by restoring RF stability, but not HR competency (Ray Chaudhuri et al, Nature 2016). Thus, maintaining RF stability and HR competency appear to represent two independent means by which *Brca1*-deficient cells may acquire resistance to PARPi/cisplatin. To further test this idea, we characterized another *Brca1* mutant mouse model where exon 2 is deleted (*Brca1* $^{\Delta 2/\Delta 2}$). This mutation results in the production of a truncated Brca1 protein lacking its N-terminal RING domain (RING-less Brca1). Notably, a major *Brca1* founder mutation in the Ashkenazi Jewish population (185delAG) also produces a structurally similar RING-less Brca1 protein. Our analyses showed that cells expressing RING-less Brca1 are highly sensitive to PARPi even though they are apparently proficient in RF protection (**Fig. 8B, C**). However, RING-less Brca1 is severely defective for HR (**Fig. 8D**). Thus, RF stabilization is not a prerequisite for chemoresistance. Rather, HR competency and RF protection can each afford chemoresistance to PARPi/cisplatin. Likewise, loss of HR and RF protection can induce chemosensitivity independently of one another. We further speculate that certain cell-intrinsic and/or external factors can compromise HR and/or RF protection to drive tumorigenesis in patients carrying germline *Brca1* mutations.

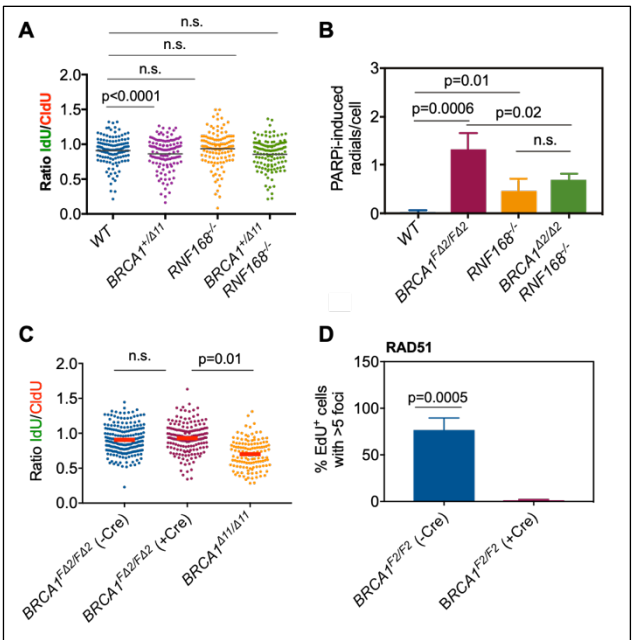

Fig.8. HR promotes chemoresistance independently of replication fork protection (A) Replication fork stability, as measured by the ratio of IdU vs CldU incorporation in hydroxyurea-treated B cells of the indicated genotypes. B) Quantification of chromosomal aberrations in MEFs of the indicated genotypes after exposure to PARPi for 16 h. (C) The ratio of IdU vs CldU incorporation in hydroxyurea-treated MEFs of the indicated genotypes. (D) Quantification of IR-induced RAD51 foci formation in BRCA1F2/F2 MEFs induced or not to express Cre. In C and D, Cre expression results in the deletion of exon 2 of the *Brca1* gene.

<u>Major Task 8</u>: Evaluate POLQ and PARP inhibitor resistance in murine xenograft and GEMM models (Site 2, DFCI, Dr. D'Andrea)

As described in Aim 2, the D'Andrea laboratory has identified a research tool compound, novobiocin (NVB), which is a specific inhibitor of the POLQ ATPase. More recently, we have

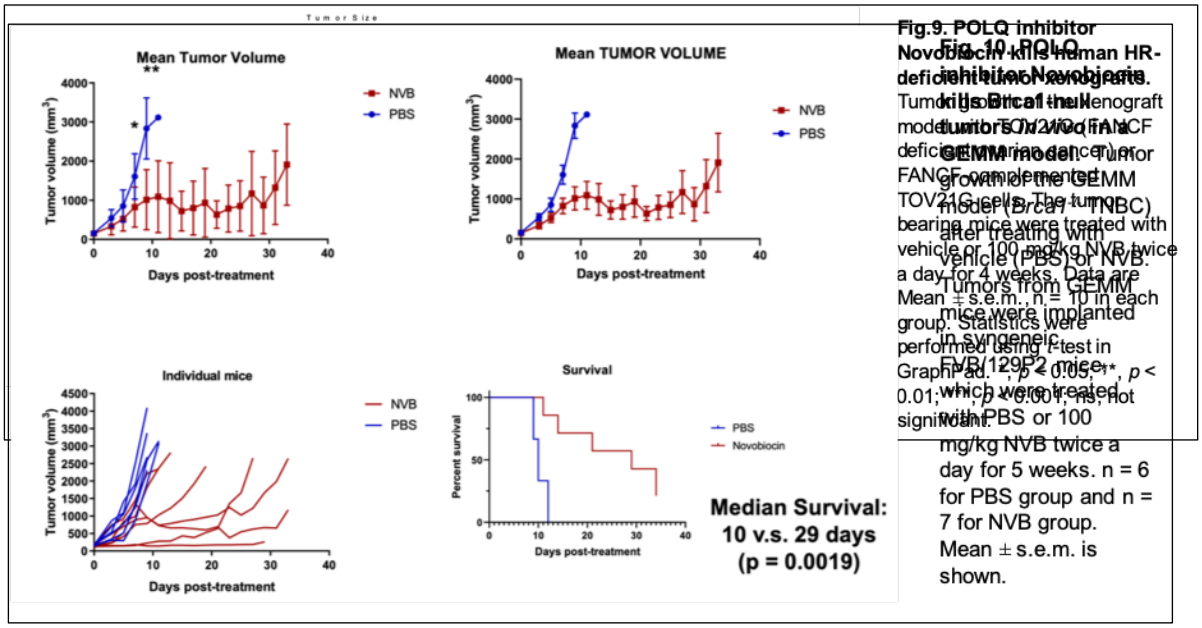

Fig.9. POLQ inhibitor Novobiocin kills human HR-deficient tumor xenografts. Tumors from the xenograft model with TOV21G (*BRCA2*-deficient ovarian cancer) or FANCF-complemented TOV21G cells. The tumor-bearing mice were treated with vehicle or 100 mg/kg NVB twice a day for 4 weeks. Data are Mean ± s.e.m., n = 10 in each group. Statistics were performed using *t*-test in GraphPad. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

Fig. 10. POLQ inhibitor Novobiocin kills Brca1-null tumors *in vivo* in a GEMM model. Tumor growth of the GEMM model (*Brca1*$^{-/-}$;*Trp53*$^{-/-}$;*Nf1*$^{-/-}$) after treating with vehicle (PBS) or NVB. Tumors from GEMM mice were implanted in syngeneic FVB/129P2 mice, which were treated with PBS or 100 mg/kg NVB twice a day for 5 weeks. n = 6 for PBS group and n = 7 for NVB group. Mean ± s.e.m. is shown.

evaluated this compound for its ability to kill BRCA1-deficient tumors *in vivo*. In **Figure 9**, we have evaluated an HR-deficient tumor called TOV21G. *In vivo*, the NVB is highly effective in killing these HR-deficient tumor cells.

In **Figure 10**, we have further evaluated the NVB for its ability to kill a BRCA1-deficient mammary tumor, using a GEMM model. Again, NVB strongly eliminates these BRCA1-null tumor cells *in vivo*, further demonstrating 1) the synthetic lethality of BRCA1 and POLQ and 2) the potential development of NVB as an anti-cancer drug.

Summary of the key research accomplishments: We have demonstrated unequivocally the functional importance of PTIP-53BP1 interaction in regulating homologous recombination (HR), and in doing so identified a new mechanism by which 53BP1 can suppress HR directly by blocking RAD51 loading post-resection. We have found that distinct domains of Brca1 counteract 53BP1 to support end resection and RAD51 loading, which improves our understanding of how different Brca1 mutations may promote genome instability and cancer. Moreover, it explains why tumors harboring different *Brca1* mutations, some of which don't appear to affect end resection, can acquire chemoresistance by inactivating 53BP1. We have also determined that restoration of replication fork (RF) protection and HR represent two independent means by which *Brca1/2*-deficient cells can become chemoresistance. We have published two manuscripts describing these results.

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?

The major goal of our project is to identify novel molecular mechanisms of PARPi resistance in BRCA1/2 mutated breast cancer. Therefore, during the upcoming grant funding year, we will continue to investigate how 53BP1, Shieldin, POLQ, BRCA1 and BRCA2 interplay to coordinate DNA repair and maintain replication fork stability. We also plan to conduct high throughput CRISP-based screening to identify additional factors that can confer chemoresistance in *Brca1/2*-deficient cells.

4. IMPACT:

Nothing to Report

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

We have shown that chemosensitivity in *Brca1/2*-deficient cells can result from either loss of RF protection or loss of HR (and perhaps both in some cases). Conversely, restoration of RF stability or HR can independently confer chemoresistance. These findings have a high impact on our ability to identify patient populations who may/may not benefit from chemotherapy and may open new avenues for targeted intervention. We have additionally shown that mutations in different domains of Brca1 can have distinct effects on HR. This suggested that all *Brca1* mutant tumors may not respond to chemotherapy in the same manner.

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.

1. ***53BP1 Enforces Distinct Pre- and Post-resection Blocks on Homologous Recombination.*** **Molecular Cell**. 2019 (In press)
   Elsa Callen, Dali Zong, Wei Wu, Nancy Wong, Andre Stanlie, Momoko Ishikawa, Raphael Pavani, Lavinia C. Dumitrache, Andrea K. Byrum, Carlos Mendez-Dorantes, Paula Martinez, Andres Canela, Yaakov Maman, Amanda Day, Michael J. Kruhlak, Maria A. Blasco, Jeremy M. Stark, Nima Mosammaparast, Peter J. McKinnon, André Nussenzweig

2. ***BRCA1 haploinsufficiency is masked by RNF168-mediated chromatin Ubiquitylation.*** **Molecular Cell**. 2019;73(6):1267-81.
   Dali Zong, Salomé Adam, Yifan Wang, Hiroyuki Sasanuma, Elsa Callén, Matilde Murga, Amanda Day, Michael J. Kruhlak, Nancy Wong, Meagan Munro, Arnab Ray Chaudhuri, Baktiar Karim, Bing Xia, Shunichi Takeda, Neil Johnson, Daniel Durocher, André Nussenzweig

3. ***USP1 Is Required for Replication Fork Protection in BRCA1-Deficient Tumors.*** **Mol Cell.** 2018 Dec 20;72(6):925-941.e4. doi: 10.1016/j.molcel.2018.10.045.Lim KS, Li H, Roberts EA, Gaudiano EF, Clairmont C, Sambel LA, Ponnienselvan K, Liu JC, Yang C, Kozono D, Parmar K, Yusufzai T, Zheng N, D'Andrea AD.

4. ***TRIP13 Regulates DNA Repair Pathway Choice through REV7 Conformational Change*. Nature Cell Biol,** 2019 (In Press). Clairmont CS*, Sarangi P*, Ponnienselvan K, Galli LD, Csete I, Moreau L, Adelmant G, Chowdhury D, Marto JA, D'Andrea AD.

5. ***Polymerase Theta Inhibition Overcomes PARP Inhibitor Resistance.*** 2019 (Submitted). Zhou J, Gelot C, Pantelidou C, Li A, Yücel H, Davis RE, Farkkila A, Shapiro GI, Blagg BSJ, Ceccaldi R, D'Andrea AD.

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

Nothing to Report

Other publications, conference papers, and presentations.

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

1. Invited Speaker, Understanding Sequence-Specific Mutations in Cancer, Memorial Sloan Kettering Cancer Center, New York, October 2018
2. Invited Speaker, Oncology Programme of the Institute for Research in Biodedicine (IRB), Barcelona, Spain, October 2018
3. Invited Speaker, workshop on Chromosome Architecture and Topological Stress, Baeza, Spain, October 2018
4. Invited Speaker, Department of Cellular and Molecular Medicine at University of California School of Medicine, San Diego, CA, February 2019.
5. Invited Speaker, Saint Louis University School of Medicine, Saint Louis, MO, February 2019.
6. Keynote Speaker, Gordon Research Conference on Mammalian DNA Repair, Ventura, CA, US, February 2019.
7. Invited Speaker, 3rd Exploring DNA Repair Pathways as Targets for Cancer Therapy conference, Nassau, Bahamas, February 2019.
8. Invited Speaker, Department of Genetics and Development, Columbia University Medical Center, New York, NY, April 2019.
9. Invited Speaker, 18th. Ataxia-Telangiectasia Workshop, Houston, TX, May 2019.
10. Keynote Speaker, 21st. Annual Midwest DNA Repair Symposium, Saint Louis, MO, May 2019.
11. Invited Speaker, Institut de Biologie de L'Ecole Normale Superieure (IBENS), Paris, France, May 2019.
12. Invited Speaker, Institut de Genetique et de Biologie Moleculaire et Cellulaire (IGBMC), Strasbourg, France.
13. Keynote Speaker, Mechanisms and Consequences of Chromosomal Translocation in Cancer Meeting, Institute Pasteur, Paris, France, May 2019.
14. Keynote Speaker, 13th. Edition of the French 3R Meeting, Replication-Recombination-Repair: From Molecular Mechanisms to Clinical Applications, Presqu'ile De Giens, France, May 2019.
15. Invited Speaker, Eukaryotic DNA Replication & Genome Maintenance Conference, Cold Spring Harbor, NY, September 2019.

16. Invited Speaker, 50th. Annual Meeting of the Environmental Mutagenesis and Genomics Society (EMGS), Washington D.C., September 2019.
17. Keynote Speaker, ASBMB-BSC Symposium on the Interplay between Epigenetic Regulation and Genome Integrity, Suzhou, China, October 2019.
18. Invited Speaker, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China, October 2019.
19. Invited Speaker, Department of Cancer Genetics and Epigenetics, Beckman Research Institute, City of Hope, Duarte, CA, October 2019.

Dr. Alan D'Andrea (Site 2, DFCI)

1. Memorial Sloan Kettering Cancer Center Think Tank: Understanding Sequence-Specific Mutations in Cancer, October 1, 2018, New York, NY
2. Beth Israel Deaconess Medical Center Standard of Cure Symposium, October 17, 2018, Boston, MA
3. Invited Seminar: Sun Yat-sen University Cancer Center, October 31, 2018, Guangzhou, China
4. icBEST/isDDRHD Meeting, November 1-4, 2018, Shenzhen, China
5. Weinman Symposium, November 29-30, 2018, Honolulu, HI
6. Ernest Beutler Lecture, ASH Annual Meeting, December 1-4, 2018, San Diego, CA
7. Keystone Symposium: DNA Replication and Genome Instability: From Mechanism to Disease, January 13-17, 2019, Snowbird, Utah
8. Stand Up To Cancer Scientific Summit, January 27-29, 2019, Santa Monica, CA
9. DNA Damage Response Therapeutics Summit, January 30-31, 2019, Boston, MA
10. Gordon Research Conference on Mammalian DNA Repair, February 11-14, 2019, Ventura, CA
11. ESMO Targeted Anticancer Therapies Conference, February 25-27, 2019, Paris, France
12. AACR Annual Meeting, March 30-April 3, 2019, Atlanta, GA
13. Perspectives in Science Seminar, Fred Hutchinson Cancer Research Center, May 20-22, 2019, Seattle, Washington
14. Aspen Cancer Conference, July 13-16, 2019, Aspen, CO
15. AACR Advances in Ovarian Cancer Research Conference, September 13-15, 2019, Atlanta, GA
16. isDDRHD Meeting, November 7-10, 2019, Shenzhen, China

- **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):	0000-0003-0037-7898
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:	Dali Zong
Project Role:	Scientist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6

Contribution to Project:	Dr. Zong has been a major contributor to the laboratory's research efforts in understanding the mechanisms of DNA repair pathway choice. He has been responsible for conducting fundamental studies on the molecular mechanisms of DNA damage/repair and has developed a number of sophisticated genetic, biological, biochemical and especially imaging assays to further these studies.
Funding Support:	Dr. Zong 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?

Dr. Shinoda, a post-doctoral fellow with Dr. Nussenzweig, is no longer associated with this project and is contributing to research on unrelated matters.

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