AWARD NUMBER: W81XWH-16-1-0391

TITLE: Inhibition of 53BP1: Potential for Restoring Homologous Recombination in Ovarian Cancer Cells

PRINCIPAL INVESTIGATOR: Maria Victoria Botuyan

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REPORT DATE: August 2017

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE
August 2017

2. REPORT TYPE
Annual

3. DATES COVERED
1 Aug 2016 - 31 Jul 2017

4. TITLE AND SUBTITLE

Inhibition of 53BP1: Potential for Restoring Homologous Recombination
In Ovarian Cancer Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-1-0391

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

In this Pilot Award, we explore at the molecular level how the relocation of DNA damage response (DDR) protein 53BP1 to chromatin harboring DNA double-strand breaks (DSBs) can be negatively regulated. 53BP1 is a natural inhibitor of homologous recombination (HR), a DNA repair pathway that is often inhibited in ovarian cancer cells. Therefore, by preventing the chromatin recruitment of 53BP1 one could in principle activate HR. Our idea is that by understanding how 53BP1 recruitment is regulated we could design ways to block 53BP1 chromatin recruitment in ovarian cancer cells, and by this means correct the HR defect and prevent ovarian tumor formation. We have three aims. For this first year of funding, our work for Aim 1 has revealed how a DDR protein can block the 53BP1-binding surface on the nucleosome, the smallest subunit of chromatin. Our studies for Aim 2 show how a regulatory protein blocks the chromatin-binding domain of 53BP1. For Aim 3, our data reveal how small synthetic organic chemicals trigger the dimerization of 53BP1 and thereby mask the chromatin-binding surface of 53BP1. The significance of our combined work is that it shows how 53BP1 chromatin recruitment can be inhibited by blocking the 53BP1-binding site in chromatin (Aim 1), or by blocking the chromatin-binding domain of 53BP1 (Aims 2 and 3). In future studies, we will probe the effectiveness of 53BP1 inhibition in correcting HR defects in ovarian cancer cells.

15. SUBJECT TERMS
DNA damage response; Homologous recombination; 53BP1 inhibition; Ovarian cancer; X-ray crystallography; NMR spectroscopy; Calorimetry

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	11	19b. TELEPHONE NUMBER *(include area code)*

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

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1. INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

In this research project, we explore the mechanisms that regulate 53BP1 recruitment to DNA damage sites in chromatin. 53BP1 is a DNA damage response protein that modulates DNA repair by promoting the error-prone non-homologous end-joining (NHEJ) pathway and inactivating the error-free homologous recombination (HR) pathway. Loss of 53BP1 was shown to restore HR DNA repair in HR-defective cells. Hence, our central hypothesis is that inhibition of 53BP1 recruitment to DNA double-strand breaks (DSBs) should correct the HR defect observed in certain ovarian cancer cells, such as those harboring an inactive BRCA1 protein. In particular, to test our hypothesis, we want to understand how 53BP1 is naturally prevented from being recruited to DSBs as a regulatory mechanism in cells. In three specific aims, we will examine how 53BP1 recruitment to DSBs can be inhibited by blocking the nucleosome surface accessed by 53BP1 or by masking the chromatin-binding domain of 53BP1. Small molecule chemical inhibitors of 53BP1 will notably be tested. The information gained from our studies is expected to help us design ways to inhibit 53BP1 recruitment to DSBs as a means to correct the HR inactivation observed in some forms of ovarian cancer cells. The new knowledge gained from our research is expected to have an important positive impact for the development of drugs that could restore the HR DNA repair pathway in carriers of a mutated *Brca1* gene whose susceptibility to develop ovarian cancer is high. In the long-term, the devastating effects of HR-inactivating *Brca1* mutations could be prevented with the prophylactic use of 53BP1 inhibitors.

2. KEYWORDS: Provide a brief list of keywords (limit to 20 words).

DNA damage response; Homologous recombination; 53BP1 inhibition; Ovarian cancer; X-ray crystallography; NMR spectroscopy; Calorimetry

3. ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

Our proposal has three main goals represented by three aims. The three aims are related to the inhibition of 53BP1 recruitment to DNA double-strand breaks, which correlates with reactivation of HR DNA repair. In Aim 1, we will elucidate the molecular basis for the inhibition of 53BP1 interaction with mono-ubiquitylated histone H2A (H2AK15ub) by DNA damage response protein RNF169. In Aim 2, we will elucidate the molecular basis for the inhibition of the interaction of 53BP1 with di-methylated histone H4 (H4K20me2) by a new regulatory protein. In Aim 3, we will characterize small molecule inhibitors of 53BP1 that block the interaction of 53BP1 with H4K20me2. We are working on the three aims in parallel as originally planned. Each aim combines structural studies using X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy and functional studies in a cellular setting. Significant progress has been made for all aims as detailed in the next section.

What was accomplished under these goals?

The first part of Aim 1, the three-dimensional (3D) structure determination of RNF169 in complex with histone H2A-H2B mono-ubiquitylated at H2A lysine 15 (H2A^{K15ub}-H2B), has been completed. We also characterized the interaction of RNF169 with the nucleosome core particle mono-ubiquitylated at H2A lysine 15 (NCP^{H2AK15ub}) in great detail. Using isothermal titration calorimetry (ITC) we were able to show that RNF169 binds NCP^{H2AK15ub} with an affinity that is approximately two orders of magnitude higher than that of 53BP1 for NCP^{H2AK15ub}. Our work explains how RNF169 inhibits the interaction of 53BP1 with chromatin and the recruitment of 53BP1 to DSBs. We validated the RNF169–H2A^{K15ub}-H2B and RNF169–NCP^{H2AK15ub} structures by probing the effects of mutations in RNF169, H2A, H2B and ubiquitin on the interaction of RNF169 with H2A^{K15ub}-H2B and NCP^{H2AK15ub} using ITC. A manuscript reporting this work was published in Molecular Cell in May 2017. The PI, Maria Victoria Botuyan, is co-first author of this study. In future studies, we will assess the effect of mutations in mammalian cells (the second part of Aim 1).

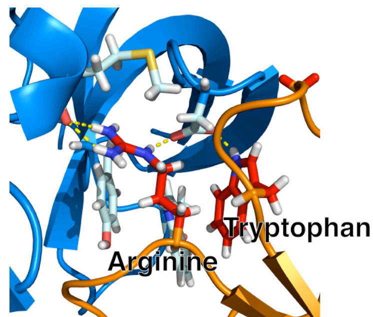

Figure 1: RNBP3/TIRR interaction. Detailed view of the RNBP3/TIRR (orange)–53BP1-TT (blue) interface highlighting the arginine and tryptophan residues key for interaction.

Aim 2 is well underway but data are unpublished. During this first year of funding, we were able to determine the crystal structure of putative RNA-binding protein RNBP3 (also called TIRR) in complex with 53BP1 tandem Tudor domains (53BP1-TT) (unpublished). We are particularly excited about this work as, to our knowledge, our high-resolution structure is the first of an inhibitory complex of a methyl-lysine reader protein (53BP1), or any post-translational modification reader. We have validated the structure *in vitro* by testing the effect of several point mutations at the intermolecular interface (data not shown). Studies in cells are ongoing. The most striking and unexpected feature of the structure is the recognition of an arginine and a tryptophan in RNBP3/TIRR by 53BP1 (**Figure 1**). These two residues are essential for binding as we verified by mutagenesis. We would like to publish this work as soon as possible and have started to draft a manuscript. We hope to submit a manuscript by the end of summer 2017.

Our first objective in Aim 3 is to determine 3D structures of 53BP1-TT in complex with small molecule inhibitors. At the time we received this grant we had solved one crystal structure 53BP1-TT bound to small molecule UNC3474. During the first year of funding, we completed the refinement of the 53BP1-TT–UNC3474 structure (**Figure 2A**) and we determined two additional crystal structures of 53BP1 bound to two other small molecules UNC2991 and UNC3351 (53BP1-TT–UNC2991 and 53BP1-TT–UNC3351). In addition, we also determined the solution structure of 53BP1-TT–UNC3474 by solution NMR spectroscopy. Our NMR spectroscopy studies also allowed us to quantify the equilibrium exchanges of the different 53BP1-TT–small molecule complexes (**Figure 2B**).

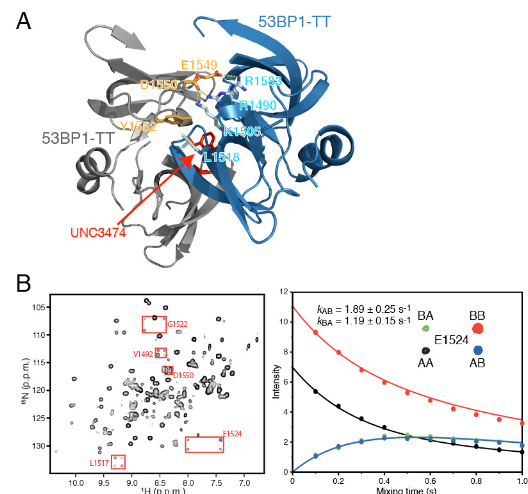

Figure 2: Crystallography and NMR spectroscopy of 53BP1 interaction with small molecule inhibitors. (**A**) Triggered dimerization of 53BP1-TT by UNC3574. (**B**) Characterization of 53BP1-TT conformational exchange using zz-exchange NMR spectroscopy.

Remarkably, in the three complex structures, the histone-binding surface of 53BP1-TT is buried at the interface of 53BP1-TT homodimer (**Figure 2A**). In other words, it is as if the small molecules trigger the dimerization of 53BP1-TT, thereby blocking the histone-binding surface of 53BP1-TT. These observations led us to discover that, in solution, there is a small population of 53BP1-TT dimer corresponding to an "auto-inhibited" state of 53BP1-TT. The small molecules dramatically shift the 53BP1-TT monomer/dimer equilibrium towards the dimeric form of 53BP1, the conformation that cannot bind chromatin in response to DNA damage. Because of this unusual inhibitory mechanism, the small molecules we tested are highly specific for 53BP1. The results summarized above for Aim 3 are unpublished. In future studies, we will test these small molecule inhibitors in cells.

What opportunities for training and professional development has the project provided?

Nothing to Report.

How were the results disseminated to communities of interest?

Nothing to Report.

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

For Aim 1, we will assess the effect of several RNF169 mutations on the recruitment of 53BP1 to DNA damage sites in cells and reactivation of HR DNA repair. Similarly, for Aim 3 we will probe the effect of small molecule inhibitors on 53BP1 recruitment in a cellular setting and reactivation of HR DNA repair. As indicated above, for Aim 2 we are currently doing a lot of work to complete a manuscript. Since we have completed the structural aspect of the work (the crystallographic studies of 53BP1 interaction with RNBP3/TIRR), we will focus on completing the testing of single point mutations *in vitro* and in mammalian cells. In Aim 2, we will also search for non-coding RNAs that regulate the formation of the aforementioned 53BP1 complex.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

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

In the process of characterizing the RNF169–H2A^{K15ub}-H2B and RNF169–NCP^{H2AK15ub} interactions, we developed a new procedure of preparing the nucleosome core particle ubiquitylated at a defined site on a large scale. It is likely that this approach will be useful to other scientists in the DNA damage response field who wish to probe how other proteins recognize ubiquitylated nucleosomes or chromatin. In fact, we have already received requests for reagents from other investigators who want to reconstitute the ubiquitylated nucleosome core particle.

What was the impact on other disciplines?

The approach mentioned above should be useful to structural biologists and cell biologists working in the broad field of chromatin, not necessarily related to the DNA damage response field.

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: The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

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

We have been trying to hire a postdoctoral fellow who would help us with the proposed studies. It has been challenging to identify someone who has the appropriate expertise. We interviewed a number of candidates. A postdoctoral fellow should be hired later this year.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Not applicable.

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

Not applicable.

Significant changes in use of biohazards and/or select agents

No changes to Report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications

Hu Q*, Botuyan MV*, Cui G, Zhao D, Mer G. Mechanisms of ubiquitin-nucleosome recognition and regulation of 53BP1 chromatin recruitment by RNF168/169 and RAD18. Mol. Cell 2017; 66(4):473-487. *Co-first authors.
Federal support was acknowledged (yes).

Botuyan MV, Hu Q, Mer G. Regulation of 53BP1 recruitment to DNA damage sites. This is an invited *Extra View* article to be published in the journal Nucleus. Federal support will be acknowledged. In preparation.

Books or other non-periodical, one-time publications

Nothing to Report.

Other publications, conference papers, and presentations

Nothing to Report.

- **Website(s) or other Internet site(s)**

Nothing to Report.

- **Technologies or techniques**

As indicated above, we have developed a method of reconstituting the ubiquitylated nucleosome core particle. All plasmids needed to produce the ubiquitylated nucleosome core particle are available upon request.

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

Nothing to Report.

- **Other Products**

The atomic coordinates for two three-dimensional structures were deposited in the Protein Data Bank (PDB) under accession codes 5VEY and 5FV0. The atomic coordinates of five crystal structures (as part of Aims 1 and 2) will be deposited in the PDB prior to publishing these studies.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Maria Victoria Botuyan, Ph.D.
Project Role: Principal Investigator
Researcher Identifier (e.g. ORCID ID): Not available
Nearest person month worked: 6

Contribution to Project: In addition to overseeing the entire project, Dr. Botuyan performed a large part of the work presented in this Annual Report, including protein purification of RNF169 and histone proteins, ubiquitylation method development, and structure determination of several protein complexes.

Name: Georges Mer, Ph.D.
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): Not available
Nearest person month worked: 2

Contribution to Project: Dr. Mer performed the majority of the isothermal titration calorimetry measurements for Aims 1, 2 and 3.

Funding Support: Mayo Clinic

Name: James Thompson, Ph.D.
Project Role: Research Scientist
Researcher Identifier (e.g. ORCID ID): Not available

Nearest person month worked: 3

Contribution to Project: Dr. Thompson contributed to the crystallographic studies (Aims 2 and 3). Dr. Thompson manages the crystallography facility at Mayo Clinic.

Funding Support: Mayo Clinic

Name: Gaofeng Cui, Ph.D.
Project Role: Research Associate
Researcher Identifier (e.g. ORCID ID): Not available
Nearest person month worked: 1

Contribution to Project: Dr. Cui contributed to the crystallographic and NMR studies.

Funding Support: Mayo Clinic

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?

Organization Name: University of North Carolina
Location of Organization: Chapel Hill, North Carolina
Partner's contribution to the project: In-kind support. We obtained several small molecules that are putative inhibitors of 53BP1.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

Not applicable.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

Not applicable.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

No appendices are provided with this report.