AWARD NUMBER: W81XWH-21-1-0159

TITLE: Translational Targets of Ribosomal Protein RPL13 as Novel Cardiac Drivers of Differentiation in Drosophila and Human iPSCs: Implications for CHD

PRINCIPAL INVESTIGATOR: Analyne Schroeder

CONTRACTING ORGANIZATION: Sanford Burnham Prebys Medical Discovery Institute
La Jolla, CA

REPORT DATE: March 2022

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
March 2022	Annual	01Mar2021-28Feb2022

4. TITLE AND SUBTITLE

Translational Targets of Ribosomal Protein RPL13 as Novel Cardiac Drivers of Differentiation in Drosophila and Human iPSCs: Implications for CHD

5a. CONTRACT NUMBER
W81XWH-21-1-0159

5b. GRANT NUMBER
W81XWH-21-1-0159

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Analyne Schroeder

E-Mail: aschroeder@sbpdiscovery.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Sanford Burnham Prebys Medical Discovery Institute
10901 North Torrey Pines Road
La Jolla, CA 92037-1005

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development 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

There is a need to identify novel genetic networks and pathways driving Congenital Heart Disease (CHD). Our research is aimed at utilizing an unconventional gene involved in translation, the large ribosomal subunit RpL13, to extract and identify novel players and mechanisms in heart development with implications for CHD. During year one, we performed preliminary staining of fly embryos using various cardiac markers and consistent with our hypothesis, we observed changes in the proportion of cell types, suggesting that cells are undergoing cell fate switches. We therefore refined our single-cell RNAseq protocol to collect and enrich for fly cardioblasts from controls and RpL13 knockdown flies and set parameters for FACS sorting. We created 10X Genomic libraries which are currently being sequenced. We will analyze the data as soon as we receive them. While we have not been able to move forward with experiments in human Multipotent Cardiac Progenitors due to lack of access, we have recently secured a new source for these cells and are excited to perform transcriptomic and proteomic analysis on these cells. We have also come up with an alternative approach that develops a new genetic tool in Drosophila that will enable us to capture the translatome with up to single-cell resolution. This would allow for better comparisons between transcriptomics and translatomic changes in fly cardioblasts.

15. SUBJECT TERMS

Congenital Heart Disease, RpL13, ribosome, translation, differentiation, cell identity, profiling, diagnosis, single-cell RNAseq, Drosophila, iPSCs

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

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1. **INTRODUCTION:** Our research is focused on identifying novel genes and pathways involved in Congenital Heart Disease pathogenesis by focusing on the role of the large ribosomal subunit *RpL13.* We had demonstrated that the knockdown of large Ribosomal Protein RpL13 in the *Drosophila* heart and human Mulitpotent Cardiac Progenitors (MCPs) led to cardiac-specific defects, and therefore, we hypothesized that *RpL13* could be used as an unconventional gene candidate to identify a novel genetic network regulating cardiac development and pathogenesis. This proposal aims to 1.) identify the consequences of *RpL13* knockdown on the transcriptome and 2.) to identify the translational targets of *RpL13.*

2. **KEYWORDS:** Congenital Heart Disease, RpL13, ribosome, translation, differentiation, cell identity, profiling, diagnosis, single-cell RNAseq, Drosophila, iPSCs

3. **ACCOMPLISHMENTS:**

   - **What were the major goals of the project?**

     The major goal under AIM1 is to obtain a transcriptomic profile of *Drosophila* cardioblasts (Major Task 1) and human Multipotent Cardiac Progenitors (Major Task 2) with single-cell resolution to track emerging cell identities that are altered by RpL13 knockdown. Analysis of the transcriptomic profiles will uncover altered expression of genes and pathways induced by RpL13 knockdown, resulting in changes in cell fates and heart morphogenesis.

     The major goal of AIM2 is to use proteomic methods to identify the changes in overall translation (Major Task 3 and 4) and to identify the specific translational targets affected by RpL13 knockdown (Major Task 5). This will inform us of the selectivity of RpL13-bound ribosomes in targeting translation and will provide a snap shot of the resulting translatome/proteome.

Specific Aim 1: Single-cell transcriptomics for population mapping of FACS-sorted *Drosophila* cardioblasts and human Multipotent Cardiac Progenitors, to track emerging cell identities and how they are altered as a result of RPL13 knockdown.	**Timeline**	**Site 1**	**Progress (%)**
Major Task 1: Molecular Characterization of *Drosophila* cardioblasts by single cell-RNAseq	Months	SBP	
Subtask 1. **RNA-seq of Stage 16-17 embryonic cardioblasts.** To determine whether cardioblasts have undergone a transformation in molecular/cardiac identity following *RpL13* knockdown.	1-3	Dr. Schroeder	50
Subtask 2. **Single-cell RNA-seq of Stage 16-17 embryonic cardioblasts.** Obtain molecular signatures of individual cells and determine whether subpopulations of cells respond differentially to the KD of *RpL13*.	2-5	Dr. Schroeder	100 (02/22)
Subtask 3. **Computational Analysis of scRNA-seq data, generation of genetic map and verification of candidate genes by ISH and antibody staining.** Select differentially expressed genes as indicated by RNA-seq and visualize gene expression changes in the embryo.	5-9	Dr. Schroeder	

Milestone(s) Achieved: Identified pathways and genes that are altered in expression following *RPL13* KD in Drosophila cardioblasts.			
Major Task 2: Molecular characterization of cardiac progenitors in Multipotent Cardiac Progenitors by single cell-RNAseq			
Subtask 1. **Single-cell RNA-seq of Multipotent Cardiac Progenitors.** Time course following *RPL13* siRNA treatment to determine changes in the transcriptomic landscape of the heterogenous cell population.	6-10	Dr. Schroeder	10
Subtask 2. **Computational Analysis of scRNA-seq data** and comparison of pathways affected by knockdown of *RPL13* between Drosophila and human MCPs.	10-14	Dr. Schroeder	
Milestone(s) Achieved: Identify various subpopulations of cells in MCP cultures and attach a molecular signature to each population. Identify key pathways altered by *RPL13* knockdown. Compare pathways between Drosophila and MCPs and look for parallels between species.			
Specific Aim 2: Polysome Profiling to identify direct and indirect translational targets of *RPL13* in human Multipotent Cardiac progenitors, leading to construction of a *RPL13*-centric genetic network driving cardiac differentiation.			
Major Task 3. Measure overall Protein Translation in MCP and *Drosophila* Cardioblasts following *RPL13* knockdown. Puromycin protein synthesis quantification assay to measure protein production following *RPL13* knockdown.	12	Dr. Schroeder	20
Milestone Achieved: Determined how *RPL13* KD affected overall protein translation in MCPs and *Drosophila* cardioblasts.			
Major Task 4. **Ribosomal Protein Quantification in MCPs**			
Subtask 1. **Polysome isolation from MCP cells treated with RPL13 siRNA.** Optimize conditions and select appropriate markers/antibodies for FACS sorting.	13-14	Dr. Schroeder	20
Subtask 2. **Quantification of ribosomal subunit levels and stoichiometry between controls and *RPL13* siRNA treated MCPs using liquid chromatography coupled to tandem mass-spectrometry LC-MS/MS).** Determine whether subunits are enriched in monosomes vs. polysome.	14-15	Dr. Schroeder	
Milestone(s) Achieved: Determined changes in the levels and stoichiometry of ribosomal proteins following *RPL13* knockdown. Determined enrichment of ribosomal proteins in the various polysome fractions.			
Major Task 5. **Polysome Profiling with RNA-seq in MCP cells**			
Subtask 1. **Polysome Profiling to identify mRNAs targeted by ribosomes in MCPs and how the mRNA targets shift following *RPL13* siRNA treatment.**	14-20	Dr. Schroeder	10

Timecourse following siRNA treatment. RNA-seq followed by data analysis and interpretation.			
Subtask 2. **Identify mRNA species that are directly bound by RPL13 loaded ribosomes.** Polysome fractionations will be subject to a pulldown using RPL13 antibody, to isolate ribosomes loaded with RPL13. Bound mRNA will be processed for RNA-seq to identify and analyze RPL13 targets.	18-22	Dr. Schroeder	10
Subtask 3. **Gather RNA-seq data and perform Computation Analysis and gene network design.**	22-24		
Milestone(s) Achieved: Identified direct and indirect mRNA targets of *RPL13* in differentiating MCPs. Developed genetic maps by computational analysis that describe and predict *RPL13* involvement in cardiac differentiation pathways.			

- **What was accomplished under these goals?**

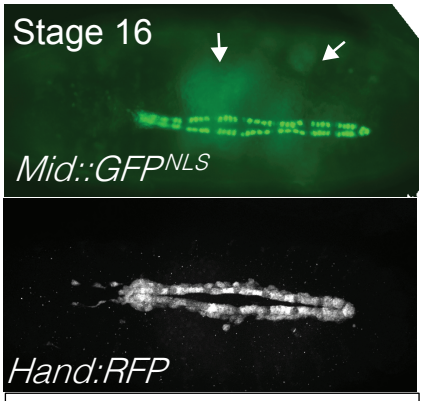

Figure 1: ***Top:*** Fluorescent images of embryo expressing *midline::*GFP shows GFP labelled cardioblasts in the heart. GFP is also detected in other extra-cardiac tissues (arrows), which would reduce the proportion of acquired cardioblasts during FACS sorting. ***Bottom*:** Fluorescent images of embryo expressing *Hand*::RFP shows RFP signal in the heart cells and bordering pericardial cells, with minimal signal from other tissues. This will enrich for cardiac relevant cells during FACS sorting.

We are in the process of completing the sub-tasks of Major Task 1 aimed at identifying the transcriptomic profile of Drosophila cardioblasts following *RpL13* knockdown (KD). During our first attempt with the single-cell RNAseq (sc-RNAseq) experiments, we encountered some technical and experimental design issues, which we spent time addressing before continuing with the experiment. First, the fluorescent marker we were using (*midline::GFP*) was labeling tissues outside the heart, which may significantly reduce the number of cardiac relevant cells collected during sorting for sc-RNAseq analysis (**Figure 1**). This would also make the results of bulk RNA-seq (Major Task 1; Sub-Task 1) less reliable if other cell types were included in the sorted sample. We therefore selected and tested a more cardiac specific fluorescent marker for FACS sorting of cardioblasts in the heart. The new marker includes a Red Fluorescent Protein driven by the promoter of the cardiac transcription factor *Hand* (*Hand::*RFP) which displays clear expression in hearts and cardiac relevant tissues such as pericardial cells, with minimal autofluorescence in other tissues (**Figure 1**). Second, I had proposed to use an embryonic driver *TinD*-GAL4 to KD RpL13 in the embryonic cardioblasts. In a separate proof-of-concept experiment, we performed scRNA-seq on sorted cardioblasts following *tinman* KD using *TinD*-GAL4 to look for changes in the transcriptome. No differences were detected (*data not shown*). We concluded that the KD using *TinD*-GAL4 may be insufficient to produce an effect during the collection timepoints at Stage 16-18. We therefore designed a new driver line that combines two broader mesodermal drivers (*Twist*-GAL4 and *24B*-GAL4) to induce RNAi KD earlier and stronger to be able to observe effects in embryos. To confirm that this driver would be sufficient to induce changes in embryos, we performed immunostaining of embryos using established cardiac markers (*neuromancer* and *tinman*) and observed changes in the proportion of cell types in the heart through both increases and decreases in cell numbers (**Figure 2**). This was confirmation of our hypothesis that *RpL13* KD was indeed having effects on cell fate early on in the embryo, and not just inducing cell

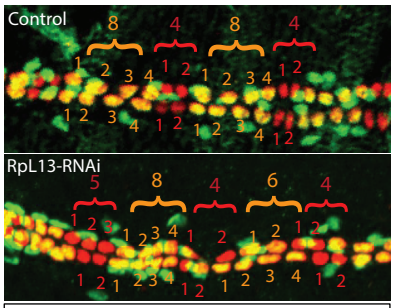

Figure 2: *RpL13* KD alters the proportion of cell types in the heart suggesting reprogramming and changes in identity. In control hearts, 8 myocardial cells (orange) and 4 seven-up ostial cells (red) are found in each segment. *RpL13* KD altered the proportions with both increased and decreased numbers of cell types.

death. We therefore felt confident moving forward with this new genotype to knockdown *RpL13* and perform scRNAseq. We also decided to collect embryos throughout development, and therefore capture cardioblasts beginning as early as Stage 12 when Hand starts being expressed. Through quantitative means, we will be able to follow trajectories (over 4-5 hours) of cell development where we hope to capture younger cells with minimal and early changes and older cells with more significant changes in transcriptome and trace the trajectory of cells through their development. We have collected cells and created RNAseq libraries of control and *RpL13* KD samples, and the RNA is currently being sequenced. Upon receipt of the sequencing data in approximately 2-3 weeks, we will thoroughly analyze the results and begin to look for cell subpopulations with altered gene networks that correlate with the change in identity. Pending these results, we will re-evaluate whether bulk-RNA-seq (Major Task1; Sub task 1) will be necessary, as we could obtain whole population information from the single-cell RNAseq.

For the other tasks partially completed, we have the reagents and tools ready to move forward with the experiment, and just awaiting the MCPs. For example, we have obtained *RpL13* and control siRNAs. We have puromycin antibody to move forward with the protein synthesis assay (Deliu LP, *et al.* 2017 *Biol. Open.)* and we are about to measure puromycin incorporation in embryonic hearts following RpL13 knockdown using a temperature-mediated inducible system (Major Task 3). We are testing methods to permeabilize the outer vitelline membrane of the embryo to allow for drug incorporation followed by fixing. We have recently secured an alternative source of MCP cells from a collaborator (see below) and will be starting experiments in cells soon.

- **What opportunities for training and professional development has the project provided?**
  The Sanford Burnham Prebys Medical Discovery Institute (SBP) Office of Education, Training & International Services (OETIS) oversees and coordinates an annual individual development planning (IDP) process for all postdocs at the Institute. The focus of the IDP process at SBP is the career goal of the postdoc; identification of what skills, knowledge, and accomplishments will be necessary for the postdoc to obtain a desired independent position following training; and identification of training and professional development opportunities that are available for the postdoc to obtain the necessary skills and knowledge. The SBP Office of Education, Training & International Services provides guidance and advising to both postdocs and PIs throughout the postdoc's training with respect to developing IDPs and preparing for a successful transition to independence post-training. The SBP Office of Education, Training & International Services also maintains webpages containing comprehensive resources on career path identification, career planning, and creating an IDP that can be utilized in conjunction with the formal annual IDP process.

  The SBP IDP process includes two components:

  1) <u>First-Year IDP (effective in 2014)</u>. Within the first 3 months of beginning postdoctoral training at SBP, all postdocs receive and fill out an initial "planning and expectations" document to discuss with their PI. This document serves as the foundation for their postdoctoral IDP and is designed to facilitate discussion between the PI and new postdoc regarding goals and expectations for the first year of training, as well as stimulate initial discussions about long-term career goals and training plans.

2) <u>Postdoctoral IDP (effective January 2013)</u>. At the end of the first year of training SBP postdocs receive notification that it is time to update their IDP, and they receive the information they included in their first-year planning and expectations document in the form of a full IDP that they can update with their accomplishments over the past year and their goals for the coming year, mid-term future, and long-term future. Each subsequent year of their postdoctoral training, postdocs will receive notification and the previous year's IDP form to update and expand. The IDP forms are designed to build upon each previous year as well as provide a solid foundation from which a postdoc can easily build his or her CV/resume.

The SBP Office of Education, Training & International Services also maintains webpages containing comprehensive resources on career path identification, career planning, and creating an IDP that can be utilized in conjunction with the formal annual IDP process.

Though the most recent review has not been conducted for Dr. Analyne Schroeder, Dr. Rolf Bodmer plans to ensure that all IDPs are updated over the coming year to be consistent with SBP IDP process for Postdocs.

- **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?**

In the coming weeks, we will receive our scRNA-seq data of Drosophila cardioblasts and we will analyze the data to identify transcriptomic changes as a result of *RpL13* knockdown.

Due to short-staffing (and covid restrictions), we have been unable to obtain Multipotent Cardiac Progenitors from our collaborator at SBP. In the meantime, another collaborator Dr. Mercola at Stanford University has offered to provide us with similar cells described in the proposal. We are arranging for their transfer, and we will move forward with the experiments described in Major Task 2,3 and 4.

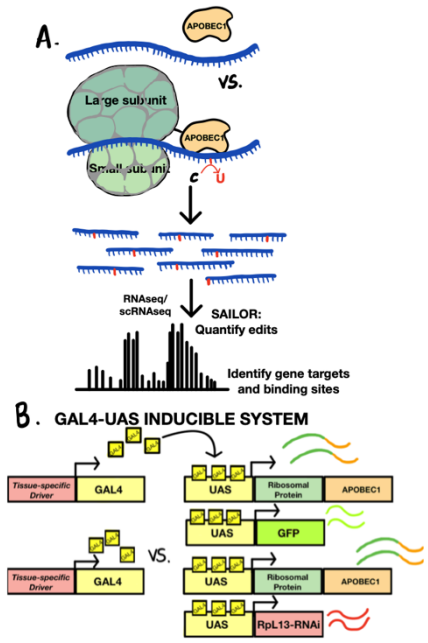

Figure 3: **A**.APOBEC1 is fused to ribosomal subunits to modify bound mRNAs by conversion of Cytosine to Uracil. These modified mRNAs are sequenced by bulk or single-cell RNAseq (scRNAseq). The SAILOR analysis pipeline quantifies the edits to identify gene targets and binding sites of the ribosome providing a snapshot of the translatome. **B.** The Ribo-STAMP will be adapted for *in vivo* use in *Drosophila*. We will use Heart specific drivers to induce the expression of various ribosomal proteins fused to APOBEC1, and test the changes in translatome upon RpL13 knockdown. The constructs and genetic combinations are depicted.

We will be amending the methodology for Major Task 5 to reduce our reliance on MCPs. Instead of using polysome profiling in MCPs, we will develop a novel methodology that will allow us to obtain a proteomic/translatomic profile of cardiac cells subject to *RpL13* knockdown in *Drosophila* embryonic cardioblasts with up single-cell resolution. In collaboration with Dr. Gene Yeo (listed as a collaborator), I plan to adapt the Ribo-STAMP methodology (Brannan K. et al. 2021 *Nature Methods)* developed in cell culture for use in an *in vivo Drosophila* model system (**Figure 3A**). Ribo-STAMP utilizes the APOBEC1 enzyme fused to a ribosomal subunit to modify ribosome-bound mRNA by converting Cytosine into Uracil. The mRNAs are sequenced by RNAseq and the SAILOR analysis pipeline identifies the modifications on actively translated mRNA and detects enrichment of binding sites. I am creating *Drosophila* lines of APOBEC1 fused to different ribosomes, particularly subunits close to the nascent

chain exit site like ***RpL13***, which will determine the contribution of individual ribosomes to the translatome (**Figure 3B**). Differences in the translatome could demonstrate that ribosome subunit composition and heterogeneity control translation of different subsets of *mRNAs* in the heart. Next, I will determine how the knockdown (KD) of *RpL13* alters selective translation of mRNA with single-cell resolution of embryonic cardioblasts and whether the translatome reflects the reprogramming and cardiac defects observed. The translatome will be compared to ongoing single-cell transcriptomics to look for correlations between the two profiles involved in reprogramming. Lastly, I will perform pull-down assays using *RpL13* as bait, to identify bound cofactors that could confer specificity in ribosome activity in *Drosophila* cardioblasts.

I believe that this novel approach to identify translational changes and targets of RpL13 knockdown *in vivo* using *Drosophila* flies will provide interesting results with higher single cell resolution, something not possible with polysome profiling. Furthermore, we will be able to more directly compare the single-cell translatomic profiles with the transcriptomic profile being generated in flies. This will also provide an opportunity to create a new genetic tool for the fly community to explore translational mechanisms in any tissue type at any developmental stage.

We will move forward and explore RpL13 translatomic targets in the cardioblasts of Drosophila by this novel method. We are close to creating the constructs and will soon inject them into fly embryos.

4. **IMPACT:**

- **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:**

- **Changes in approach and reasons for change**

  Nothing to Report

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

  As we were beginning to plan the execution of our cell culture experiments, our collaborator lost 2 out of 3 key personnel responsible for generating and maintaining the human Multipotent Stem Cells. Despite efforts to hire new personnel, our collaborator has been short-staffed since with just 1 postdoc running the lab. They have been unable to provide the cells, nor able to adequately train me to generate the cultures myself. Therefore, experiments in stem cells have been delayed. Recently, we contacted another collaborator, Dr. Mark Mercola from Stanford University and a former professor at Sanford Burnham Prebys. He will be supplying similar

progenitor cells, and I will be able to take these cells and move forward with the experiments myself. We have also come up with an exciting change in methodologies to achieve the goals of this project (no change in scope) (described above), that will be able to identify translational changes caused by the knockdown of *RpL13* in *Drosophila* cardioblasts.

- **Changes that had a significant impact on expenditures**.

  Nothing to Report

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

  Nothing to Report

- **Significant changes in use or care of human subjects**.

  Nothing to Report

- **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**
  - **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:	Analyne Schroeder
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-7537-458X
Nearest person month worked:	7.32
Contribution to Project:	Dr. Schroeder has designed and performed the experiments, obtained necessary fly stocks and performed fly husbandry. Collected embryos, troubleshoot scRNA-seq experiments, acquired data and performed data analysis of immunostaining.
Funding Support:	N/A

Name:	Arthur Bautista
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1.12
Contribution to Project:	Assisted with the maintenance of fly stocks, preparation of reagents.
Funding Support:	N/A

Name:	Marco Antonio Tamayo
Project Role:	Lab Coordinator
Researcher Identifier (e.g. ORCID ID):	0000-0001-9891-0755
Nearest person month worked:	2.25
Contribution to Project:	Assisted with the maintenance of fly stocks, embryo collections, preparation of reagents and constructs.
Funding Support:	N/A

- **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?** *Nothing to Report*

- **Organization Name:** *Nothing to Report*

8. **SPECIAL REPORTING REQUIREMENTS**

Nothing to Report

9. **APPENDICES:** *Nothing to Report*