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TITLE: RbFox Genes in Congenital Heart Disease and Cardiomyopathy

PRINCIPAL INVESTIGATOR: Caroline Burns

CONTRACTING ORGANIZATION: Boston Children's Hospital, Boston, MA

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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 None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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1. INTRODUCTION:

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.

2. KEYWORDS:

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

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Appendix 1: SOW with actual completion dates or percentages of completion.

What was accomplished under these goals?

Appendix 2: See attached document.

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

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 have included 1) mentoring a research technician (Celia Harding) to perform experiments in zebrafish, 2) receiving 1:1 guidance to interpret RNAseq and alternative splicing data from Xiaoran Zhang, 3) improving her 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 her HLHS data at our monthly Longwood Medical Area (LMA) cardiovascular seminar series, 4) preparing a manuscript including text and figures for publication, 5) presenting her data on HLHS to our lab in a weekly lab meeting, and 6) and training in grant writing. She is currently supported by an American Heart Association fellowship. 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. We have created an Independent Development Plan (IDP) to support her training.

Celia Harding: Ms. Harding is the lab manager and research technician that is pursuing 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.

Xiaoran Zhang: Dr. Zhang is a trainee in laboratory of Dr. William Pu who is the Chief of our research division in the department of cardiology at BCH. His laboratory is next to mine. Xiaoran performed all the bioinformatics in our manuscript on the role of rbfox in HLHS that is currently in revision. She also received new training in rMATS to perform isoform analysis from RNAseq data from Dr. Vincent Butty at MIT. In addition, she prepared data in figure format for our publication and attended our departmental weekly seminar series on cardiovascular development and disease, and our monthly Longwood Medical Area (LMA) cardiovascular seminar series to improve her fund of knowledge. She has created an IDP with Dr. Pu.

Alexander Akerberg: Dr. Akerberg is a senior member of our laboratory. He was promoted from postdoctoral fellow to Instructor during the budget year. joined our laboratory in October 2019 as a postdoctoral fellow. He has performed some of the experiments in Aim 1 and trained Mengmeng Huang to use zebrafish. He is a co-first author on our manuscript describing a zebrafish rbfox model of HLHS. Specific training opportunities have 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. His postdoctoral fellowship from the AHA ended in January 2022. He submitted a career transitional award to the Additional Ventures research foundation to continue

studying the role of RNA binding proteins in the heart. We have created an Independent Development Plan (IDP) to support his training.

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. We have created an Independent Development Plan (IDP) to support his training.

Katherine Copenhaver: Ms. Copenhaver was our lab manager and research technician. She trained Celia Harding to take over her role as she pursues her MD/PhD from Louisiana State University (LSU).

How were the results disseminated to communities of interest?

I have been contacted by 2 local high schools, Monomoy Regional and Newton North to give a seminar to students interested in STEM careers that will enhance their understanding of what a career in basic science research looks like. I also tend to attract women to these seminars as they are naturally drawn to seeing a woman in science talking about her career trajectory. The Monomoy seminar is scheduled for April 29th. We are finalizing the date of the Newton North seminar for some time in May 2022. In addition to these outreach activities, we are also hosting 3 high school students in our laboratory over the summer of 2022 for an internship experience.

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

In the next funding period (Year 3), we will publish our manuscript detailing the data collected in Years 1 and 2 under Specific Aims 1 and 3. This manuscript is currently in revision at Nature Communications where it received very positive reviews.

In addition, we will complete experiments in Specific Aim 2. We are finalizing our data comparing control to *rbfox2* mutant hearts, which we expect to be complete by November 2022. In addition, we are proposing to analyze the hearts of *rbfox1l+/-;rbfox2-/-* because we think that their cardiomyopathy phenotype might arise earlier than that of *rbfox2-/-* animals. We do not anticipate a problem in finalizing the SOW proposed in Specific Aim 2 by 05/01/2023 (grant end).

We will also concentrate our efforts on specific tasks in Specific Aim 3 as outlined in the SOW. Specifically, we will complete Major Tasks 2, 3, and 50% of 4.

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

It is currently unclear if we will detect differences in exercise capacity between control and *rbfox2-/-* zebrafish at 9 months of age. In addition, we are still in the process of quantifying the metrics proposed in Aim 2 for 9 month old *rbfox2-/-* adult hearts. In order to increase our chances of detecting a strong cardiomyopathy phenotype, we propose to evaluate *rbfox2* mutants prior to 3 months of age and *rbfox11+/-;rbfox2-/-* adults using the same approaches outlined in Aim 2. We do not believe that this constitutes a significant change, but rather a more comprehensive analysis of pre-approved experiments.

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

Actual Problem with Specific Aim 3, Major Task 2 and Major Task 3: RIPsequencing from zebrafish embryonic hearts and IP/Mass Spec from zebrafish embryonic hearts: As described in the application and the SOW, we proposed to identify the RNAs and other proteins that physically associate with Rbfox in the zebrafish embyronic heart. At the time that the application was submitted, we did not know which cardiac cell type (cardiomyocyte or endocardium) we would need to assess. Our studies from Aim 1 have revealed that we need to examine cardiomyocytes as Rbfox proteins are essential in this tissue for heart development. We have also learned that we are not able to generate single cell suspensions from embryonic zebrafish hearts as the cardiomyocytes die when they are dissociated from each other. Moreover, it has become clear that we cannot obtain enough tissue from dissected hearts to successfully perform the RIPsequencing or the IP/Mass Spec.

Action Plan to Resolve the Problem in Budget Year 3: To address these issues, we have turned our attention to adult zebrafish ventricles as the tissue source for our experiments. We recently performed a mock RIP experiment from adult zebrafish ventricles that was successful. We are currently performing RIPs using either Rbfox1l or Rbfox2 antibodies and will send the RNA that comes down with each protein off for sequencing. We anticipate submitting our samples by 07/01/2022. We will also attempt the IP/Mass Spec experiment using both Rbfox1l and Rbfox2 antibodies this summer (2022) from zebrafish adult ventricles.

Changes that had a significant impact on expenditures

Nothing to report.

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

Significant changes in use or care of human subjects

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.
  Books or other non-periodical, one-time publications.

Manuscript in Revision:
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.* ^co-first authors; *co-corresponding authors.

Presentations made during the past year on the work under this award:
Local:
RNA binding proteins in the heart
Beth Israel Deaconess Medical Center Dept. of Cardiology Seminar Series.
Boston, MA
December 9, 2021

RNA binding proteins in the heart
Sterling Drug Visiting Seminar Speaker Series
Dept of Pharmacology and Experimental Therapuetics
Boston University School of Medicine
Boston, MA
April 6, 2022

Modelling Congenital Heart Disease in Zebrafish and human iPSCs
Boston Children's Hospital Stem Cell Day
Webinar
April 14, 2022

Women in STEM careers
STEMinist Club Seminar Series
Monomoy Regional High School
Harwich, MA
April 29, 2022

International: Mengmeng Huang, PhD's abstract on our *rbfox* HLHS zebrafish model was selected for an oral and poster presentation at the Weinstein Cardiovascular Development and Regeneration conference in Marseille, France in May, 2022. Both Dr. Huang and I will attend the conference.

Other publications, conference papers and presentations.

Other publications:
Abrial M^, Basu S^, Huang M, Butty V, Schwertner A, Jeffrey S, Jordan D, **Burns CE*, Burns CG***. Latent TGFb binding proteins 1 and 3 protect the larval zebrafish outflow tract from aneurysmal dilation. ***Disease Models & Mech.*** 2022. 15(3). ^ co-first authors; * co-corresponding authors PDF

Basu S - First Person Interview. ***Disease Models & Mech***. 2022. 15(3). PDF

Sharpe M^, González-Rosa JM^, Wranitz F, Jeffrey S, Copenhaver K, **Burns CG*, Burns CE***. Ruvbl2 suppresses cardiomyocyte proliferation during zebrafish heart development and regeneration. ***Front Cell Dev. Biol.***, 2022, 10:800594. ^ co-first authors; * co-corresponding authors PDF

In Revision:
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. BioRXV* 2021.03.08.434502. *co-corresponding authors.

- **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**

We generated new genetic zebrafish strains that will be made available upon request once our manuscript describing the lines are *in press*. The lines include: *rbfox1l*^{chb5}, *rbfox2*^{chb6}, *Tg(myl7:rbfox1l)*^{chb7}.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Caroline Burns
Project Role:	PI
Researcher Identifier (ORCID ID):	0000-0003-1565-7489
Nearest person month worked:	2 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:	12
Contribution to Project:	Dr. Huang generated the data in our manuscript this is *in revision*.
Funding Support:	AHA postdoctoral fellowship

Name: Hui-Min Yin
Project Role: Postdoctoral Fellow
Researcher Identifier (ORCID ID): 0000-0002-4059-9914
Nearest person month worked: 1
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 that is *in revision.*

Name: Warlen Pereira Piedade
Project Role: Postdoctoral Fellow
Researcher Identifier (ORCID ID): 0000-0002-6080-8701
Nearest person month worked: 8
Contribution to Project: Dr. Pereira Piedade worked with Mengmeng Huang to generate data and helped with training Celia Harding. He received training on how to use zebrafish to study and model HLHS. He has also maintained zebrafish lines relevant to this project.

Name: Katherine Copenhaver
Project Role: Research Technician
Researcher Identifier (ORCID ID): 0000-0001-6336-8708
Nearest person month worked: 1
Contribution to Project: Ms. Copenhaver was our lab manager and research technician. She trained Celia Harding to take over her role as she pursues her MD/PhD from Louisiana State University (LSU).

Name: Alexander Akerberg
Project Role: Instructor in Pediatrics
Researcher Identifier (ORCID ID): 0000-0001-9385-3739
Nearest person month worked: 3
Contribution to Project: Dr. Akerberg performed experiments with Dr. Mengmeng Huang and is co-first author on our manuscript that is *in revision*.

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.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

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.	2-8	Complete, Year 2 *Changed to determining body length and body weight for CTRL and *rbfox2-/-* at 3 and 7 months of age. Will perform on *rbfox1l+/-;rbfox2-/-* in

		Year 3.
Subtask 2: Stain histological sections from control and *rbfox2-/-* hearts and image.	6-12	18-36; 20% complete
Milestone(s) Achieved: Identification of cardiomyopathy subtype (hypertrophic, dilated, fibrotic) present in rbfox2-/- adults.	12	18-36; 20% complete
Major Task 2: Cellular/Subcellular assessment of *rbfox2-/-* adult ventricles		
Subtask 1: Quantify cardiomyocyte length, width, and area from single cells	18-24	24-36 Add: Control and *rbfox1l+/-;rbox2-/-*
Subtask 2: Quantify sarcomere integrity in cardiomyocytes from control and *rbfox2-/-* hearts.	18-24	24-36 Add: Control and *rbfox1l+/-;rbox2-/-*
Subtask 3: Determine cardiomyocyte nucleation and ploidy from control and *rbfox2-/-* hearts.	18-24	24-36 Add: Control and *rbfox1l+/-;rbox2-/-*
Milestone(s) Achieved: Document whether alterations in cell size, sarcomeric structure, ploidy, or nucleation accompany heart failure in rbfox2-/- adults.	24	24-36; 20% complete
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
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	20% Complete. Add *rbfox1l+/-;rbox2-/-* in Year 3.
Subtask3: Record animal numbers bi-weekly for 3 months to generate a Kaplan-Meier survival graph.	12-24	20% complete. Add *rbfox1l+/-;rbox2-/-* in 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 for 3 month old fish. Will perform on the same fish at 9 months old. Will also perform the swim test on 3-4 month old control and *rbfox1l+/-;rbox2-/-* in Year 3.
Milestone(s) Achieved: Identification of the cellular mechanism underlying the rbfox2-mediated cardiomyopathy	30	Ongoing.
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	*Change to adult ventricles.
Subtask 2: Perform RIP experiments, isolate RNA, and send to the Harvard Biopolymers Core for quality control, library construction and sequencing.	6-12	24-36
Subtask 3: Bioinformatics analysis of RNA immunoprecipitating with Rbfox1l and Rbfox2.	12-18	24-36
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	Change to adult ventricles
Subtask 2: Perform IP experiments, run gel, and send unique bands to the MassSpec core for identification.	6-12	24-36
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.
Subtask 2: Hypothesis-driven follow-up experiments will be performed in the adult based on data gathered in Subtask 1.	18-36	24-36
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 2 include 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, 2 published manuscripts and 1 manuscript *in revision* related to other grants and 1 manuscript *in revision* 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, developments, and conclusions:**

   Overarching Summary:

   Over the past year, we have learned that more than half of the predicted number of *rbfox2-/-* animals are missing when we genotype at 3 months. We suspect that this cohort is dying. We have new fish from an rbfox2+/- incross growing and we will monitor their hearts much earlier than anticipated – starting at 2 weeks through 8 weeks of age. Based on the expression pattern of rbfox2, we hypothesize that the mutants are dying from a cardiac or skeletal muscle defect.

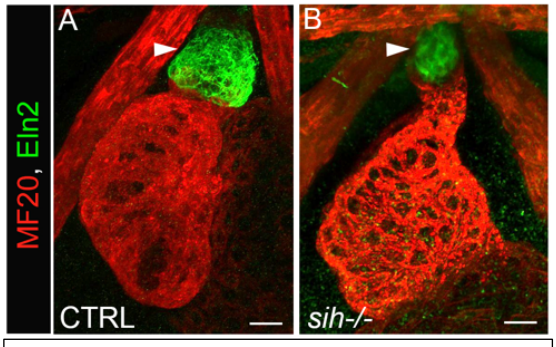

Figure 1. Functional deficits lead to secondary aortic stenosis. (A,B) Confocal projections of 72 hpf control or sih mutant embryos following immunostaining for MF20 (red) or Eln2 (green). White arrowhead shows the base of the aorta, which is more narrow in *sih* than control. Sample Size: n=8 for each group. Scale bar: 20 µm.

We also learned that taking away 1 allele of the *rbfox2* family member, *rbfox1l*, results in much sicker animals that we believe are developing cardiomyopathy with higher penetrance and expressivity. In Year 3, we will finalize our analysis on *rbfox2-/-* hearts and include an analysis of *rbfox1l+/-; rbfox2-/-* adults to increase the likelihood of discovering a genetic condition leading to cardiomyopathy.

Specific Aim 1 has been completed.

MAJOR TASK 4: We analyzed aortic diameter in wild type control and *silent heart (sih)* mutants that don't have a heartbeat due to mutations in a gene that encodes a critical component of the sarcomere. To visualize the heart and the aortic root, we

performed immunofluorescence with antibodies that recognize striated muscle (MF20; red) and smooth muscle of the aorta (Eln2; green). We then qualitatively compared the width of the green signal between cohorts. As hypothesized, we found that the aortic root is stenotic (too narrow) in *sih* mutants (n=8) compared to control siblings (n=8) (Fig. 1A,B).

Major Conclusion: Compromised pump function, which we found is the primary defect in *rbfox* mutants, leads to secondary aortic stenosis.

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 and *rbfox2-/-* animals at 3 and 7 months of age. We found that body length and mass are significantly lower in *rbfox2-/-* males and females at 3 months compared to sibling controls that were raised under the same conditions (Fig. 2A). We analyzed these parameters at 7 months (in the same fish) and that reduced growth persists (Fig. 2B,C). We plan to measure and weigh these fish again at 9 months prior to challenging them in the swim tunnel test. Following completion of the test, we plan to euthanize the animals, embed their hearts in paraffin, and perform histology. We will use ImageJ software to quantify the area of the ventricle and atrium in the section where each chamber appears the largest (near the middle of the heart) and divide by the body mass in order to generate a ventricle size:body weight ratio, which can be compared between cohorts.

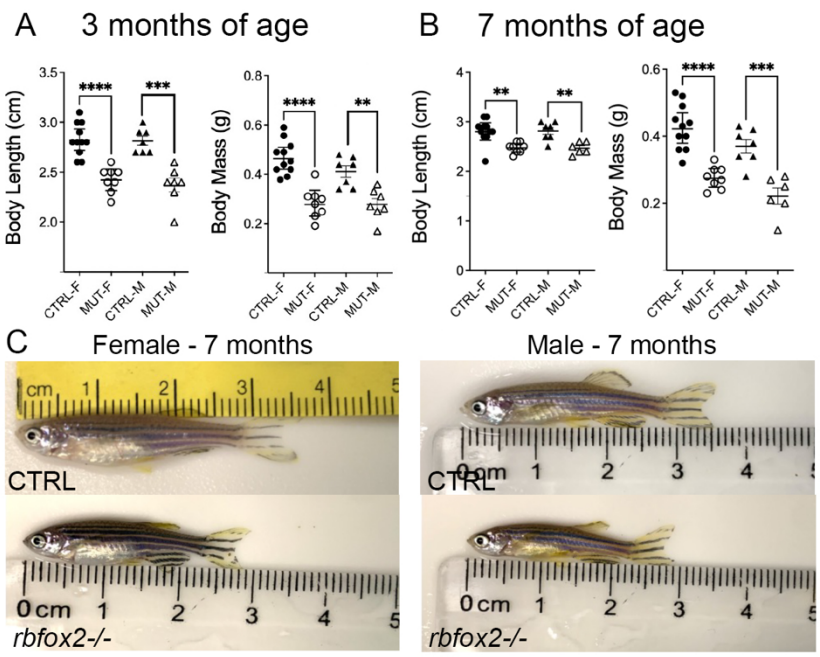

Figure 2. Rbfox2 is required to support normal growth rates in zebrafish. (A,B) Male and female adult zebrafish were measured and weighed at 3 months of age, and then again at 7 months of age. Graphs depict our findings, which show significant reductions in both parameters in male and female zebrafish. (C) Representative photographs of 7 month old control and *rbfox2-/-* zebrafish.

Major Conclusion: Rbfox2 is essential for supporting normal growth rates in zebrafish. We are working to determine if the ventricle size:body size and atrium size:body size ratios are altered in *rbfox2* mutant adults at 9 months of age.

Subtask 2: We proposed to stain histological sections from control and *rbfox2-/-* hearts and image. We have imaged sections from 2 control and 2 *rbfox2* mutants at 4 months. We find that the ventricles appear smaller in size, which can be indicative of heart failure (Fig. 3 A,B). However, the sample size is small, chamber size has not been normalized to body weight, and sex has not been analyzed. We plan to use the adults from the experiment shown in Figure 2 at 9 months of age to do the following: (1) conduct a swim test challenge, (2) measure their body length and mass, (3) euthanize the animals and dissect their hearts for histology where chamber area will be normalized to body weight for both males and females. We have decided to terminate the experiment at 9 months.

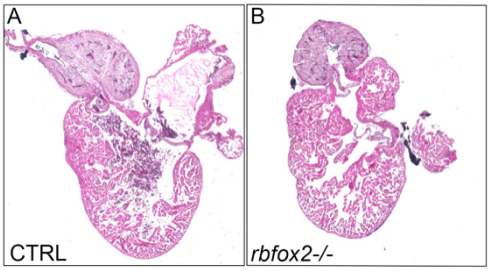

Figure 3. Histological sections of control and *rbfox2-/-* hearts at 4 months of age.

Major Conclusion: We cannot yet conclude whether the *rbfox2* mutants that survive to 9 months develop a cardiomyopathy. See below regarding those that succumb prior to 9 months.

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

Subtasks 2 and 3: We proposed to genotype animals from *rbfox2* heterozygous incrosses at 6 weeks of age and generate a Kaplan-Meier survival graph by monitoring survival biweekly. Because the animals were very small at 6 weeks of age, we waited until 12 weeks (3 months) to genotype. We found that *rbfox2* mutants are underrepresented at 3 months of age. Specifically, we observed 6 mutants of 53 total animals (11.3%) instead of the expected 13 animals (25%) according to Mendelian genetics. In a separate family, we similarly observed 27 mutants of 172 total animals instead of the expected 43 (25%). Therefore, we conclude that roughly half of *rbfox2-/-* animals are dying prior to 3 months. We have now set up a new experiment where we will be able to generate a bona fide Kaplan Meier graph based on the following: we know that *rbfox2* mutants are much smaller than their siblings at 3-4 weeks of age. We will separate these fish from the larger sibling controls and track survival. We will perform confirmation genotyping when the animals are large enough (hopefully by 8 weeks/2 months) and carry the experiment to 9 months.

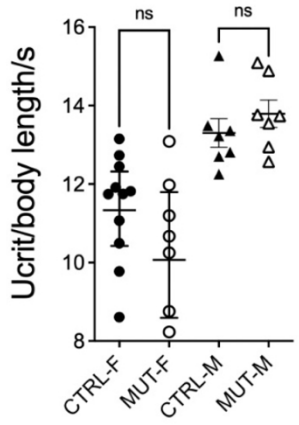

Figure 4. No significant difference in exercise capacity was observed between control and rbfox2 mutants at 3 months of age.

Major Conclusion: Roughly half of *rbfox2* mutants are dying prior to 3 months of age.

Subtask 4: We proposed to perform swim tunnel challenges on 3 month old 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 (Fig. 4), suggesting that cardiac pump function is NOT severely compromised at 3 months of age. We will repeat the challenge on these same fish at 9 months of age to learn whether cardiac function decreased over time. The biggest caveat with this experiment is that we inadvertently selected the healthiest *rbfox2* mutants as roughly half of the mutant population died before the test was administered. Based on what we learn from our survival analysis (when do the most affected mutants die?), we will determine whether an earlier test can be administered or whether we are confined to the older population that is arguably healthier.

Major Conclusion: *Rbfox2* mutants show no significant difference in exercise capacity compared to their control siblings at 3 months of age.

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. We have 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 are now performing the RIPseq for Rbfox1l and Rbfox2 from adult hearts.

Major Conclusion: We have validated that the RIPseq and IP/MassSpec for Rbfox1l and Rbfox2 RNA targets and complex members is feasible from adult zebrafish ventricles.

4. Other Achievements:

Publications:

Abrial M^, Basu S^, Huang M, Butty V, Schwertner A, Jeffrey S, Jordan D, **Burns CE*, Burns CG***. Latent TGFb binding proteins 1 and 3 protect the larval zebrafish outflow tract from aneurysmal dilation. ***Disease Models & Mech.*** 2022. 15(3). ^ co-first authors; * co-corresponding authors PDF

- Basu S - First Person Interview. ***Disease Models & Mech***. 2022. 15(3). PDF

Sharpe M^, González-Rosa JM^, Wranitz F, Jeffrey S, Copenhaver K, **Burns CG*, Burns CE***. Ruvbl2 suppresses cardiomyocyte proliferation during zebrafish heart development and regeneration. ***Front Cell Dev. Biol.***, 2022, 10:800594. ^ co-first authors; * co-corresponding authors PDF

Manuscripts in Revision:

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. BioRXV* 2021.03.08.434502. *co-corresponding authors.

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.* ^co-first authors; *co-corresponding authors.