AWARD NUMBER: W81XWH-20-1-0165

TITLE: RbFox Genes in Congenital Heart Disease and Cardiomyopathy

PRINCIPAL INVESTIGATOR: Caroline Burns

CONTRACTING ORGANIZATION: Boston Children's Hospital

REPORT DATE: JULY 2023

TYPE OF REPORT: Final Report

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 2023	Final Report	1APR2020 - 31MAR2023

4. TITLE AND SUBTITLE

RbFox Genes in Congenital Heart Disease and Cardiomyopathy

5a. CONTRACT NUMBER
W81XWH-20-1-0165

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Caroline E. Burns, PhD

E-Mail: Caroline.Burns@childrens.harvard.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Children's Hospital Corporation (DBA Boston Children's Hospital
Office of Sponsored Programs
300 Longwood Avenue
Boston, MA 02115

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: Hypoplastic left heart syndrome (HLHS) is a devastating form of congenital heart disease (CHD) that is caused by underdevelopment of the left side of the heart. Whole exome sequencing identified mutations in the RNA splicing factor *Rbfox2* that segregate with HLHS in newborns. While these mutations are likely to be causal, this hypothesis has yet to be tested. Moreover, Rbfox2 has not been linked previously to cardiac development. As such, its mechanism of action is unknown. We created the first clinically relevant zebrafish model of HLHS by mutating the *rbfox* orthologs, *rbfox1l* and *rbfox2*. Specifically, we found that Rbfox double mutant embryos die within 4 days of life from severe cardiovascular abnormalities that mirror HLHS in newborns. While heart development is normal in single mutant zebrafish, progressive heart failure develops in Rbfox2 adults that is lethal by 5 months of age, implicating Rbfox2 as a risk factor for early onset cardiomyopathy. We propose to exploit our unique system over three years to gain new mechanistic insights into the roles of Rbfox in developing and maintaining the heart. In Aim 1, we will study the cardiovascular defects in Rbfox double mutant embryos in more detail and distinguish primary from secondary malformations. In Aim 2, we will study the heart failure observed in Rbfox2 mutant adults. In Aim 3, we will discover the molecular targets of Rbfox to learn how mutations in this gene lead to cardiovascular defects in both our HLHS embryonic model and our adult heart failure model.

15. SUBJECT TERMS

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

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

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

Hypoplastic left heart syndrome (HLHS) is a devastating form of congenital heart disease (CHD) that is caused by underdevelopment of the left side of the heart. Whole exome sequencing identified mutations in the RNA splicing factor *Rbfox2* that segregate with HLHS in newborns. While these mutations are likely to be causal, this hypothesis has yet to be tested. Moreover, Rbfox2 has not been linked previously to cardiac development. As such, its mechanism of action is unknown. We created the first clinically relevant zebrafish model of HLHS by mutating the *rbfox* orthologs, *rbfox1l* and *rbfox2*. Specifically, we found that Rbfox double mutant embryos die within 4 days of life from severe cardiovascular abnormalities that mirror HLHS in newborns. While heart development is normal in single mutant zebrafish, progressive heart failure develops in Rbfox2 adults that is lethal by 5 months of age, implicating Rbfox2 as a risk factor for early onset cardiomyopathy. We propose to exploit our unique system over three years to gain new mechanistic insights into the roles of Rbfox in developing and maintaining the heart. In Aim 1, we will study the cardiovascular defects in Rbfox double mutant embryos in more detail and distinguish primary from secondary malformations. In Aim 2, we will study the heart failure observed in Rbfox2 mutant adults. In Aim 3, we will discover the molecular targets of Rbfox to learn how mutations in this gene lead to cardiovascular defects in both our HLHS embryonic model and our adult heart failure model.

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

Rbfox, RNA binding protein, congenital heart disease, hypoplastic left heart syndrome, HLHS, zebrafish, cardiomyocyte, disease model, heart defect, cardiomyopathy, mitochondrial biogenesis, MICOS

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

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

Appendix 1: SOW with completion dates.

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

Appendix 2: See attached document.

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

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional

development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Professional Training Opportunities:
Mengmeng Huang, PhD: Dr. Huang joined our laboratory in October 2019 as a postdoctoral fellow. She performed the majority of experiments in our manuscript describing a zebrafish *rbfox* model of HLHS. Specific training opportunities included 1) continued mentoring a research technician (Celia Harding) to perform experiments in zebrafish, 2) improving her fund of knowledge by reading the literature surrounding HLHS, 3) attending and presenting twice at our departmental weekly seminar series on cardiovascular development and disease, 4) presenting her data on HLHS to our lab in a weekly lab meeting, 5) giving an oral presentation on her work at the Weinstein Cardiovascular Development and Regeneration conference in Marseille, France, 6) attending, giving an oral presentation, and sitting on an expert panel at the Additional Ventures Single Ventricle Disease conference in Baltimore, MD, 7) and training in grant writing. She has submitted a revision for an NIH K99/R00 Career Transition Award and will apply for an Additional Ventures Catalyst to Independence Award. We have created an Independent Development Plan (IDP) to support her training.
Hui-Min Yin, PhD: Dr. Yin is a postdoctoral fellow in our laboratory that that has benefited from 1:1 training in zebrafish genetics and cardiac development. Specific training opportunities have included 1) receiving 1:1 training by a more senior fellow in the lab to perform experiments in zebrafish, 2) improving her fund of knowledge by reading the literature surrounding cardiomyocyte proliferation, 3) attending and presenting once at our departmental weekly seminar series on cardiovascular development and disease and attending our monthly Longwood Medical Area (LMA) cardiovascular seminar series, 4) presenting her data to our lab in a weekly lab meeting, 5) delivering an oral presentation at the Weinstein Cardiovascular Development and Regeneration conference in San Diego, CA, and 6) training in grant writing for an American Heart Association postdoctoral fellowship application. We have created an Independent Development Plan (IDP) to support her training.
Celia Harding: Ms. Harding is the lab manager and research technician that pursued the adult experiments outlined in Specific Aims 2 and 3 with oversight from Mengmeng Huang. Specific training opportunities have included 1) receiving 1:1 training by Dr. Huang to perform experiments in zebrafish pertaining to Aims 2 and 3, 2) improving her fund of knowledge by reading relevant literature, attending our departmental weekly seminar series on cardiovascular development and disease, and attending our monthly Longwood Medical Area (LMA) cardiovascular seminar series, and 4) presenting her data to our lab in a weekly lab meeting. This past year, Celia applied to graduate school, and after being accepted to many, decided to attend Johns Hopkins.
Alexander Akerberg: Dr. Akerberg, a former senior member of our laboratory, was a co-first author on our manuscript describing a zebrafish rbfox model of HLHS. Specific training opportunities included 1) mentoring Dr. Huang to perform experiments in zebrafish, 2) receiving 1:1 guidance to interpret RNAseq and alternative splicing data from Xiaoran Zhang, 3) improving his fund of knowledge by reading the literature surrounding HLHS, attending and presenting twice at our departmental weekly seminar series on cardiovascular development and disease, and presenting data at our monthly Longwood Medical Area (LMA) cardiovascular seminar series, 4) preparing figures for publication, 5) presenting his data to our lab in a weekly lab meeting, and 6) and training in grant writing. He recently accepted a position as "Stem Cell Senior Research Scientist" at Vertex in Boston, MA.
Warlen Pereira Piedade: Dr. Pereira Piedade is a postdoctoral fellow in our laboratory that that has benefited from 1:1 training in zebrafish genetics and cardiac development. Specific training opportunities have included 1) receiving 1:1 training by more senior fellows in the lab to perform experiments in zebrafish, 2) improving his fund of knowledge by reading the literature surrounding congenital heart disease and HLHS, 3) attending and

presenting once at our departmental weekly seminar series on cardiovascular development and disease and attending our monthly Longwood Medical Area (LMA) cardiovascular seminar series, 4) presenting his data to our lab in a weekly lab meeting, and (5) training in grant writing. Dr. Pereira Piedade was awarded a two year AHA postdoctoral fellowship that began in January 2022. He will be leaving the lab to go back to Brazil to start his own group in Sept. 2023. We have created an Independent Development Plan (IDP) to support his training.

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

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

I gave a seminar at The Rivers School in Weston, MA on what it is like to run a biomedical research lab in academia in February 2022. In addition, we are also hosting 2 high school students in our laboratory over the summer of 2023 for an internship experience.

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

Nothing to Report.

4. IMPACT: D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to report.

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

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

Nothing to report.

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

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

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

Nothing to report.

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

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:
- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

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

Nothing to report.

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

Actual Problem with Specific Aim 3: We encountered issues with either the amount of starting material or with the Rbfox antibodies for the RIPseq and IP/MassSpec. We will be trouble shooting these problems next.

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

Nothing to report.

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

Significant changes in use or care of human subjects

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: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.
  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*
  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Publications during the past year on the work directly under this award:
Huang M^, AkerbergAA^, Zhang X, Yoon H, Joshi S, Nguyen C, Pu WT, Haigis M, Burns CG*, and **Burns CE***. Myocardial-intrinsic defects underlie an Rbox-mediated zebrafish model of hypoplastic left heart syndrome. *Nature Commun. 2022. 13:5877.* ^co-first authors; *co-corresponding authors.

Presentations made during the past year on the work directly under this award:
National:
RNA binding proteins in the heart
MCW Cardiovascular Seminar Series
Medical College of Wisconsin, Milwaukee, WI
October 12, 2022

International:
RNA binding proteins in the heart
Molgen Graduate Student Seminar Series
Molecular Genetics, University of Toronto
October 21, 2022

Local:
Careers in Academic Biomedical Research
The Rivers School
Weston, MA
April 29, 2022

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

Akerberg AA, Trembley M, Butty V, Schwertner A, Zhou L, Beerens M, Liu X, Mahamdeh M, Yuan S, Boyer L, MacRae C, Nguyen C, Pu WT, **Burns CE***, and Burns CG*. RBPMS2 is a conserved regulator of alternative splicing that promotes myofibrillar organization and optimal calcium handling in cardiomyocytes. *Circ Res* 2022. 131: 980 – 1000. *co-corresponding authors.

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

Nothing to report

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

Nothing to report

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

Nothing to report

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

We generated new genetic zebrafish strains that are now publicly available upon request. The lines include: *rbfox1l*^{chb5}, *rbfox2*^{chb6}, *Tg(myl7:rbfox1l)*^{chb7}.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

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

Example:
Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): 12345
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: Caroline Burns
Project Role: PI
Researcher Identifier (ORCID ID): 0000-0003-1565-7489
Nearest person month worked: 3 months
Contribution to Project: Dr. CE Burns has overseen the scientific progress of the funded research program, disseminated information through speaking engagements, co-mentored trainees performing the research, and written and edited drafts of the manuscript describing the funded research. She has also trained Mengmeng Huang, Alex Akerberg, and Warlen Pereira Piedade in grant writing.

Name: C. Geoffrey Burns
Project Role: PI
Researcher Identifier (ORCID ID): 0000-0002-5812-6621
Nearest person month worked: 2 months
Contribution to Project: Dr. CG Burns has jointly overseen the scientific progress of the funded research program, co-mentored the trainees performing the research, and edited drafts of the manuscript describing the funded research.

Name: Mengmeng Huang
Project Role: Postdoctoral Fellow
Researcher Identifier (ORCID ID): 0000-0002-2265-3489
Nearest person month worked: 1
Contribution to Project: Dr. Huang generated the data in our *Nature Commun*. manuscript.

Name: Hui-Min Yin
Project Role: Postdoctoral Fellow
Researcher Identifier (ORCID ID): 0000-0002-4059-9914
Nearest person month worked: 3
Contribution to Project: Dr. Yin has helped to train members of the Burns lab working on the award.

Name: Celia Harding
Project Role: Research Technician
Researcher Identifier (ORCID ID): 0000-0001-8137-7763
Nearest person month worked: 9
Contribution to Project: Ms. Harding has supported the wet bench research and managerial side of this project. She has performed most of the experiments in Appendix 2 with oversight from Dr. Huang.

Name: Xiaoran Zhang
Project Role: Postdoctoral Fellow
Researcher Identifier (ORCID ID): 0000-0003-0979-7100
Nearest person month worked: 1
Contribution to Project: Dr. Zhang has performed the bioinformatic analysis presented in our manuscript.

Name: Hakan Coskun
Project Role: Postdoctoral Fellow
Researcher Identifier (ORCID ID): 0000-0002-7581-3060
Nearest person month worked: 3
Contribution to Project: Dr. Coskun helped with training Celia Harding. He also received training on how to use zebrafish to study and model congenital heart defects, including HLHS. He has also maintained zebrafish lines relevant to this project.

Name: Alexander Akerberg
Project Role: Instructor in Pediatrics
Researcher Identifier (ORCID ID): 0000-0001-9385-3739
Nearest person month worked: 9
Contribution to Project: Dr. Akerberg performed experiments with Dr. Mengmeng Huang and is co-first author on our manuscript.

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

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

New Active Award:

Title: Cardiovascular disease in fetal alcohol spectrum disorder

Major Goals: The long-term goal of this research is to decipher cardiovascular health outcomes in adults with FASD and to identify novel molecular mechanisms and biomarkers for ethanol-induced congenital heart defects and subsequent cardiac dysfunction in adulthood. We propose to accomplish these goals through a retrospective study on adult patients with and without FASD and by evaluation of a zebrafish model of embryonic alcohol exposure.

Status of Support: Active

Project Number: RFA-AA-21-010 , U01 Research Project (Cooperative Agreements)

Name of PI: Caroline Burns, C. Geoffrey Burns

Source of Support: National Institute on Alcohol Abuse and Alcoholism

Dates: 06/01/2022 – 5/51/2027

	Year (YYYY)	Person Months (##.##)
1.	2022	1.1
2.	2023	1.1
3.	2024	1.1
4.	2025	1.1
5.	2026	1.1
6	2027	1.1

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

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

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

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

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Appendix 1: SOW report
Appendix 2: Major Activities

STATEMENT OF WORK – July 11, 2019
PROPOSED START DATE April 1, 2020

Performance Site:	Boston Children's Hospital (BCH) Harvard Medical School Department of Cardiology 300 Longwood Avenue, EN13 Boston, MA 02115
	PI: Caroline E. Burns, PhD

Research-Specific Tasks:	**Proposed Months:**	**Actual Completion**
Specific Aim 1: To test the hypothesis that the *rbfox* DKO ventricular hypoplasia results from cell autonomous deficiencies in cardiomyocyte sarcomere assembly that decrease overall cell size.		
Major Task 1: Organ-wide phenotypic analysis of *rbfox* DKO hearts		
Subtask 1: Quantify ventricular chamber area and myocardial wall thickness in 72 hpf control and DKO embryos.	1-6	Completed, Year 1
Subtask 2: Calculate percent fractional shortening in control and DKO animals.	1-6	Completed, Year 1
Subtask 3: Quantify aortic diameter in 72 hpf control and DKO embryos.	1-6	Completed, Year 1
Subtask 4: Examine valve development in 72hpf control and DKO hearts.	1-6	Completed, Year 1
Subtask 5: Evaluate endocardial fibroelastosis in 72 hpf control and DKO hearts.	1-6	Completed, Year 1
Milestone(s) Achieved: Uncover the full array of cardiovascular phenotypes in DKO hearts that are shared with human HLHS patients at birth.	6	Completed, Year 1
Major Task 2: Phenotypic rescue with wildtype and mutant human *rbfox2*		
Subtask 1: Order wildtype human rbfox2 cDNA from AddGene and *In Vitro* Transcribe mRNA from human *rbfox2* cDNA.	1-6	Completed, Year 1
Subtask 2: Microinject zebrafish embryos from *rbfox1l+/-;rbfox2+/-* incrosses with vehicle control or human *rbfox2* mRNA and quantify ventricular area and aortic diameter.	3-8	Completed, Year 1
Subtask 3: Perform site-directed mutagenesis to create the reported human variants in Rbfox2 that segregate with HLHS in the human rbfox2 cDNA clone. *In Vitro* Transcribe mRNA from human *rbfox2* variant cDNA.	6-12	Completed, Year 1
Subtask 4: Microinject zebrafish embryos from *rbfox1l+/-;rbfox2+/-* incrosses with wildtype or variant human *rbfox2* mRNA and quantify ventricular area and aortic diameter.	12-18	Completed, Year 1
Milestone(s) Achieved: Implicate the human variants that segregate with HLHS in newborns as causal for disease pathogenesis.	18	Completed, Year 1

Major Task 3: Determine cardiomyocyte cell size and the myocardial proliferative index in control and DKO ventricles		
Subtask 1: Analyze cardiomyocyte circularity and area at the arterial pole of the linear heart tube at 24 hpf and in both the inner and outer curvatures of the ventricle at 48 and 72 hpf in control and DKO embryos carrying the *myl7:GFP*.	3-12	Completed, Year 1
Subtask 2: Quantify the number of ventricular cardiomyocytes at 72 hpf in control and DKO hearts.	3-8	Completed, Year 1
Subtask 3: If less cardiomyocytes are observed in DKO ventricles at 72 hpf, then we will determine the ventricular myocardial proliferative index using a standard 48 hpf BrdU pulse and 72 hpf chase.	6-12	Completed, Year 1
Milestone(s) Achieved: Define the cellular mechanism underlying the rbfox-mediated ventricular hypoplasia.	12	Completed, Year 1
Major Task 4: Rbfox1l and Rbfox2 cardiac localization, primary function, and cell-intrinsic roles		
Subtask 1: Localize Rbfox1l and Rbfox2 proteins to specific tissues in the developing heart using double immunohistochemistry.	18-24	Completed, Year 1
Subtask 2: Measure aortic diameter in wildtype and *silent heart* mutants	18-24	Completed, Year 2
Subtask 3: Generate two transgenes where the zebrafish *rbfox2* cDNA is driven off of either the *myl7* or *kdrl* promoter.	6-12	Completed, Year 1
Subtask 3: Inject each transgene into embryos from *rbfox1l+/-* incrosses, raise the babies to adulthood, and screen animals for germline transmission.	6-12	Completed, Year 1
Subtask 3: Cross *rbfox1l+/-;Tg(myl7:rbfox2)*^{MOSAIC} adults with *rbfox2+/-* adults to create double heterozygotes carrying the *myl7:rbfox2* or *kdrl:rbfox2* transgene.	18-24	Completed, Year 1
Subtask 4: Evaluate ventricular chamber area and aortic width in DKO and DKO;transgenic embryos.	24-36	Completed, Year 1
Milestone(s) Achieved: Distinguish primary from secondary phenotypes caused by Rbfox mutations.	36	Completed, Year 1
Specific Aim 2: To test the hypothesis that mutations in Rbfox2 result in cardiomyopathies that are characterized by loss of ventricular muscle mass due to sarcomere destabilization.		
Major Task 1: Organ-wide assessment of *rbfox2-/-* adult hearts		
Subtask 1: Determine ventricle:body weight ratio and atrium:body weight ratio in control and *rbfox2-/-* animals. *Changed to determining body length and body weight for CTRL and *rbfox2-/-* at 3, 7, and 9 months of age.	24-36	Completed, Year 3.
Subtask 2: Stain histological sections from control and *rbfox2-/-* hearts and image.	6-12	Complete, Year 3.
Milestone(s) Achieved: Identification of cardiomyopathy subtype (hypertrophic, dilated, fibrotic) present in rbfox2-/- adults.	12	Complete, Year 3.

Major Task 2: Cellular/Subcellular assessment of *rbfox2-/-* adult ventricles		
Subtask 1: Quantify cardiomyocyte length, width, and area from single cells	18-24	Incomplete.
Subtask 2: Quantify sarcomere integrity in cardiomyocytes from control and *rbfox2-/-* hearts.	18-24	Incomplete.
Subtask 3: Determine cardiomyocyte nucleation and ploidy from control and *rbfox2-/-* hearts.	18-24	Incomplete.
Milestone(s) Achieved: Document whether alterations in cell size, sarcomeric structure, ploidy, or nucleation accompany heart failure in rbfox2-/- adults.	24	Incomplete.
Major Task 3: Mortality Rates and Swim Test Challenge for Control and *rbfox2-/-* adults.		
Subtask 1: Purchase and set up the Loligo Swim Tunnel Respirometer	1-8	Completed, Year 1.
Subtask 2: Genotype animals from three *rbfox2* heterozygous incrosses that produce at least 80 embryos each at 6 weeks of age and divide each group into separate tanks keeping each family separate.	6-12	Completed, Year 3.
Subtask3: Record animal numbers bi-weekly for 3 months to generate a Kaplan-Meier survival graph.	12-24	Completed, Year 3.
Subtask 4: Perform cardiac stress tests on each *Rbfox2* genotypic cohort at 2 months of age and quantify Ucrit values.	15-30	Completed, Year 3.
Milestone(s) Achieved: Identification of the cellular mechanism underlying the rbfox2-mediated cardiomyopathy	30	Completed, Year 3.
Specific Aim 3: To identify cardiomyocyte-specific Rbfox1l and Rbfox2 complex components and RNA targets in zebrafish.		
Major Task 1: Bulk RNA sequencing from embryonic hearts		
Subtask 1: Isolate hearts from 40 wildtype and DKO embryos at 48 hpf. Pool 10 hearts per replicate.	1-3	Completed, Year 1
Subtask 2: Extract RNA from each sample and send to the Harvard Biopolymers Core for quality control, library construction and sequencing (Bulk RNAseq).	3-6	Completed, Year 1
Subtask 3: Bioinformatics analysis of gene expression changes and alternatively spliced transcripts.	6-12	Completed, Year 1
Milestone(s) Achieved: Identification of the gene expression changes and alternatively spliced transcripts in rbfox DKO embryonic hearts.	12	Completed, Year 1
Major Task 2: RIPsequencing from embryonic hearts		
Subtask 1: Isolate hearts from 200 wildtype embryos at 48 hpf.	3-6	Completed, Year 3.

Subtask 2: Perform RIP experiments, isolate RNA, and send to the Harvard Biopolymers Core for quality control, library construction and sequencing.	6-12	Incomplete.
Subtask 3: Bioinformatics analysis of RNA immunoprecipitating with Rbfox1l and Rbfox2.	12-18	Incomplete.
Milestone(s) Achieved: Identification of cardiac RNA targets bound to Rbfox proteins in vivo.	18	36
Major Task 3: IP/MassSpec from embryonic hearts		
Subtask 1: Isolate hearts from 200 wildtype embryos at 48 hpf.	3-6	Completed, Year 3
Subtask 2: Perform IP experiments, run gel, and send unique bands to the MassSpec core for identification.	6-12	Incomplete.
Milestone(s) Achieved: Identification of proteins bound to Rbfox proteins in the embryonic heart.	12	36
Major Task 4: Follow-up Hypothesis-driven Experiments		
Subtask 1: Hyopothesis-driven follow-up experiments will be performed in the embryo based on data gathered in Major Tasks 1-3 in this Aim.	12-36	Completed, Year 2.
Subtask 2: Hypothesis-driven follow-up experiments will be performed in the adult based on data gathered in Subtask 1.	18-36	Incomplete.
Milestone(s) Achieved: Discovery of the molecular mechanism leading to ventricular hypoplasia in rbfox-deficient embryos and potentially ventricular failure in rbfox2-/- adults.	36	36

1. Major **Activities**: The major activities during budget Year 3 included completing Specific Aim 1, part of Specific Aim 2, and part of Specific Aim 3 as proposed in the SOW (see section 3 below), training opportunities for 7 individuals in my lab, four invited talks on the zebrafish HLHS model, and 1 published manuscript on the work described in Aims 1 and 3 regarding our zebrafish model of HLHS.

2. Specific Objectives: Our specific objectives remain unchanged from the original application.

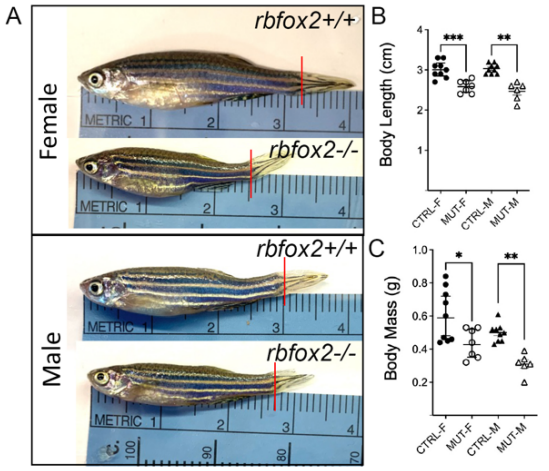
Specific Aim 1. To test the hypothesis that ventricular hypoplasia in *rbfox* DKO embryos results from cell autonomous deficiencies in cardiomyocyte sarcomere assembly that decrease overall cell size. (COMPLETED in Budget Year 1)

Specific Aim 2. To test the hypothesis that mutations in Rbfox2 result in cardiomyopathies that are characterized by loss of ventricular muscle mass due to sarcomere destabilization. (PARTIALLY COMPLETE)

Specific Aim 3. To identify cardiomyocyte specific Rbfox1l and Rbfox2 complex components and RNA targets in zebrafish. (PARTIALLY COMPLETE)

3. Significant Results or Key Outcomes including major findings and conclusions:

Overarching Summary: Over the past year, we have learned that Rbfox2 mutant adults do not reliably develop cardiomyopathy, nor do they show consistent growth reductions or increased mortality rates. It is unclear why, but variations in phenotypic penetrance and expressivity have caused issues with aim completion. In addition, when we did see a statistically significant difference in one read-out, a different read-out would show no difference. Some of the primary data are shown below. We conclude that studying Rbfox2-induced cardiomyopathy is not reliable in the zebrafish model.



the anus (red line). (B,C) Male and female adult zebrafish were measured and weighed at 9 months of age. Graphs depict our findings, which show significant reductions in both parameters in both sexes.

Specific Aim 1 has been completed.

Specific Aim 2 is partially complete.

The major goal of this aim was to test the hypothesis that mutations in *rbfox2* result in cardiomyopathies that are characterized by loss of ventricular muscle mass due to sarcomere destabilization.

MAJOR TASK 1: Organ-wide assessment of *rbfox2-/-* adult hearts.

Subtask 1: We proposed to determine ventricle:body weight ratio and atrium:body weight ratio in control and *rbfox2-/-* animals. This was not feasible with the sensitivity of our analytical scale as zebrafish hearts are extremely small. Therefore, we altered our original plan and quantified body length and body weight in control (CTRL) and *rbfox2-/-* animals at 3 and 7 months of age (See Year 2 report). To summarize, we found that body length and mass were significantly lower in *rbfox2-/-* males and females at 3 and 7 months compared to sibling controls that were raised under the same

conditions. In Year 3, we measured and weighed the same fish again at 9 months prior to a swim test challenge. We found that growth rates remained deficient in *rbfox2* mutant male and female fish (Fig. 1A-C).

Major Conclusion: Rbfox2 is essential for supporting normal growth rates in zebrafish through at least 9 months of age.

Subtask 2: We proposed to stain histological sections from control and *rbfox2-/-* hearts and image. Although sectioning and staining was not performed, the hearts of the 9 month old animals shown in Figure 1 were dissected and imaged so that ventricular area could be determined. If a heart is failing, then we normally see a gross reduction in ventricular size and large expansion of the atrial chamber. Neither were observed in *rbfox2* male or female mutants. While quantitative measures confirmed this for the female cohort, we found a statistically significant reduction in ventricular area for the male population. These data suggested that 9 month old *rbfox2* mutant zebrafish are not reliably developing cardiomyopathy as anticipated.

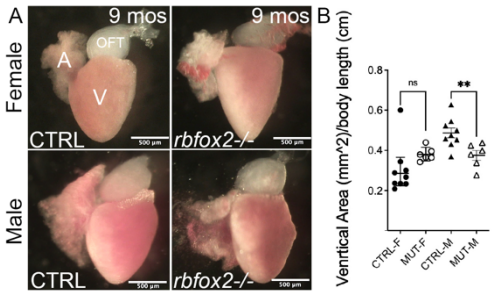


Figure 2. Cardiac areas are not grossly altered in rbfox2 mutants compared to controls at 9 months.

Major Conclusion: Rbfox2 mutant adults survive to 9 months but show relatively normal heart structures with no evidence of cardiomyopathy.

MAJOR TASK 2: Cellular/Subcellular assessment of *rbfox2-/-* adult ventricles. INCOMPLETE. We did not complete Subtasks 1, 2, or 3 because we did not find evidence of cardiomyopathy in 9-month-old *rbfox2* mutant zebrafish as anticipated.

MAJOR TASK 3: Mortality rates and swim test challenge for control and *rbfox-/-* adults

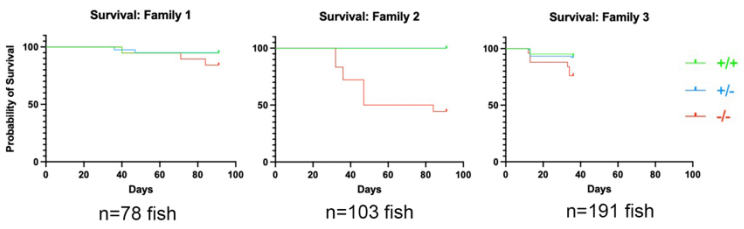


Figure 3. Survival was affected in some rbfox2 mutant families but not all.

Subtasks 2 and 3: We generated Kaplan-Meier curves for 3 different *rbfox2* heterozygous incrosses, called family 1, 2, and 3. We found very different survival data with no difference in mortality between controls and mutants in family 1 at 100 days, and about 50% mortality at 30 days in family 2. Overall, the reduced mortality displayed in some families was not reproducible and could not be studied in a systematic way.

Major Conclusion: Decreased survival was not a fully expressive or penetrant phenotype leading to an inability for further study.

Subtask 4: We proposed to perform swim tunnel challenges on control and *rbfox2* mutants to learn if cardiac function is compromised. As described in the application, we used a swim tunnel challenge and calculated a Ucrit value for each individual. Briefly, Ucrit = $U_f + U_s$ X (t_f/t_s) where U_f is the speed of the water at fatigue, U_s is the water speed step increase (0.05 meters/second), and t_f is the time spent on the last step of the trial (the last flow increase) before fatigue. We normalized the Ucrit values to body length. The lower the Ucrit/body lenth/s, the worse the

animal did in the challenge. We found no significant difference between control and mutant females or males at 3 months (Year 2 report), suggesting that cardiac pump function is NOT severely compromised at this timepoint. We repeated the challenge on these same fish at 9 months of age to learn whether cardiac function decreased over time. We found a statistically significant reduction in exercise capacity in the females but no difference in the males (Fig. 4).

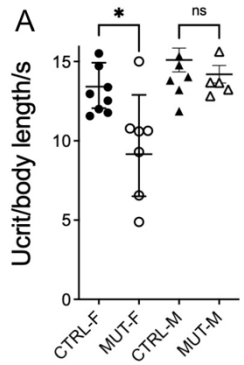


Figure 4. No significant difference in exercise capacity was observed between control and *rbfox2* mutants at 9 months of age.

Major Conclusion: *Rbfox2* females show reduced exercise capacity at 9 months, which is not seen in males.

Specific Aim 3 is partially complete.

Major Task 2: We proposed to perform RIPseq from embryonic hearts to identify RNAs that physically associate with Rbfox1l and Rbfox2 *in vivo*. We have learned that we are unable to isolate enough RNA from 200 wildtype hearts at 48 hpf. Therefore, turned our attention to using adult zebrafish ventricles as our starting material instead of the embryonic hearts. We performed a RIPseq for a different RNA binding protein (RBPMS2) to learn if this method would successfully recover RNA to be sent off for sequencing. This method was successful, so we performed the RIPseq for Rbfox1l and Rbfox2 from adult hearts. Unfortunately, we did not recover sufficient RNA from this approach for sequencing. We plan to troubleshoot the protocol in the future.

Major Task 3: INCOMPLETE.

Major Task 4: We completed subtask 1, but not subtask 2 due to the unreliable nature of the cardiomyopathy phenotype in adult *rbfox2* mutants.

Major Conclusion: The zebrafish model is not idea for finding cardiomyocyte-specific direct RNA targets of RBFOX2. We have moved to the human iPSC-derived cardiomyocyte system to address the molecular underpinnings of the observed cardiac phenotypes.

4. Other Achievements:

Publications:

Akerberg AA^, Trembley M, Butty V, Schwertner A, Zhao L, Beerens M, Liu X, Mahamdeh M, Yuan S, Boyer L, MacRae C, Nguyen C, Pu WT, **Burns CE***, Burns CG*. RBPMS2 Is a Myocardial-Enriched Splicing Regulator Required for Cardiac Function. ***Circ Res.*** 2022. 131:980-1000. * co-corresponding authors. PDF

Huang M^, Akerberg AA^, Zhang X, Yoon H, Joshi S, Hallinan C, Nguyen C, Pu WT, Haigis MC, Burns CG*, **Burns CE***. Intrinsic myocardial defects underlie an Rbfox deficient zebrafish model of hypoplastic left heart syndrome. ***Nat Commun*** 2022. 13: 5877. ^ co-first authors; * co-corresponding authors. PDF