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TITLE: Understanding Tumor Dormancy as a Means of Secondary Prevention

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14. ABSTRACT The purpose of this collaborative project is to establish a molecular definition of the dormant state of a cancer cell. In doing this we hope to understand how this dormancy is broken, ultimately leading to recurrence in a patient that was stably in remission. Once our understanding of this is more complete it is hoped that we can devise strategies for secondary prevention. This funding year we have developed new methods to track dormant cells in vivo, which will also help with RNA extraction and thus transcriptome sequencing. We have also modified our methods to construct mice with markers for tumor niche cells, using the latest technology, and progressed with our work on single chain antibodies, with the intention to use these to identify dormant cells in human biopsies.					
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1. INTRODUCTION

The primary purpose of our collaborative research project is to discover the genes and pathways that regulate breast cancer dormancy. This information will allow us to generate hypotheses about the mechanisms underlying dormancy maintenance as well as how it is broken. We will use models of breast cancer wherein murine or human breast cancer cells establish and progress through dormancy. We will utilize innovative methods to perform laser capture microdissection of cancer cells in the dormant state as well as cancer cells that are progressing from dormancy. These cells, which are at distinct stages of cancer progression within their native environments, will be molecularly profiled. We will also profile various cell-types within the micro-environment that contact cancer cells. This molecular description of the dormant state will allow us to perform loss-of-function and gain-of-function studies of candidate drivers and suppressors of dormancy progression. Ultimately, we anticipate the identification of genes that could serve as attractive targets of therapeutic inhibition.

While the Tavazoie lab has been working towards human breast cancer profiling, The Hannon lab has been optimizing conditions for identifying and profiling dormant cells. In the last 3 years of funding we have been working towards two goals. One is the ability to locate dormant cells and the second is the ability to capture and profile, using RNA seq, single cells.

2. KEYWORDS

Dormancy, cancer, breast, DiD, laser capture, Flow cytometry, RNA seq, low input

3. ACCOMPLISHMENTS

What were the major goals of the project? And what was accomplished under these goals?

Subtask 1a.

In task 1, we had proposed to characterize the molecular features of dormant cells by identifying these cells in metastatic micro-environments through the use of automated serial two-photon tomography (STPT).

The Hannon lab has been working towards this goal by developing and optimizing the methodology required for this.

1. As described in previous reports, during these funding years we have established the necessary techniques for perfusion, fixation and agarose embedding of mouse tissue prior to processing with the two-photon Microscope (TPM). In the previous year we had been working with GFP-H2B labeled 4T1 mouse breast cancer cells, and different injections methods to simulate metastasis. As mentioned in our previous report we identified the limitations of using GFP labeled cells for metastasis experiments as the immune response to GFP was restricting the ability for the cancer cells to metastasis. We are currently in the process of comparing 4 different florescent proteins and observing how the immune response behaves towards these cells, initial trials were not 100% conclusive on the best florescent protein to use, however they did demonstrate that the alternative florescent proteins are better able than GFP to allow for metastasis. This year we replaced the virus promoter to a far more effective one from our previous work, and we are also comparing how the antibiotic selection marker also affects the immune response. It is important that we use a cancer model that is not hindered by the labeling method that we chose.

2. As a potential alternative to using virus infected fluorescently labeled tumor cells, we have also been working on using other methods to both label the cells for visualization purposes, and also as a measure of dormancy. Last year we began work with EdU using the Click-IT chemistry. This proved useful, however one hindrance of this technique was the fact that cells needed to be fixed with PFA before they could be visualized based on their EdU content. This is not an issue when we are only observing, which might be the case when we seek to look for correlations between dormant cells and the cells that surround them, but as previous work of ours has shown, cells exposed to PFA are very hard to obtain good quality RNA from. We therefore started work on a new method, using a membrane stain, previously reported to be a useful indicator for dormant cells (A novel method for monitoring tumor dormancy using fluorescent dye DiD, Yumoto et.al. 2014). We have been testing the DiD lipophilic

fluorescent dye for this purpose. We have had promising results with this, initially in cell culture, where we optimized the staining protocol to ensure maximum cell brightness, and minimum intensity variation between cells. We also tracked the brightness of cells over time and saw a gradual decrease in intensity as cells divided, and this loss of intensity could be slowed when using methods to reduce cell division rate, e.g. lowering the FBS in the media. There is also no loss of signal when the cells are treated with PFA (as they would need to be for imaging on the Two-photon microscope).

We went on to test the utility of this in vivo to ensure we could identify DiD positive cells against background tissue, and also to ensure that the DiD was not effected by the digesting of the tissue. Injecting cells into the fat pad of a mouse and extracting shortly after demonstrated that neither of these things were a problem (FIG 1) .

We also tested the performance of these DiD stained cells in tumor development and metastasis's. So far we have no reason to believe that metastasis is effected and we can identify with some confidence the presence of DiD+ cells within a tumor after 2.5-3 weeks (tumor growth ~ 1cm) and with slightly less confidence the presence of DiD+ cells in organs after this time (with both Flow cytometry (FIG 2) and imaging.) We will be more confident of the DiD positive cells in the organs once we have labeled all 4T1 cells with a fluorophore.

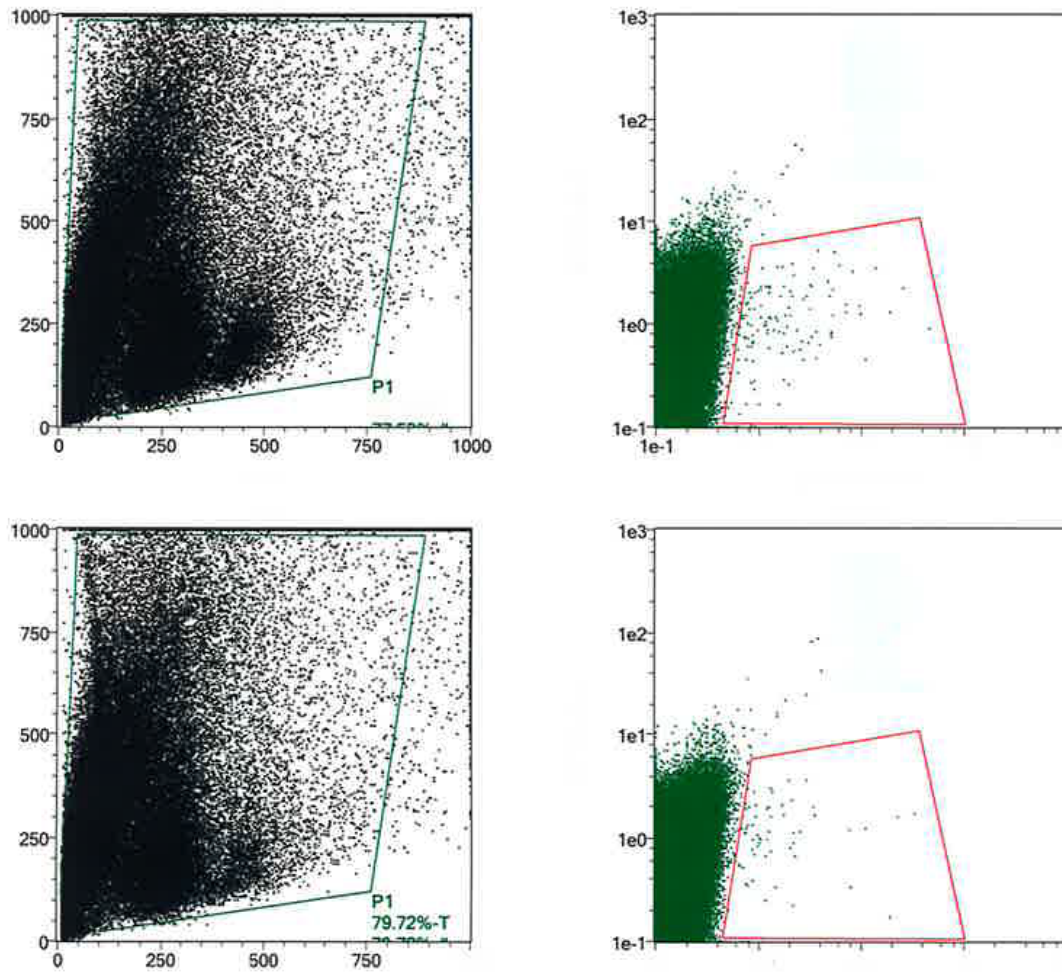


FIG 1: DiD positive cells (red gated) after removal and digestion of mouse fatpad. Plots show 200 thousand events. Left plots show FSC/SSC . right plots show APC (DiD channel) on X axis and FITC on right axis. Upper plots are with 100 thousand 4T1 cells injected, lower plots are with 50 thousand cells injected.

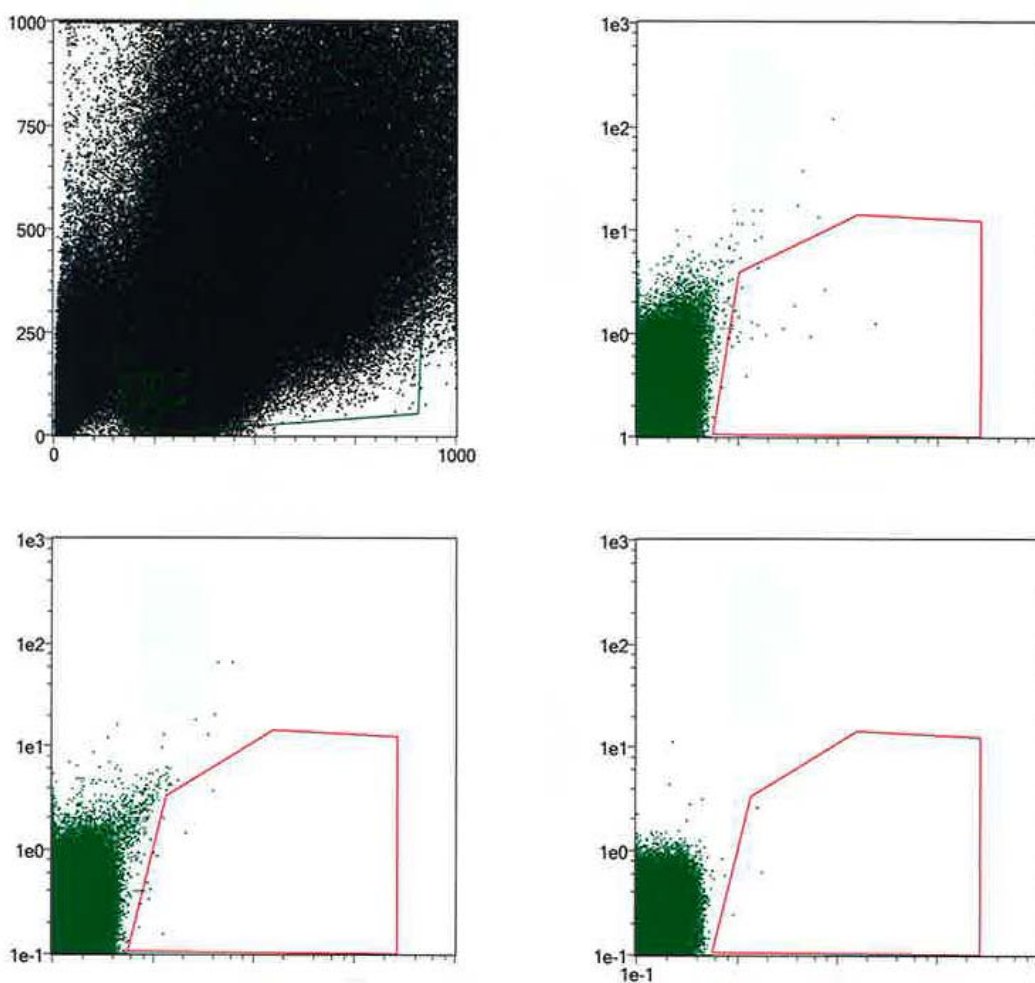


FIG 2: DiD positive cells after 2.5 weeks. Top right is digested tumor. Bottom left is digested liver, bottom right is digested lung

We are currently monitoring these cells and their presence in organs over a time series 1 week post injection, 2 weeks etc. . Initial work suggests that cells are dispersed early as we see DiD+ cells in organs after 1 week. This will be useful for when we progress with removing the tumor (in the early stages) and then monitoring for more long term dormant cells. This method will however select for dormant cells which leave the

primary tumor early, however, this in combination with our other techniques should allow a good representation.

3. Because the DiD+ cells do not need to be fixed with PFA, this allows a better recovery of RNA. We are currently in the process of testing the performance of these cells for RNA extraction/RNA sequencing post single cell sorting and from frozen section dissected with the LCM. This might not be possible however as resolution on frozen sections may not be good enough to detect single cells. Using the two photon microscope still requires the fixation of the tissue, so we must still seek to find ways to construct good quality libraries from fixed tissue. The DiD stain has the benefits however of not requiring any secondary treatment to visualize so will work well if we collect multiple “dormant” cells from sections imaged by the serial Two photon microscope, thus allowing for a better RNAseq library.

4. This funding year we have also progressed with a collection device for the tissue sections that result from the STPT imaging. We have interacted with a lab in London who have designed and built a device for collecting ordered individual sections, and we are working on how to implement this in our lab.

5. Continuing the construction of BACs for micro-environment cell specific / cell status, markers we have opted to use the more recent technology of CRISPR to create these, as the typical recombineering methods were proving challenging. We are currently designing the strategy and it is hoped this will prove more successful than the prior method.

Subtask 1b.

In this subtask, we had proposed to interrogate human biopsy samples for dormant tumor cells and determine if the expression levels of genes identified to be over-expressed in the transition from dormant to micro-metastases stratify patients into those that relapse and those that do not. Although we have not yet established a gene expression list for clinical validation analyses, we are working on combining the

techniques we are establishing in mice with more human tissue specific techniques. We have searched the literature and found two proteins thought to specifically label human breast cells, with higher expression in breast tumor cells, these being Mammaglobin and MUCL1. We have tested their expression using immunohistochemistry and QPCR, in 6 human cell lines and for controls, 2 pancreatic cell lines and 2 prostate cell lines. We are also developing whole mount staining techniques to stain mouse organs containing human breast cells that can then be visualized using TPM, with the intention that we will locate human dormant breast cancer cells in human biopsies. This year we have been continuing work on the development of a single chain antibody for mammaglobin, using alpacas. We have completed the protocol (which takes several months of antigen presenting to the alpaca) and part constructed the libraries for sequencing. We are in the process of deciphering the sequencing data, identifying the variable regions and looking for sequences that have grown more abundant over the course of the antigen exposure. It is hoped that the reduced size for the single chain antibody will allow whole mount and slide staining to work far more efficiently and allow us to fluorescently tag the antibody, thereby reducing immunostaining times and improving RNA recovery.

Subtask 2b.

In this subtask, we had proposed to functionally test the roles of genes identified as being differentially expressed in micro-metastatic tumor cells relative to dormant cells through loss-of function and gain-of-function analyses. In order to initiate these studies, we first had to characterize and optimize the human breast cancer in vivo dormancy systems that will be subsequently used for the profiling methods. In the coming year, we anticipate the identification of genes differentially expressed by these cancer cell populations and their micro-environments and will proceed to functional testing of these genes.

Subtask 3a.

In this subtask, we had proposed to modulate, through over-expression or knockdown, genes that are predicted to maintain dormancy cell survival. This subtask will be

pursued in future years of the funding period upon identification of differentially expressed genes that are candidate dormancy regulators.

Subtask 3b.

As per above, This subtask will be pursued in future years of the funding period upon identification of differentially expressed genes that are candidate dormancy regulators.

Subtask 3c.

As per above, This subtask will be pursued in future years of the funding period upon identification of differentially expressed genes that are candidate dormancy regulators.

Subtask 3b.

As per above, This subtask will be pursued in future years of the funding period upon identification of differentially expressed genes that are candidate dormancy regulators.

4. IMPACT

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

During the transition we are currently training a new technician in Cambridge UK, Sophie Watcham, who will become incorporated onto the project once we have petitioned for the grant to be moved to the UK. Meagan Keane, is still involved in the project and continues her training at CSHL.

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?

It is our intention to start molecular profiling the lone cells and “dormant” cells and create an initial list of relevant genes.

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

Nothing to report

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:

During the lab transition to the UK, we have had some initial set up delays. However, work has mainly been continuing as normal in CSHL and as lead post doc on the grant Clare Rebbeck has now moved to the UK, but still continues her role in both the UK and CSHL.

6. PRODUCTS:

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project (reporting period 9/15/14 – 9/14/15)?

Name: Greg Hannon

Project Role: PI

Nearest Person month worked: 1 calendar month

Contribution to project: no change

Name: Clare Rebbeck

Project Role: Post Doc

Nearest Person month worked: 11 calendar month

Contribution to project: no change

Funding Support:

Name: Elena Rozhkova

Project Role: Post Doc

Nearest Person month worked: 11 calendar month

Contribution to project: no change. Elena Rozhkova has left the lab.

Name: Elvin Wagenblast

Project Role: Post Doc

Nearest Person month worked: 10 calendar month

Contribution to project: Elvin Wagenblast has left CSHL. His effort will be replaced by Simon Knott beginning October 15, 2015

Name: Simon Knott

Project Role: Post Doc

Nearest Person month worked: 0

Contribution to project: Simon will be resuming the work previously performed by Elvin Wagenblast as of October 15, 2015

Name: Meagan Keane

Project Role: Technician

Nearest Person month worked: 7 calendar month

Contribution to project: no change

Name: Sun Kim

Project Role: Technician

Nearest Person month worked: 6 calendar month

Contribution to project: Sun Kim has left CSHL. Her effort is replaced by Meagan Keane

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

The following previously active grants have closed:

- 5 R21 DA035612-02
- Howard Hughes Medical Institute
- National Philanthropic Trust
- PEW Charitable Trust
- 5 R01 HG005238-04

Greg Hannon has been replaced as the Primary Investigator on the 5 P01 CA013106-44

For complete details, please refer to Other Support documentation in the Appendix

What other organizations were involved as partners?

Organization Name: CRUK Cambridge University

Location of Organization: UK

Partner's contribution to the project - Collaboration

8. APPENDICES: Greg Hannon Other Support

OTHER SUPPORT

HANNON, G.

ACTIVE

5 P01 CA013106-44 (Stillman) 01/01/77 – 12/31/16 2.40 calendar

NIH

CSHL Cancer Research Center - Project 4, microRNAs in Human Cancer

The major goals of this project are: To assess the minimal complement of cellular signaling pathways that may be altered to convert a normal human cell into a cancer cell.

Role: Project Leader

OVERLAP: None

5 P01 CA013106-44 (Stillman) 01/01/77 – 12/31/16 0.60 calendar

NIH

CSHL Cancer Research Center

Core B: Modulation of gene expression through RNAi

The major goal of this project is: to provide essential services and facilities utilized by all of the projects of the program.

Dr. Hannon does not receive any research support for his lab from this grant.

Role: Core Director

2 R37 GM062534-14 (Hannon) 09/01/09 – 08/31/18 1.20 calendar

NIH/NCI

Roles of small RNAs in guarding germ cell genomes

The major goal of this project is: To understand the mechanistic basis of dsRNA-induced gene silencing using a biochemical approach.

Role: Primary Investigator

OVERLAP: None

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Rockefeller Univ PI Tavazoie

DOD Collaborative Scholars Award

Understanding tumor dormancy as a means for secondary prevention

The major goal of this project is: to characterize the molecular and cellular basis of breast cancer dormancy and dormancy progression.

Role: Co-Primary Investigator

OVERLAP: None

1 U01 MH106035-01 (Hannon) 09/26/14 – 06/30/17 1.2 calendar

NIH

The major goal of this project is: to develop a broad optogenetic toolkit, based on covalent protein tags and photoreleasable compounds, enabling the recovery of genetic material, the alteration of gene expression, and the insertion of transgenes to any cell of the brain with high spatial precision.

OVERLAP: None

STARR (Hannon/Atwal/Darnell) 01/01/14-12/31/15 0.24 calendar

17-A723

Functional analysis of ectopic germline gene expression in cancer

The major goal of this project is: to systemically investigate signature of ectopic germline gene expression in non-germline cancers and their function in oncogenesis

Role: Co-Primary Investigator

OVERLAP: None

COMPLETED

5 P30 CA045508-27 (Hannon) 08/15/05 – 04/01/15 0.60 calendar
NIH/NCI

CSHL Cancer Center Support

The major goal of this project is: Cancer Genetic Program Leader on the CSHL Cancer Center

Dr. Hannon does not receive any research support for his lab from this grant.

Role: Program Leader

OVERLAP: None

5 P01 CA013106-44 (Hannon) 01/01/77 – 07/09/2015 0.24 calendar
NIH

CSHL Cancer Research Center

Core A: Administration

The major goal of this project is: Administrative Services

Dr. Hannon does not receive any research support for his lab from this grant.

Role: Administrator

5 R01 HG005238-04 (Smith USC) 07/01/10 – 06/30/15 0.60 calendar
NIH

Analytic tools to examine high resolution and genome scale DNA methylation data

The major goal of this project is: to develop analytical technology to complement cutting edge experimental technologies for examining DNA methylation.

Role: Sub-contractor

OVERLAP: None

2010-000702-001 (Hannon) 01/01/11 – 12/31/15 0.00 calendar*
PEW Charitable Trust

Glaucoma Research

The major goal of this project is: to carry out research to understand further the biology of retinal ganglion cell loss.

Role: Primary Investigator

OVERLAP: None

NBCC (Hannon) 10/10/13 – 10/09/15 0.00 calendar*
National Philanthropic Trust

Research and analysis activities in support of the Artemis Project

The major goal of this project is: to produce large-scale transcriptome datasets that will describe the molecular events that occur in DCIS

Role: Primary Investigator

OVERLAP: None

Howard Hughes Medical Institute (Hannon) 09/01/05 – 08/31/15 N/A

This support provides general research and scholarship support to the laboratory and is not designated for use in any given project.

Role: Investigator

5 R21 DA035612-02 (Hannon) MSSM PI Ellis-Davies NIH	04/01/13-03/31/15	0.24 calendar
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A light regulated protein tagging method to study local translation in neurons
The major goal of this project is: to develop an enabling technology for studying biological processes with subcellular resolution.

Role: Co-Primary Investigator

OVERLAP: None

5 U01 CA105388-10 (Lowe) NIH	09/01/09 – 08/31/14	0.60 calendar
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Identifying driver mutations and tumor dependencies by comparative oncogenomics
The major goal of this project is: to use RNAi in mice to silence cancer relevant genes to various degrees.

Role: Investigator/Senior Personnel

NBCC (Hannon) National Philanthropic Trust	10/10/13 – 10/09/14	0.00 calendar*
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Research and analysis activities in support of the Artemis Project
The major goal of this project is: to produce large-scale transcriptome datasets that will describe the molecular events that occur in DCIS

Role: Primary Investigator

STARR (Perrimon/Hannon) I5-A505	08/01/11 – 07/31/14	0.24 calendar
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Genomic and epigenetic changes occurring during carcinogenesis: a fly perspective
The major goal of this project is: to provide a comprehensive view of how genomes, with specific oncogene and tumor suppressor-activated signaling pathways, coordinate their genetic and epigenetic responses towards a transformed state.

Role: Co-Primary Investigator

Team Award (Hannon/Hammell/Vakoc) Melanoma Research Alliance	08/01/11 – 07/31/14	0.24 calendar
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A functional approach to targeted melanoma therapy
The major goal of this project is: to use a non-biased genome-wide approach to identify candidate targets for melanoma therapies.

Role: Co-Primary Investigator

*Please note that no designated level of professional effort is required by the funding organization for these projects, however, Dr. Hannon has 3.6 calendar months of effort currently available at his discretion to apply toward the oversight of these research endeavors and any other research or administrative responsibilities. At no time will his effort exceed 12 person months in any year.