AWARD NUMBER: W81XWH-18-1-0182

TITLE: Structural and Functional Studies of Androgen Receptor and Its Cofactors

PRINCIPAL INVESTIGATOR: Elizabeth Wasmuth

CONTRACTING ORGANIZATION: Sloan Kettering Institute for Cancer Research
New York, NY 10065

REPORT DATE: June 2019

TYPE OF REPORT: Annual

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
June 2019	Annual	15 May 2018 – 14 May 2019

4. TITLE AND SUBTITLE

Structural and Functional Studies of Androgen Receptor and Its Cofactors

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-18-1-0182

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Elizabeth Wasmuth

E-Mail: Wasmuthe@mskcc.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Sloan Kettering Institute for
Cancer Research
1275 York Avenue, New York NY
10065-6007 USA

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

Enter a brief (approximately 200 words) unclassified summary of the most significant finding during the research period.
The androgen receptor (AR) is the master transcription factor that governs prostate identity. In castration resistant prostate cancer (CRPC), AR signaling is hyperactivated and drives advanced disease. AR inhibitors are used to treat CRPC; though initially effective, half of patients eventually acquire resistance through further AR amplification, necessitating a need for more potent AR inhibitors. One novel approach is to disrupt disease-specific cofactor interactions that activate AR. Normally not expressed in prostate, the ETS transcription factor ERG is overexpressed in over half of patients with CRPC, and expands the AR transcriptome through a process that is not fully understood. Though one of these avenues may be through a direct AR-ERG interaction, molecular details are lacking primarily due to difficulty in isolating active AR protein.

We optimized AR expression and purification, and reconstituted an AR/ERG/DNA complex using recombinant proteins. We found that in the absence of ERG, AR exhibits N-terminal dependent autoinhibition. However, association with ERG stimulates AR's ability to bind DNA through a direct interaction within the ERG ETS domain. This stimulation is maintained in the presence of the AR inhibitor, enzalutamide. These findings may extend to other ETS factors whose expression is altered in CRPC. Finally, we have initiated structural studies of this complex using single particle cryo-electron microscopy to resolve the structure of multidomain AR and define the region of interaction between AR and ERG.

15. SUBJECT TERMS

Key words or phrases identifying major concepts in the report.
Protein/nucleic acid complex, biochemistry, cryo-electron microscopy

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	14	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

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

The androgen receptor (AR) is the driver and drug target of metastatic prostate cancer. While AR targeting drugs initially work, all patients develop resistance necessitating a need for more potent drugs. A bottleneck limiting development of new AR therapies is the lack of biochemical and structural understanding of AR and regulatory cofactors involved in advanced disease. Using purified proteins, we have reconstituted and biochemically characterized a complex between AR and ERG, an ETS transcription factor amplified in half of men with advanced disease and have initiated structural studies of this complex by single particle cryo-electron microscopy (cryoEM).

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

Protein/nucleic acid complex, biochemistry, cryo-electron microscopy, androgen receptor, ETS transcription factors

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

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: Biochemical reconstitution and characterization of active androgen receptor (AR) bound to DNA and protein cofactors important in prostate cancer, and structural characterization of one or more of these nucleoprotein complexes	**Months**	**% completion**
Major Task 1: Reconstitution and biochemical characterization of stable AR/cofactor complexes		
Subtask 1: Recombinantly express in *E. coli* and optimize purification and reconstitution of AR and its protein cofactors	1-4	100
Subtask 2: Determine the effects of protein cofactors on AR binding to DNA using fluorescence polarization	2-4	100
Milestones Achieved: Production of various AR/cofactor complexes bound to DNA suitable for structural studies, and understanding of how cofactors influence AR activity	4	
Major Task 2: Structural determination of agonist-bound AR		
Subtask 1: Crystallization and structure determination of ΔNTD AR/protein cofactor complexes using x-ray crystallography	4-21	10
Subtask 2: Structural studies of full-length AR/protein cofactor/DNA complexes using single particle cryo-electron microscopy	8-21	40
Milestone Achieved: Determination of an atomic resolution model of an agonist-bound multidomain AR	21	
Major Task 3: Mutational analyses of critical residues identified in the AR structure(s)		
Subtask 1: Validation of the intra- and intermolecular contacts observed in tasks 2.1 and 2.2 in recombinant mutant proteins using fluorescence polarization	21-24	30
Subtask 2: Validation of the intra- and intermolecular contacts observed in tasks 2.1 and 2.2 in AR-driven prostate cancer cells lines Cell lines used: LNCaP, LNCaP-AR, VCaP	21-24	0
Milestones Achieved: Structural and functional of studies agonist-bound AR and interactions with its DNA and protein cofactors; publication of 1-2 peer-reviewed papers	24	

Specific Aim 2: Biochemical and structural determination of the mechanism of AR inhibition by the drug, enzalutamide		
Major Task 1: Isolation and biochemical characterization of inhibited, enzalutamide-bound AR		
Subtask 1: Develop an approach to express and purify AR-bound enzalutamide in *E. coli*	1-2	100
Subtask 2: Determine the direct, biochemical effects of the anti-androgen, enzalutamide, on AR binding to its cognate DNA and protein cofactors from Specific Aim 1 using fluorescence polarization	3-6	100
Milestone(s) Achieved: Production of enzalutamide-bound AR and a biochemical understanding of its role on AR interactions	6	
Major Task 2: Structural determination of antagonist-bound AR		
Subtask 1: Crystallization and atomic resolution structure determination of enzalutamide-bound ΔNTD AR or its ligand binding domain using x-ray crystallography	3-24	0
Subtask 2: Determining the global changes (if any) caused by enzalutamide binding in full-length AR using single particle cryo-electron microscopy	8-24	0
Milestone(s) Achieved: Characterization of effects of the anti-androgen, enzalutamide, on AR activity and structure; structural insight to design of a next-generation AR-targeting drug; publication of a peer reviewed paper	24	

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

The objectives for this period were to 1) optimize AR purification, and develop methods to biochemically stabilize AR through various means for biochemical and structural studies; 2) develop a reconstitution protocol for a complex between AR, ERG and DNA; 3) biochemical characterization of this complex; 4) initiation of structural studies by cryo and negative stain electron microscopy.

Due to the symbiotic nature between structural biology and biochemistry, continuous feedback from these different objectives helped shape the overall progress in the outcomes described below.

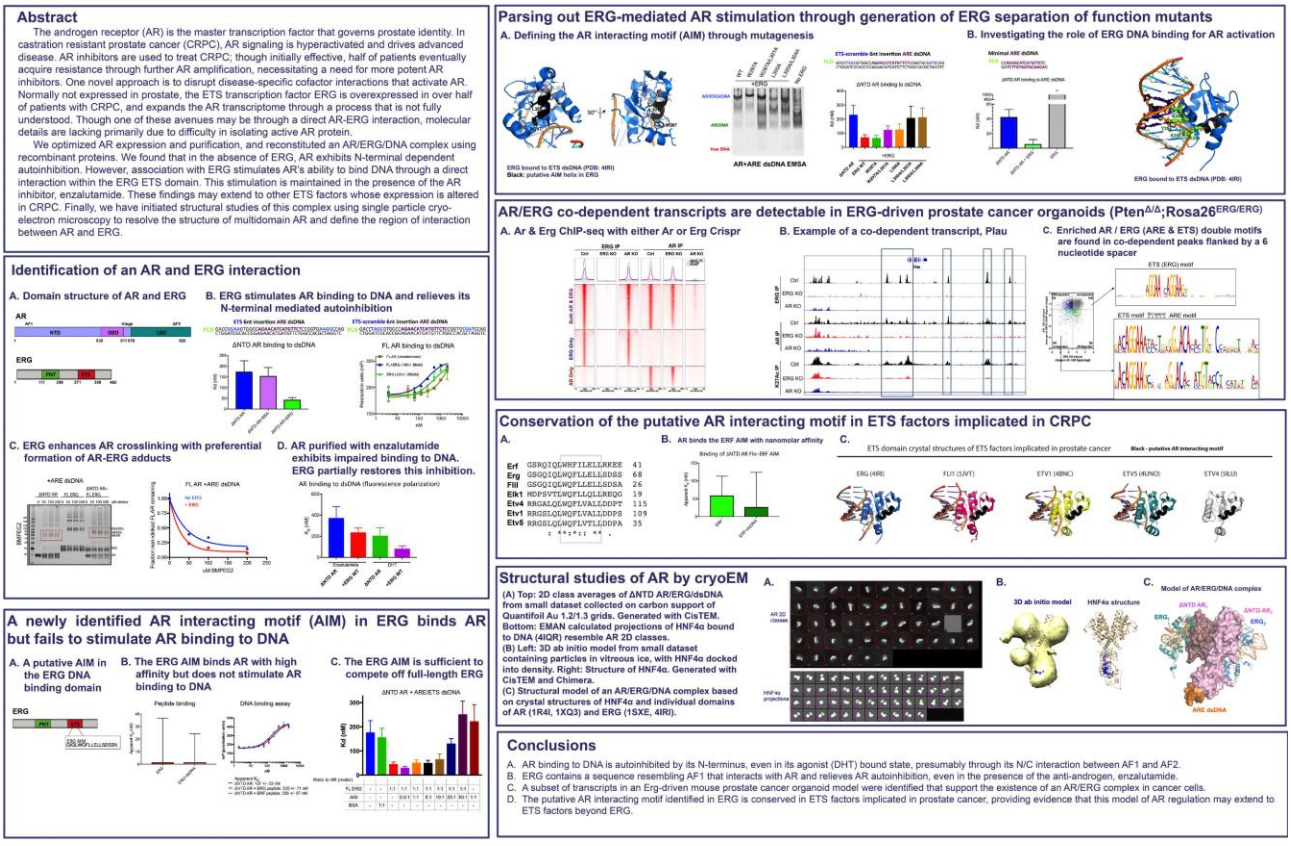
Some of the approaches used to accomplish these objectives included:

1) Identification of protein cofactors that are both relevant in disease and appropriate for structural studies. The ETS transcription factor, ERG, was selected among 4 other candidates due to its biological relevance (it is aberrantly amplified in 50% of men with advanced prostate cancer and expands the AR cistrome through a mechanism not fully understood), and its favorable behavior in vitro, including its stability at low salt making it advantageous for DNA binding studies.
2) Development of a covalent crosslinking assay to stabilize complex formation between AR, ERG, and DNA
3) Detergent screening to find conditions where AR stably and productively binds DNA
4) DNA substrate screening for structural studies by cryoEM

Significant results:
The reagents and methodology developed during this reporting period allowed us to perform the first biochemical study of AR binding to DNA using purified recombinant proteins. We characterized how AR binds DNA in its ligand activated (DHT) and inhibited (enzalutamide) state, and parsed out how its 3 individual domains contribute to its own allosteric regulation: the N-terminus plays an inhibitory role in DNA binding in the DNA binding domain (DBD) while the ligand binding domain stimulates the DBD when ligand bound. We also identified an example of intramolecular modulation of AR's DNA binding activity conferred by other protein cofactors involved in disease through direct DNA-independent interactions with AR.

We have focused on a complex between AR, ERG and DNA for structural by cryo- and negative stain electron microscopy and have spent much of the past reporting period optimizing the protein purifications and grid preparations for data collection. Though low resolution, our preliminary results have yielded 2D classes and ab initio models that resemble structures of known nuclear hormone receptors. This funding period, we aim to improve resolution using very recent advances we made in grid and sample preparation, in addition to newly generated fusion proteins that can be extensively purified to homogeneity.

Methodology employed includes the use of recombinant proteins overexpressed in E. coli to purify WT, mutant and fusion proteins; DNA binding assays via native gel shifts (EMSAs) and fluorescence polarization; protein crosslinking/mass spectrometry; protein crystallography; negative stain and cryo-electron microscopy.



What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Training:
NYSBC training courses in cryoEM
Use of and training at cryoEM facilities at Rockefeller University, Memorial Sloan Kettering Cancer Center, New York University Langone, New York Structural Biology Center, Janelia Research Campus (HHMI)

Professional development:
2019 AACR annual meeting (attended)
2019 Geoffrey Beene Retreat (poster prize recipient)

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Manuscript describing the biochemical reconstitution and characterization of the AR/ERG complex is in preparation and will be submitted in the next one to two months

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

After publication of the pending biochemical manuscript, my major focus will be directed towards structural studies of the ERG/AR/DNA complex using cryo and negative stain electron microscopy employing the novel strategies I have implemented over the past year. These include new protein constructs and purification methods for preparation of more stable and homogenous samples, as well as favorable conditions for imaging the complex in vitreous ice as well as in negative stain. I will also try to identify residues involved the AR/ERG interaction through covalent crosslinking/mass spectrometry These contacts identified through a structural model and mass spectrometry will then be validated through mutagenesis biochemically using recombinant proteins and in prostate cancer cells.

4. **IMPACT:** D*escribe 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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and

research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Structural and biochemical understanding of nuclear hormone receptors, particularly the type I variety (including the androgen receptor [AR]), is lacking mostly due to the challenges in obtaining substantial quantities of active, well-behaved, and pure protein. During this reporting period, I made substantial progress in optimizing the expression and purification of active and inhibited AR, suitable enough that we are now publishing the first biochemical study using recombinant proteins which documents how AR binds DNA and the direct effects of the chemotherapy, enzalutamide on DNA binding. I was able to identify an interaction between AR and the transcription factor, ERG, which is aberrantly expressed in over 50% of men with advanced prostate cancer. Our studies this past year suggest that ERG may contribute to disease through its interaction with AR, which in turn stimulates AR's ability to bind DNA; the reconstitution of this complex has enabled our current initiative to solve the structure of an AR/ERG/DNA complex by cryoEM. The findings from a structure of this complex will help design new drugs for men with advanced prostate cancer, including the 50% who have aberrant ERG amplification.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

The biochemical findings from this period have direct consequence on translational cancer biology. The ETS transcription factor ERG is amplified in 50% of men with metastatic prostate cancer and is associated with more aggressive disease. Its amplification is associated with selective expansion of the AR cistrome through a mechanism that has remained a mystery to cancer biologists. A concurrent study in our lab has recently discovered that a subset of these transcripts is composed of AR/ERG co-dependent genes that encode closely spaced AR and ERG consensus sites within their enhancers. The biochemical analyses reported within this past year have reconciled the spacing between these two consensus sites and explain how these two transcription factors can interact and modulate each other's DNA binding activity. Whether these co-dependent transcripts are important in disease progression is currently unknown. However, structural elucidation of the AR/ERG interaction surface could have downstream implications in the development of a therapy that disrupts the AR/ERG interaction.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official 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

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report

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

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

N/A

Significant changes in use or care of vertebrate animals

N/A

Significant changes in use of biohazards and/or select agents

Nothing 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. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to report this period (manuscript in preparation to be submitted in next few months)

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to report

  Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

  Geoffrey Beene Cancer Research Center Annual Retreat (2019): poster presenter & poster prize recipient

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

  Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Development of a means to purify and assay enzalutamide inhibited androgen receptor Development of a robust DNA binding assay for purified AR in its active and inhibited state

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Data (manuscript in preparation) Research material (bacterial cell lines, purified recombinant proteins)

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: M. Jason de la Cruz
Project role: MSK CryoEM core facility manager/collaborator
Nearest person month worked: 1
Contribution to project: Has assisted in cryoEM data collection at MSK, NYU Langone and Janelia Research Campus & provided computational support for data storage and processing

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

1) Janelia Research Campus
Ashburn, VA
CryoEM facility (use of Krios TEM)

2) NYU Langone
New York, NY
CryoEM facility (use of Arctica TEM)

3) New York Structural Biology Center
New York, NY
CryoEM facility (use of T12, F20 TEM)

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (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.*

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.

Nothing to report.