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TITLE: Modulating cancer genetics for immune regulation and breast cancer therapy

PRINCIPAL INVESTIGATOR: Maria Spies

CONTRACTING ORGANIZATION: The University of Iowa
Iowa City, IA 52242-1316

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Modulating Cancer Genetics for Immune Regulation and Breast Cancer Therapy

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6. AUTHOR(S)

Maria Spies

E-Mail: maria-spies@uiowa.edu

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5e. TASK NUMBER

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

The University of Iowa
105 Jessup Hall
Iowa City, IA 52242-1316

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

In contrast to its important function in DNA repair, overexpression of human MSH2/6 mismatch recognition protein is driving the progression of basal like breast cancer and is associated with poor prognosis. This project will establish the molecular mechanisms of this MSH2/6 activity. During the first funding period we have established an experimental workflow to test the hypothesis that MSH2/6 protein targets methyltransferase DNMT1 to specific promoters and activates its enzymatic activity thus connecting the MSH2/6 overexpression with altered epigenetic regulation. Our preliminary data suggest that this hypothesis is correct. During the next funding period we will complete the biochemical and single-molecule studies testing this hypothesis.

15. SUBJECT TERMS

NONE LISTED

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a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
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1. INTRODUCTION:

Current treatment options for basal like breast cancer (BLBC) rely on cytotoxic dose-dense chemotherapy and surgery. The work under this award builds on our preliminary data that suggested that MSH2/6 may be a viable molecular target for BLBC therapy. Our goal is to define the role of MSH2/6 at different stages of BLBC pathogenesis and to establish MSH2/6 as a molecular target for BLBC therapy. As a partnering PI with expertise in biochemical mechanisms of DNA mismatch repair (MMR), Dr. Spies' lab is responsible for the biochemical and single-molecule experiments carried under this award.

2. KEYWORDS:

Basal like breast cancer, BLBC; DNA mismatch repair, MMR; Human MutS homolog MSH2/6, MutSα; human methyltransferase DNMT1; total internal reflection fluorescence microscopy, TIRFM; single-molecule pull-down, SiMPull

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Determine the role of MSH2/6 in BLBC pathogenesis and immunotherapy

The Spies' lab involvement in completion of this specific aim will start in year three of the project

Specific Aim 2: Determine the mechanism how MSH2/6 contributes to epi/genetics and immune modulation. Below are the tasks relevant to this reporting period

- Determine DNMT1 binding efficiency to CD274 and PVR promoters.
- Determine MSH2 and DNMT1 binding;
- DNMT1 and MSH2/6 dimers will be purified and the interaction between DNMT1 and MSH2/6 will be determined by single-molecule pull down (SiMPull).
- Determine the effect of MSH2-MSH6 on the DNMT1 enzymatic activity

What was accomplished under these goals?

During the initial funding period we have made the following progress towards the aims of this project:

Biochemical analysis of the effect of the purified MSH2/MSH6 on the enzymatic activity of human DNMT1:

DNMT1 recognizes hemi-methylated DNA and converts it to fully methylated sequence, the process important for the faithful of replication of inherited epigenetic patterns. We have proposed that MSH2-MSH6 can activate methyltransferase DNMT1 thereby enabling a crosstalk between overexpression of MSH2-MSH6 and epigenetic regulation. In addition to MSH2/6, we have expressed and purified three DNMT1 constructs, a nearly full length protein containing self-inhibition domain, an "activated" truncated form of DNMT1 lacking self-inhibition domain, and the inhibitory domain by itself. We that developed a (Föster resonance energy transfer (FRET)-based methylation assay to test the activation of MSH2/6 by DNMT1 (**Figure 1; see next page**). In this assay, the DNMT1 activity is followed by monitoring conversion of a hemi-methylated DNA into fully methylated DNA that can be cleaved by a methylation-specific restriction enzyme Gla I. Figure 1 below shows design of the substrate that contains hemi-methylated site that is recognized by an active form of DNMT1 (**a**) and upon methylation is recognized and cleaved by Gla I (**b**). The products of the cleavage by Gla I are two short oligos containing Cy3 and Cy5 FRET donor and acceptor, respectively. Due to their length, the oligos dissociate from one another, which can be detected by native polyacrylamide gel electrophoresis PAGE (inset with full length substrate and two products marked with (1), (2) and (3), respectively). Kinetics of the cleavage can be monitor using spectrofluorimeter and measuring FRET between Cy3 and Cy5 dyes as the cleavage reaction is progressing (**c**).

To monitor DNMT1 activation by MSH2/6 we have expressed and purified three DNMT1 constructs (**Figure 2; page 7**). This purification was new to the lab and had to be optimized. All purified DNMT1 constructs had activities and properties as described in literature. Gla I was from a commercial source. MSH2/6 is routinely purified in the lab and is readily binds to our new DNA substrate (**Figure 3; page 8**).

Thus far we have established conditions that are conducive to simultaneous binding of MSH2/6 and DNMT1 to the same DNA. While this study is still ongoing, our preliminary data are suggestive of the possible stimulatory effect of the mismatch bound MSH2/6 on the DNMT1 activity (**Figure 4; page 8**). The interpretation of the results was complicated by an unexpected effect of ATP on the DNMT1 activity. Nevertheless, we now have all necessary components of the assay and the controls to complete this study during the first half of the next funding period.

Two single-molecule types of MSH2/6-DNMT1 interaction analysis were planned, SiMPull (single-molecule pull-down) and the massively parallel analysis of binding specificity (a single-molecule methodology we developed to complement the ChIP; **Figure 5**). The major time consuming step in both analysis is establishing the experimental conditions and reagents. We have completed this task for both types of analyses. Currently, we have the SiMPull fully working on the model system with purified and affinity tagged proteins and during the second funding period will be transitioning this analysis to the MDA-MB-231 and 4T1 cells. Most importantly, we have a procedure at hand for labeling antibodies to surface tether MSH2/6 and to visualize DNMT1. For the second single-molecule analysis (see **Figure. 5**) we are in the process of large-scale purification of the GFP-tagged MSH2/6 dimer.

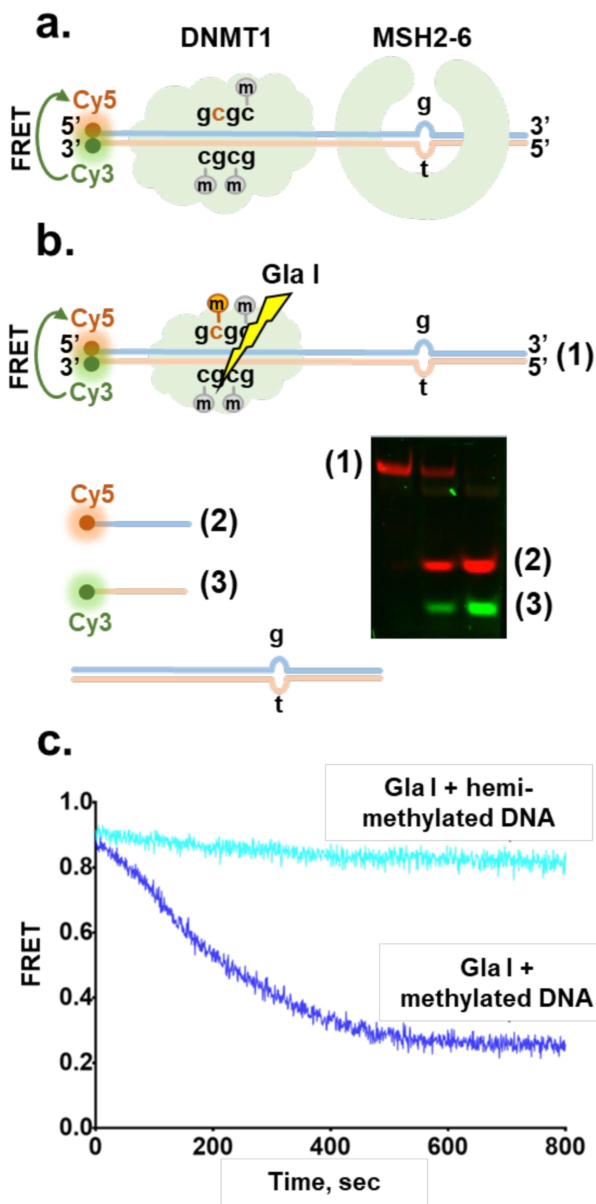
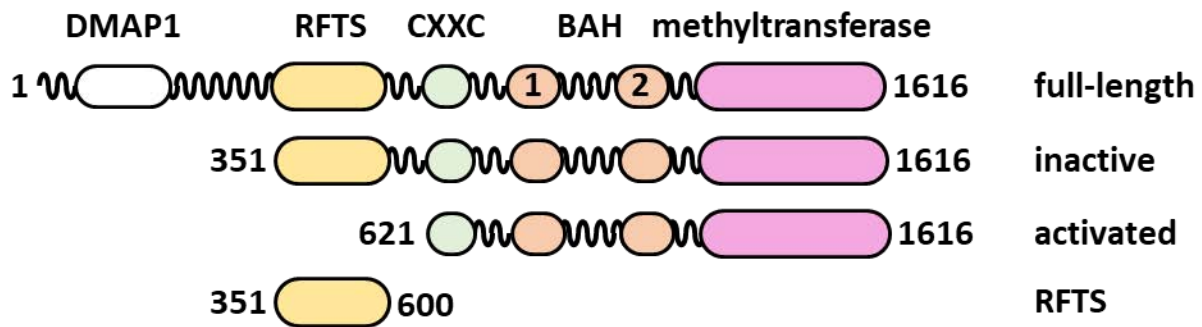


Figure 1. Development of assay to monitor DNMT1 activation by MSH2-6. **a.** DNA substrate (blue and orange lines) is assembled from two synthetic oligonucleotides, one labeled with Cy5 and the other with Cy5, which are FRET donor and acceptor, respectively. The DNA substrate is synthesized hemi-methylated (the three cytosines are marked with "m") and provides binding site for DNMT1 and a g/t mismatch for MSH2-6 binding.

b. Methylation of the recognition site by DNMT1 results in a fully methylated site that can be cleaved by Gla I. Cleavage products can be distinguished from the substrate by separating the DNA on native PAGE (inset)

c. We can also follow the reaction progression in real time by following FRET between Cy3 and Cy3 dyes. When the dyes are in close proximity, the FRET signal is high; when the end of the duplex is cleaved by Gla I, the two short oligos dissociate resulting in reduction in FRET. Comparison of the cleavage reaction of hemi- and fully-methylated DNA is shown. The rate of cleavage is much faster than rate of methylation by DNMT1 (not show), that the FRET is a proxy readout for the methylation reaction. From these kinetic measurements we calculate the rate and the extent of the reaction.



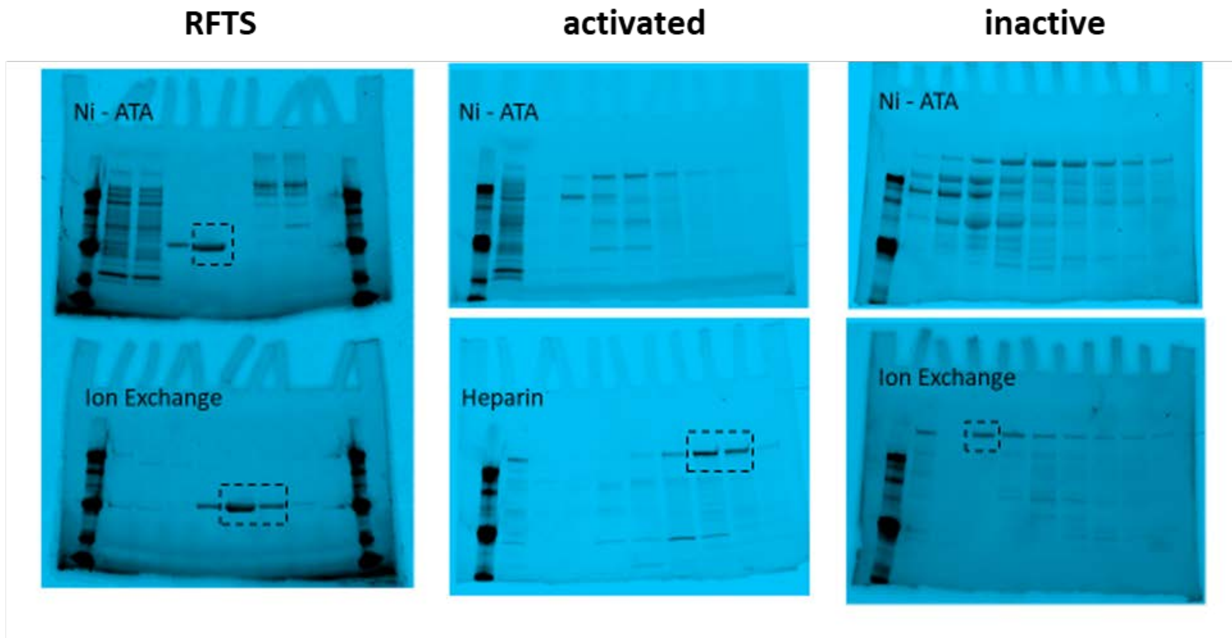


Figure 2. Purification of DNMT1 construct for activation experiment. Biochemical and single-molecule studies depend on our ability to purify large amounts of proteins. While we have worked with MSH2/6, DNMT1 is new to the lab. Full-length DNMT1 is a multi-domain protein 1616 amino acids in length. The “inactive” construct residues 351-1616 contains all relevant functional domains and when purified has only residual methyltransferase activity because the entry to the active site is blocked by RFTS domain; this construct is in a self-inhibited conformation. We hypothesized that DNA-mediated interaction with MSH2-6, which involves RFTS domain would activate methyltransferase. As controls, we have also purified constitutively activated forms of DNMT1 (621-1616) and RFTS domain (351-600). All constructs were expressed in *E. coli* and purified to homogeneity. Each construct has a 6xHis tag at the N-terminus to facilitate purification. The gels show two step purification for each construct. Proteins from fractions indicated in boxes were collected and used for biochemical analyses.

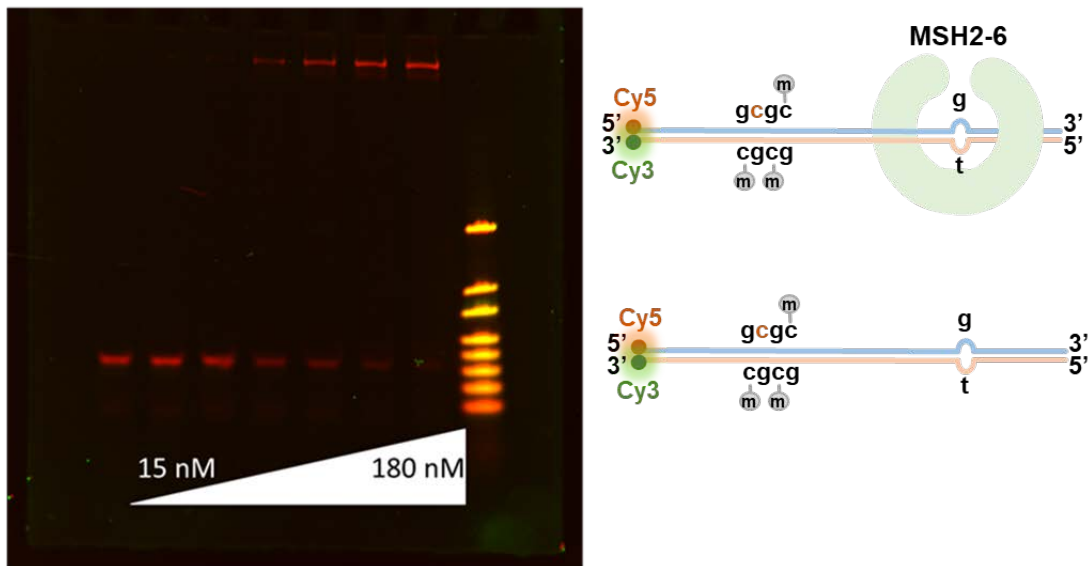


Figure 3. Binding of MSH2-6 to the DNA substrate used in DNMT1 activation experiments. Native PAGE showing shift of the substrate DNA (10 nM) migration in the presence of increasing concentrations of MSH2-6. DNA has been visualized by following Cy5 only.

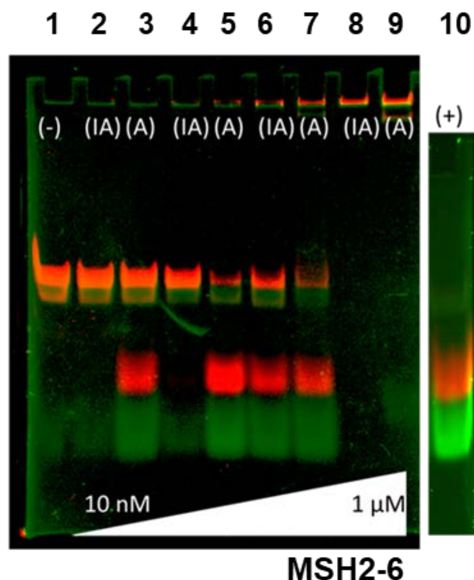


Figure 4. Preliminary DNMT1 activation experiment. Native PAGE (as in Figure 1) separates the substrate (S) and products (P).
Gla I is present in all lanes
Lane 1 – substrate only
Lane 2,4,6,8 – substrate + inactive DNMT1
Lane 3,5,7,9 – substrate + activated DNMT1
Each pair of lanes has increasing concentration of MSH2-6;
Lane 10 – substrate + inactive DNMT1 + MSH2-6 +ATP

Note that we are still optimizing conditions for the experiment to prevent accumulation of MSH2-6 in wells; the results in Lane 10 (from a different gel) and Lane 6, which show appearance of the cleaved product in the presence of inactive DNMT1 and MSH2/6 suggest that MSH2/6 can indeed activate self-inhibited DNAMT1. The following controls are being done: reproducing these analyses under experimental conditions that prevent trapping of the DNA in wells (this is important for quantification of the reaction); optimizing condition for the reaction in the presence of ATP, which converts MSH2/6 from a tightly bound clamp to a sliding clamp; experiments in the presence of RFTS domain, which is expected to compete with inactivated DNMT1 for MSH2/6 binding.

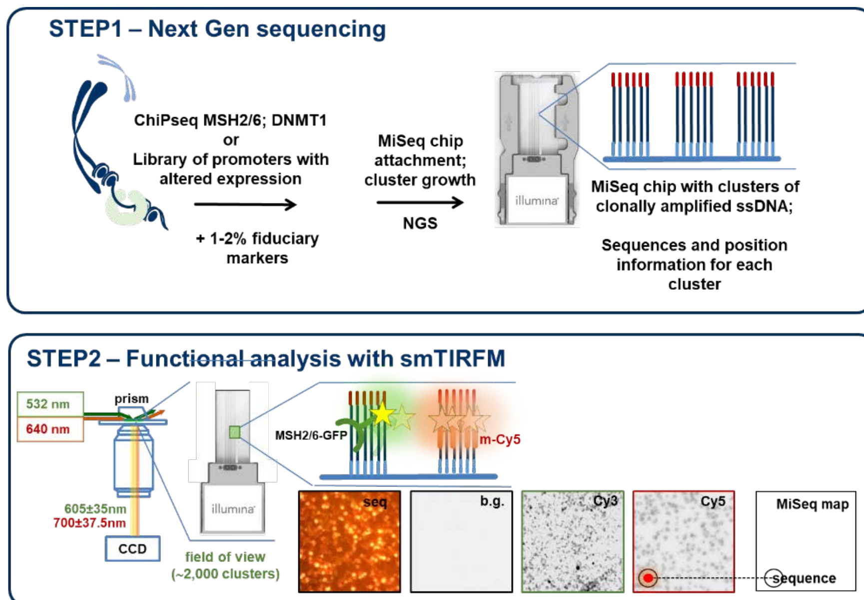


Figure 5. Developing massively parallel platform for analysis of the MSH2/6-DNA-DNMT1 interaction. The experimental system we are developing will test the hypothesis that MSH2/6 binds not only to mismatched DNA, but also to highly flexible regions found in some promotors. We are building a library of promotor sequences from genes that are the most up or downregulated in MSH2/6 overexpressing BLBC cells. This library will be amplified and subjected to sequencing using Illimina MiSeq platform (Step 1). At the end of the sequencing, we will have a MiSeq chip with clusters of clonally amplified DNA. We have adapted our total internal reflection fluorescence microscopy (TIRFM) system to image the surface of the MiSeq flow cells (Step 2). The insets show (seq) a partial field of view of the flow cell surface right after sequencing (photographed through eyepiece); (b.g.) regenerated flow cell with all fluorescent remnants of sequencing removed – DNA clusters are still present, but are invisible; (Cy3) clusters visualized by binding of a Cy3-labeled protein; (Cy5), clusters visualized by binding of a Cy5-labeled protein. Because we are spiking the library with fiduciary markers, we can correlate the position of clusters from the sequencing file with their positions observed in TIRFM. This will allow us to correlate for each cluster the binding signal for GFP-tagged MSH2/6 and Cy3-labeled DNMT1 with the DNA sequence and thereby determine binding specificity for MSH2/6 and/or DNMT1.

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

Part of the DNMT1 activation analysis was carried out as a summer research project supervised by Dr. Masayoshi Honda and carried out by Mr. Martin Yepes, an undergraduate student from St. Mary's College of Maryland under the University of Iowa Biochemistry Summer Undergraduate Research Fellowship. Dr. Honda trained Mr. Yepes in biochemical techniques (protein expression and purification, and gel-based methylation analyses) and FRET-based analyses of DNMT1 activity.

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?

We will complete the studies of DNMT1 activation by MSH2/6, MSH2/6-DNMT1 interaction and will establish the specificity of MSH2/6-promoter interactions. Experimental strategies will include biochemical, FRET-based analyses of the DNMT1 enzymatic activation by the MSH2/6 bound to a mismatch and/or a specific sequence, as well as single-molecule analyses of the DNMT1-MSH2/6 interactions and MSH2/6 sequence specificity.

4. IMPACT:

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

When completed, our work will characterize a previously unknown, pathological function of the MSH2/6 protein, which is primarily thought of as a guardian of genome integrity through its function in DNA mismatch repair.

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:

Nothing to report

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

We had a slight delay in the large scale biochemical production of MSH2/6 and MSH2/6-GFP proteins. We have now resolved these problems and expect to continue all the biochemical and single-molecule experiments along the proposed timeline. This delay also resulted in a slight change in the timeline of cell-based studies. These will be completed during the second funding period.

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

Significant changes in use or care of human subjects

N/A

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:

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

  Nothing to report

  Journal publications.

  Nothing to report

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**

Nothing to report

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

Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Maria Spies
Project Role:	Principal Investigator
Research Identifier (e.g. ORCID ID):	0000-0002-7375-8037
Nearest person month worked:	1
Contribution to Project:	Dr. Spies has coordinated work outlined in Specific Aim 2, Major Task 4
Funding Support:	No change.
Name:	Masayoshi Honda
Project Role:	Assistant Research Scientist
Research Identifier (e.g. ORCID ID):	0000-0002-9817-3908
Nearest person month worked:	8
Contribution to Project:	Dr. Honda has performed work outlined in Specific Aim 2, Major Task 4
Funding Support:	No change.

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

Please see the following changes in active support for Maria Spies:

(NEW)
The Role of Human RAD52 Protein in Genome Stability
Source: NIH R01 CA232425 (MPI: Spies, Malkova, Pichierri)
Goals of the Project: We have discovered a new function of the RAD52 DNA repair protein in protecting stalled replication forks from the excessive nucleolytic degradation. We proposed two possible roles for RAD52 in this process, as a gatekeeper or/and as a protector of the replication fork. The work under this award will develop a mechanistic understanding these two novel activities of RAD52, their importance to genome stability and the basis of the synthetic lethality between RAD52 inhibition and BRCA defects.
Specific Aims/Tasks: Aim 1 is to determine how RAD52 contributes to replication fork stability.
Aim 2 is to determine the mechanism of the RAD52 interaction with the replication fork.
Aim 3 is to characterize genetic instability resulting from RAD52 deficiency.
Overlap: There is no overlap with the proposed studies.
Start and End Date: 4-1-2019 - 3-31-2024
Level of Effort: 23%
Annual Direct Costs: $277,971/year ($162,965/year to Spies Lab)
Point of Contact at Funding Agency: Philipp Oberdoerffer, PhD, Program Official

(NEW)
Assembly and Dynamics of Molecular Machines in Genome Maintenance
Source: NIH 1R35GM131704 (PI: Spies)
Goals of the Project: This MIRA grant will replace the existing NIH 1R01GM108617 and will support our efforts to understand the role of conformational protein dynamics in the assembly of the machinery of homologous recombination and in the activities of recombinational DNA helicases.
Specific Aims/Tasks: Project 1 "Conformational dynamics in HR" will address the mechanism of the RAD51 and its regulation, which are so important for genome stability.
Project 2 "Multipurpose recombinational DNA helicases" will determine how the auxiliary domain dynamics in DNA repair helicases helicases affects their roles in controlling HR and in supporting DNA replication.
Overlap: There is no overlap with the proposed studies. MSH2/6 protein is one of the many partners of FANCJ helicase. This interaction, however, is unlikely to be relevant for the MSH2/6 role in BLBC pathogenesis.
Start and End Date: 4-1-2019 – 3-31-2024
Level of Effort: 51%
Annual Direct Costs: $250,000/year
Point of Contact at Funding Agency: Michael K. Reddy, PhD, Program Official

(NEW)
Targeting RAD51 DNA Repair Protein for Cancer Therapy: Development of a Combined Experimental/Computational Workflow
Source: University of Iowa Center for Biocatalysis and Bioprocessing Pilot Grant
Goals of the Project: To initiate a drug-discovery campaign targeting human RAD51 recombinase, an important player in homology-directed DNA repair.
Specific Aims/Tasks: Aim 1 will establish a fragment-based drug discovery work flow to generate the new RAD51 binders in the µM-mM range. In parallel, Aim 2 will use our *in silico* workflow to screen a virtual Enamine REAL library of 3 million compounds representing novel chemical space that can be readily synthesized.
Overlap: There is no overlap with the proposed studies.
Start and End Date: 7-29-2019 – 7-28-2020
Level of Effort: 0%
Annual Direct Costs: $35,000/year ($17,500/year to Spies Lab)
Point of Contact at Funding Agency: Mark A. Arnold, PhD, Director, Center for Biocatalysis and Bioprocessing, University of Iowa

(NEW)
Determination of RAD52 and Replication Fork DNA Complex
Source: PNCC (Pacific Northwest Center for Cryo-EM)
Goals of the Project: This proposal provides us with up to 480 hours annual Titan Krios and 200kV Talos Artica microscope access at the PNCC. This proposed project is built around our current model, whereby two undecameric rings of RAD52 bind to the stalled replication fork. RAD52 then remodels the fork into a structure refractory to fork regression enzymes. Our objective is to visualize this structure at atomic resolution.
Specific Aims/Tasks: Aim 1 will obtain a high resolution structure of the RAD52 undecameric ring. Aim 2 will determine the structure of the RAD52 in complex with the RPA-bound model replication fork.
Overlap: There is no overlap with the proposed studies.
Start and End Date: 2-1-2020 – 1-31-2022
Level of Effort: 0%
Annual Direct Costs: No budget/costs associated with this project. Purpose provides time on the instrument only.
Point of Contact at Funding Agency: Lauren B. Hales Beck, PNCC User Project Coordinator

THE FOLLOWING GRANTS ARE NOW INACTIVE :

Regulation of Unwinding and Remodeling Activities in FeS-DNA Helicases
Source: NIH 3R01GM108617 (PI: Spies)
Goals of the Project: To determine the mechanism by which the domain mobility controls the activities of three FeS helicases important for genome maintenance, XPD, FANCJ and RTEL1.
Specific Aims/Tasks:
Aim 1: Determine the role of ARCH domain mobility in controlling XPD activities.
Aim 2: Determine the role of ARCH domain mobility in FANCJ and RTEL1 mediated DNA unwinding and remodeling of G-quadruplexes.
Aim 3: Determine how protein partners tune the activities of FANCJ and RTEL1.
Overlap: There is no overlap with the proposed studies. MSH2/6 protein is one of the many partners of FANCJ helicase. This interaction, however, is unlikely to be relevant for the MSH2/6 role in BLBC pathogenesis.
Start and End Date: 6-1-2014 – 2-28-2019
Level of Effort: 40%
Annual Direct Costs: $190,000/year
Point of Contact at Funding Agency: Karen F. Whitaker, Grants Management Specialist

Administrative Equipment Supplement Request for Regulation of Unwinding and Remodeling Activities in FeS-DNA Helicases
Source: NIH 3 R01GM108617-05S1 (PI: Spies)
Goals of the Project: to build a micromirror TIRFM system which will complement our existing aging TIRFM
Instruments.
Specific Aims/Tasks: to build a micromirror TIRFM system which will complement our existing aging TIRFM
Instruments.
Overlap: There is no overlap with the proposed studies.
Start and End Date: N/A
Level of Effort: Supports purchase of equipment
Annual Direct Costs: $171,415 (one time supplement)
Point of Contact at Funding Agency: Karen F. Whitaker, Grants Management Specialist

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: This report reflects the work and progress of Maria Spies, PI.

QUAD CHARTS: N/A

9. APPENDICES: None