AWARD NUMBER: W81XWH-20-1-0433

TITLE: Novel In Vivo Genetic and Single-Cell Genomic Analyses for Understanding Congenital Heart Disease

PRINCIPAL INVESTIGATOR: Lisa Maves

CONTRACTING ORGANIZATION: SEATTLE CHILDREN'S HOSPITAL
SEATTLE CHILDREN'S RESEARCH INSTITUTE
SEATTLE WA

REPORT DATE: July 2021

TYPE OF REPORT: Annual Technical

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
July 2021	Annual	15Jun2020-14Jun2021

4. TITLE AND SUBTITLE
Novel In Vivo Genetic and Single-Cell Genomic Analyses for Understanding Congenital Heart Disease

5a. CONTRACT NUMBER
W81XWH-20-1-0433

5b. GRANT NUMBER
PR190841

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Lisa Maves

E-Mail: lisa.maves@seattlechildrens.org

5d. PROJECT NUMBER
0011415050

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

SEATTLE CHILDREN'S HOSPITAL
SEATTLE CHILDREN'S RESEARCH INSTITUTE
4800 SAND PT WAY NE
SEATTLE WA 98105-3901

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

This research project addresses the FY19 PRMRP Topic Area Congenital Heart Disease. Congenital heart disease (CHD) is a birth defect that is characterized by improper cardiac development, resulting in the abnormal structure and function of the heart. It is the most common birth defect, affecting approximately 1 percent of the population at varying severities. Understanding the causes of CHD is critical for military personnel and their families because undiagnosed congenital heart defects can impact health and combat effectiveness. It is estimated that previous studies have thus far accounted for only about 10-20 percent of the genetic contribution to CHDs. Thus, many more human CHD genes, likely about 400 genes, await discovery. A major hurdle that remains for understanding the causes of CHD is the identification and validation of the many human CHD genes that are as yet unknown. If a better understanding of the genetic and molecular bases of CHD could be obtained, then advances in genomic technologies now make it feasible to use genomic screening of military personnel and recruits to identify at-risk individuals. The goal of this research project is to provide increased understanding of the genetic and molecular causes of CHD, leading to more accurate diagnoses of CHD. The hypothesis of this proposal is that we can demonstrate functions for a novel set of CHD-candidate genes in heart development, using in vivo functional and genomic assays in the zebrafish animal model.

15. SUBJECT TERMS
None listed.

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	15	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. INTRODUCTION:

Congenital heart disease (CHD) is a birth defect that is characterized by improper cardiac development, resulting in the abnormal structure and function of the heart. It is the most common birth defect, affecting approximately 1 percent of the population at varying severities. It is estimated that previous studies have thus far accounted for only about 10-20 percent of the genetic contribution to CHDs. A major hurdle that remains for understanding the causes of CHD is the identification and validation of the many human CHD genes that are as yet unknown. If a better understanding of the genetic and molecular bases of CHD could be obtained, then advances in genomic technologies now make it feasible to use genomic screening of military personnel and recruits to identify at-risk individuals. The goal of this research project is to provide increased understanding of the genetic and molecular causes of CHD, leading to more accurate diagnoses of CHD. The hypothesis of this project is that we can demonstrate functions for a novel set of CHD-candidate genes in heart development, using in vivo functional and genomic assays in the zebrafish animal model. In Aim 1, we validate functions in heart development for a novel set of 128 human CHD-candidate genes, using in vivo functional screening and characterization in the zebrafish animal model. In Aim 2, we determine whether new CHD genes regulate shared or distinct gene regulatory programs in myocardial cells and other cell types, using single-cell RNA-seq analyses of whole mutant zebrafish embryos. The work proposed takes an innovative approach to discovering and classifying candidate CHD genes.

2. KEYWORDS:

Congenital heart defects, genetics, zebrafish, CRISPR, single-cell RNA-seq

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major Task 1: Screen functions of 128 genes in early zebrafish heart development, using F0 CRISPR screening. Target dates Months 1-3; 30% completion.

Major Task 2: Generate stable mutant strains for genes that show heart phenotypes in Major Task 1 F0 screen. Targets dates Months 3-12; 50% completion.

Major Task 3: Generate single-cell nuclei preps from control and mutant embryos from different classes of heart defect mutant strains. Targets dates Months 13-15; 30% completion.

Major Task 4: Analyze data from sc-RNA-seq experiments. Target dates Months 16-24; 0% completion.

Major Task 5: Preparation and submission of 2 manuscripts for publication. Target date Year 2; 25% completion.

What was accomplished under these goals?

For Major Task 1, the major activities and specific objectives have been to obtain CRISPR guides for 61 of our 128 genes (Subtask 1) and to inject and screen 40 of our 128 genes for heart defects in F0 zebrafish embryos (Subtask 2). For these activities, we synthesized CRISPR guide RNAs in vitro and then injected them into 1-cell-stage zebrafish embryos. Heart development was assessed over the first 5 days of development by imaging myl7-gfp expression in live embryos. The key outcomes are that 13/40 of the genes screened thus far showed defects in heart development. These defects fall into 3 phenotypic classes. These activities thus far are exciting findings for two reasons. First, these results show that about 33% of our candidate genes for heart defects have requirements in zebrafish heart development. Second, these results point to important roles for proteasome genes in heart development and heart defects, since our efforts identified pomp (proteasome maturation factor), psmd6 (proteasome subunit), and psma6 (proteasome subunit) as required for heart development in zebrafish.

For Major Task 2, the major activities and specific objectives have been to generate a stable mutant strain for the zebrafish pomp gene (F3 generation; Fig.1) and to raise germline mutant strains for grpel1 and psmd6 to the F1 generation (Subtask 1). We have also confirmed heart defects and genotyping protocols for the pomp mutant strain (Subtask 2; Fig.1). For these activities, we injected CRISPR guides for each gene, then we used PCR approaches to determine the efficient and active guide RNAs for each gene, then we raised generations of zebrafish carrying mutations for each gene. For the pomp mutant strain, we used genetic sequencing to determine that the mutation is a 6-bp deletion that leads to an early stop mutation (Fig.1). We have used myl7-gfp expression to confirm a heart defect phenotype in the pomp mutant strain (Subtask 3; Fig.1).

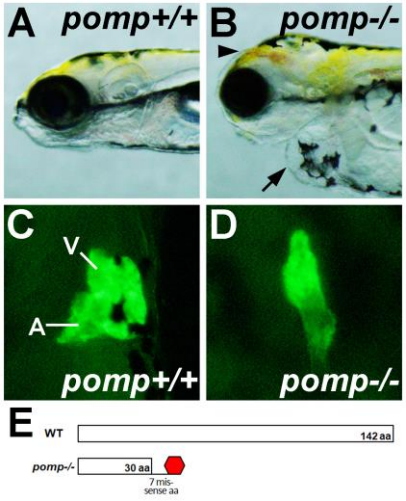

Fig.1. A-B. Control and pomp mutant 4-day-old embryos. Arrow points to pericardial swelling. C-D. myl7-gfp expression in control and pomp mutant hearts in 4-day-old embryos. A, atrium. V, ventricle. E. Cartoon illustrating pomp mutation.

We have also expanded the objectives of Major Task 2 beyond the generation of stable mutant strains that have internal gene mutations; we are now using CRISPR to make mutant strains that carry genetic deletions removing the 5' transcription start site for each gene. We thus far have F1 generation fish carrying 5'end deletions for the pomp and psmd6 genes. These 5'end deletion fish are critical for studies in the zebrafish field in order to confirm null/full knock-out phenotypes for zebrafish genes.

For Major Task 3, the major activities and specific objectives have been to optimize our preparation of single-cell nuclei from whole zebrafish embryos (Subtask 1). This is a critical step in our efforts to do sc-RNA-seq on our mutant zebrafish embryos. We obtained several different nuclei-prep protocols from other zebrafish researchers and we also obtained advice from 10X Genomics scientists. We also optimized this nuclei-prep technique to allow for embryo genotyping, which is another critical step for analyzing our different mutant strains. Although we have not yet progressed as far as we had anticipated with Major Task 3, our protocols now put us in a great position to make progress in Year 2, and to generate data in Year 2 for analysis in Major Task 4.

For Specific Aim 2, to address the objective of determining whether new CHD genes regulate shared or distinct gene regulatory programs, we are taking a new approach in addition to our proposed single-cell RNA-seq analysis. To take advantage of working remotely during the COVID pandemic, we have used the online program STRING to identify protein-protein-interaction (PPI) networks among our candidate genes for heart defects. We identified a sub-network of genes that includes the proteasome family factors (Pomp, Psmd6, Psma6) as well as two genes already known to have roles in heart development (Gli3, Notch1) (Fig.2). These findings suggest that this sub-network of genes might work together and have similar roles in heart development.

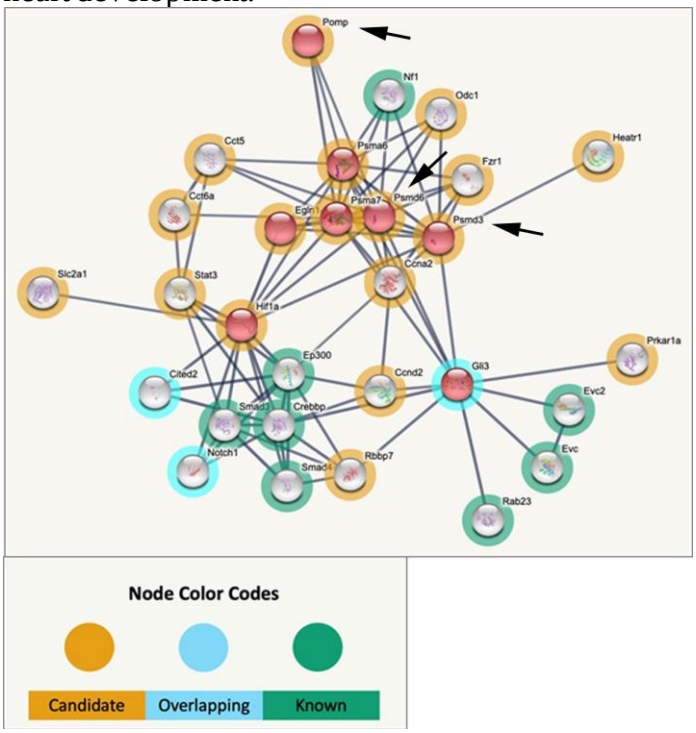

Fig.2. Protein-Protein Interaction (PPI) subnetwork generated using STRING. Using Local Cluster analysis within STRING, we identified members of the subnetwork shown here (from our larger candidate gene network) that are related to transcriptional regulation and Hedgehog 'on' state (indicated by nodes with red centers). The proteins corresponding to known heart defect genes are labeled with green halos. Our candidate genes are labeled with yellow, and those that are shared between both groups ("Overlapping") are blue. Arrows point to proteasome factors Pomp, Psmd6, and Psma6.

For Major Task 5, the major activities and specific objectives have been to prepare a draft of a manuscript for publication. This manuscript will describe our results thus far from Major Tasks 1 and 2, as well as our new PPI network analysis from Specific Aim 2. The preliminary title of the manuscript is "Systems genetics analysis identifies novel roles for proteosome factors in heart development and congenital heart defects".

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

Nothing to Report.

How were the results disseminated to communities of interest?

Nothing to Report.

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

For Specific Aim 1, we plan to complete our CRISPR screen and identify major phenotypic classes of heart development phenotypes. We also plan to engineer stable mutant strains for selected genes within these phenotypic classes.

For Specific Aim 2, we plan to perform single-cell RNA-seq and data analysis on mutant embryos from 2-3 different phenotypic classes from our screen, including from pomp mutant embryos (proteasome gene class).

For Major Task 5, we plan to submit the manuscript currently in preparation in the first half of Year 2 and prepare a second manuscript on the completion of our CRISPR screen in Year 2.

4. IMPACT:

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

Thus far, the significant impact of this project is the advancement of our understanding of the causes of congenital heart defects (CHDs). Our zebrafish CRISPR screening shows that we are identifying several new genes with likely roles in CHDs in humans. We have also identified a new family of genes, proteasome factors, that are required for proper heart development. Our network analysis suggests that these proteasome factors may interact with genes like Notch1 that are already known to cause congenital heart defects. We expect that our studies will advance our understanding of the causes and diagnoses of CHDs.

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

For Specific Aim 2, in order to address the objective of determining whether new CHD genes regulate shared or distinct gene regulatory programs, we are taking a new approach in addition to our proposed single-cell RNA-seq analysis. To take advantage of working remotely during the COVID pandemic, we have used the online program STRING to identify protein-protein-interaction (PPI) networks among our candidate genes for heart defects. This new approach allowed us to identify a novel sub-network of genes might work together and have similar roles in heart development. This approach is thus very fruitful and remains within the scope of our proposed objectives

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

Because of general delays and disruptions during the COVID pandemic, we were not able to make as much progress as originally expected on our CRISPR screening in Specific Aim 1 and on our single-cell RNA-seq studies in Specific Aim 2. We expect to be able to continue progress on these Aims in Year 2.

Changes that had a significant impact on expenditures

Our Animal Per Diem expenditures were about half of what we had projected (about $15k vs $30k). We believe this is due to delays in our CRISPR screening, leading to the generation of fewer new mutant zebrafish strains than anticipated. However, in recent weeks and months we have increased our Animal expenses and we will continue to have increased Animal expenses in Year 2.

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

Not Applicable.

Significant changes in use or care of vertebrate animals

Similar to our Animal Per Diem expenditures (above), our zebrafish Animal use was about one third of what we had projected (about 3,000 fish vs 15,000 fish). We believe this is due to delays in our CRISPR screening, leading to the generation of fewer new mutant zebrafish strains than anticipated.

We had no significant deviations, outcomes, or changes in the care of, or procedures used on, our zebrafish vertebrate animals. Recently (June 2021) we underwent a successful Triennial Review of our SCRI IACUC zebrafish animal protocol. No significant new procedures or modifications were included in the Triennial Review.

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:	Lisa Maves
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-9798-790X
Nearest person month worked:	2
Contribution to Project:	Dr. Maves has performed work in the area of Specific Aim 1 in analyzing CRISPR screening data and generating and maintaining new mutant zebrafish strains and in the area of Specific Aim 2 in obtaining single cell nuclei protocols and analyzing PPI network analyses. Dr. Maves has also worked on preparing a manuscript for publication.
Funding Support:	NIH, Brotman Baty Institute Pilot Grant, Seattle Children's Research Institute

Name: Gist Hank Farr III
Project Role: Research Associate
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 4
Contribution to Project: Mr. Farr has performed work in the area of Specific Aim 1 in preparing CRISPR guides, performing CRISPR screening, analyzing CRISPR screening data, generating genotyping protocols, and generating and maintaining new mutant zebrafish strains. Mr. Farr has performed work in the area of Specific Aim 2 in testing and optimizing single cell nuclei protocols.
Funding Support: NIH, Brotman Baty Institute Pilot Grant, Seattle Children's Research Institute

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

Below 2 previous pending grants have been activated since the beginning of the award.

Title: Three-model platform for advancing DMD epigenetic mechanisms and small molecule therapies
Time Commitment: 35% / 4.2 calendar months (Maves PI)
Funding Agency: NIH
Point of contact: Victoria C Matthews
Performance Period: 2/04/2021 – 1/31/2026
Level of Funding:

Goal: The goals of this proposal are to identify novel epigenetic small molecules that are beneficial for DMD, by demonstrating effectiveness in multiple DMD models, and, in parallel, to better characterize the disrupted transcriptional and epigenetic mechanisms underlying DMD.

Specific Aims: Aim 1: Identify the classes of epigenetic small molecules that improve the DMD phenotype. Aim 2: Validate the effectiveness, and identify potential mechanisms of action, of new epigenetic drugs for DMD. Aim 3: Validate epigenetic drug candidates through analysis in the DMD rat.

Title: Advancing DMD mechanisms and therapies using single-cell sequencing
Time Commitment: 8% / .96 calendar months (Maves PI)
Funding Agency: BBI
Point of contact: Nola Klemfuss
Performance Period: 02/01/2020 – 07/31/2021
Level of Funding:

Goal: The goals of this project are to complete one of the first organism-wide epigenetic and transcriptome maps, comparing normal and DMD mutant zebrafish embryos, and to define the early events of epigenetic and transcriptional dysregulation in two DMD models.

Specific Aims: Aim 1: Characterize the epigenetic and transcriptional perturbations during the initiation of DMD, using dmd zebrafish. Aim 2: Characterize the epigenetic and transcriptional perturbations during the initiation of DMD, using hiPSC-derived DMD skeletal muscle cultures.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

Not Applicable.

9. APPENDICES:

No appendices attached.