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PRINCIPAL INVESTIGATOR: Caroline Burns

CONTRACTING ORGANIZATION: Boston Children’s Hospital, Boston, MD

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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.

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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.

KEYWORDS:

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

2. 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 has performed the majority of experiments shown in Appendix 2. Specific training opportunities have included 1) receiving 1:1 training by a more senior fellow in the lab 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 Grand Rounds at BCH when the topic is HLHS, attending and presenting twice at our departmental weekly seminar series on cardiovascular development and disease, and attending 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 applied for and was recently awarded an American Heart Association fellowship to support her stipend from spring 2021-2023. 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 HLHS, attending Grand Rounds at BCH when the topic is HLHS, 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) preparing and publishing a review article on cardiomyocyte proliferation, 5) presenting her data to our lab in a weekly lab meeting, and 6) attending the International Zebrafish Society and Weinstein Cardiovascular Development and Regeneration conference webinars. We have created an Independent Development Plan (IDP) to support her training.
Katherine Copenhaver: Ms. Copenhaver is the lab manager and research technician that supported efforts pertaining to this grant application. 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 relevant literature, attending Grand Rounds at BCH when the topic is congenital heart disease, 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. She applied to and was accepted into the MD/PhD program at Louisiana State University where she will matriculate this summer (2021).
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 has performed all of the bioinformatics presented in Appendix 2. She has also received new training in rMATS to perform isoform analysis from RNAseq data from Dr. Vincent Butty at MIT. In addition, she has prepared data in figure format for 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.

How were the results disseminated to communities of interest?

Oral research seminars were given by me (Caroline Burns) to communities of interest over the past budget year (Year 1):

Modeling HLHS in zebrafish
Medical College of Wisconsin, Departmental Seminar Series
Milwauke, WI
Postponed because of COVID-19; April 8, 2020

Modeling HLHS in zebrafish
Beth Israel Deaconess Medical Center Dept. of Cardiology Seminar Series.
Boston, MA
Postponed because of COVID-19; June 5, 2020

Mechanisms of Cardiovascular Development, Regeneration, and Disease
Developmental & Regenerative Biology (DRB) Program
Harvard Medical School BBS Program Student/Faculty Seminar Series
Boston, MA, March 8, 2021

Modeling HLHS in Zebrafish
Extenal Scientific Advisory Committee Review
Department of Cardiology, Boston Children's Hospital
Boston, MA, April 15, 2021

Modeling Cardiovascular Disease in Zebrafish
Grand Rounds
Department of Cardiology, Boston Children's Hospital
Boston, MA, April 16, 2021

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

In the next funding period (Year 2), we will submit our data from budget year 1 for publication. In addition, we will perform experiments in Specific Aim 2, Major Task 1, Major Task 2, and Major Task 3 (Subtasks 2 and 3 and potentially Subtask 4). We will concentrate the major of our efforts on Specific Aim 3 to uncover the molecular mechanisms underlying the cardiovascular phenotypes in rbfox DKO mutants that were documented in Specific Aim 1 during this past funding year. We will work with DoD scientific officer Jonathan Ryder to learn whether we can add work in human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) to our program so that we might better accomplish Major Task 2 and 3 as outlined in Specific Aim 2 (see SOW). For more details, see section 5 below regarding CHANGES/PROBLEMS. In addition, we will perform hypothesis driven experiments in zebrafish to learn how alterations in alternative splicing of the MICOS complex members, MIC25 and MIC19, impact mitochondrial function and ATP production. We will also determine whether the mitochondrial biogenesis deficiency is responsible for the sarcomere assembly issues, or whether these 2 subcellular phentoypes arise independent from one another due to faulty splicing of transcripts encoding both MICOS (mitochondrial) and actin cytoskeletal components such as TPM1, TPM3, and PDLIM5B. If granted permission by the DoD to pursue work in human iPSC-CMs, then we will also perform RNAsequencing on control and Rbfox2 knock-out CMs and analyze the dataset for alterations in gene expression and in alternative splicing. We will then cross-reference this human analysis with our zebrafish analysis to learn which changes are most highly conserved, as these are the most likely to be important for cardiomyocyte development and function.

4. IMPACT:

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

I recently gave an oral presentation for Grand Rounds to the Department of Cardiology at Boston Children's Hospital (BCH; April 16, 2021). Most of the attendees were BCH clinicians that see many HLHS patients each year and perform their single ventricular surgeries. The clinicians in the audience were extremely interested in and impressed by our zebrafish Rbfox HLHS model because our studies suggest that HLHS can arise from a primary defect in myocardial growth. This finding in zebrafish also suggests that the same is true in humans. We speculate that this subgroup of HLHS patients is more likely to have heart failure after single ventricular surgery or succumb to sudden cardiac death (poor clinical outcome). We also speculate that not all HLHS is caused by a primary myocardial issue as there is a different cohort of patients that do very well after single ventricular surgery (better clinical outcome). Sharing our data with the clinical side of BCH has led us to begin talking with Dr. Ram Emani, a lead surgeon that performs the HLHS surgeries, about biobanking HLHS heart tissue for future studies that could more accurately predict clinical outcomes after single ventricle surgery. Although we are only in a planning phase, our mission is to ultimately impact patient lives with our findings.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report.

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

Anticipated 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 embryonic 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, the IP/Mass Spec, or any metabolic profiling assays that have become increasingly important based on our findings in Specific Aim 3, Major Task 1. In addition to these limitations in zebrafish, we have become highly interested in learning whether Rbfox2 function is conserved in human cardiomyocytes for myofibril assembly and mitochondrial biogenesis like we found in zebrafish.

Action Plan to Resolve the Problem in Budget Year 2: To address these issues, we propose to use human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) to study the cell-autonomous roles of Rbfox2 and relate our findings to HLHS. Specifically, we propose to use CRISPR-Cas9 genome editing to create deleterious mutations in both alleles of human Rbfox2, isolate single clones, and differentiate 2 distinct clones into cardiomyocytes along with control "wild-type" iPSCs. We propose to stain the iPSC-CMs with antibodies to detect sarcomere assembly and dyes to assess mitochondrial biogenesis, mitochondrial inner membrane potential, and calcium transients. We also propose to analyze control and *rbfox2* knock-out iPSC-CMs by transmission electron microscopy to assess mitochondrial morphology and sarcomere organization. In addition, we propose to perform metabolomics on the control and *rbfox2* knock-out iPSC-CMs using an Agilent Seahorse instrument and a MassSpec to learn which metabolites might be low and whether ATP levels are decreased as anticipated. **Prior to any of these proposed ideas being implemented, we will work with science officer Jonathan Ryder to go through the proper channels of approval.**

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.

Yin HM, Burns CG*, **Burns CE***. *Innate Mechanisms of Heart Regeneration*. Cold Spring Harbor perspectives. (2021) *in press*. (This review is presented as a single chapter in the CSH monolith series titled "*Regeneration*") Acknowledgement of federal support – yes.

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**

We have 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. Burns has overseen the scientific progress of funded research program, disseminated the information by speaking engagements, co-mentored the trainees performing the research, and written and edited drafts of the manuscript describing the funded research. She has also trained Mengmeng Huang in grant writing by helping her with her American Heart Association fellowship application.

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 has generated the data presented in Appendix 2.

Name: Hui-Min Yin
Project Role: Postdoctoral Fellow
Researcher Identifier (ORCID ID): 0000-0002-4059-9914
Nearest person month worked: 12
Contribution to Project: Dr. Yin has helped to generate the data presented in Appendix 2 and has written and published a review article on cardiomyocyte proliferation.

Name: Katherine Cophenhaver
Project Role: Research Technician
Researcher Identifier (ORCID ID): 0000-0001-6336-8708
Nearest person month worked: 6
Contribution to Project: Ms. Copenhaver has supported the wet bench research and managerial side of this project.

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 all of the bioinformatic analysis as described in Appendix 2.

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

APPENDIX 1

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
Subtask 2: Calculate percent fractional shortening in control and DKO animals.	1-6	Completed
Subtask 3: Quantify aortic diameter in 72 hpf control and DKO embryos.	1-6	Completed
Subtask 4: Examine valve development in 72hpf control and DKO hearts.	1-6	Completed
Subtask 5: Evaluate endocardial fibroelastosis in 72 hpf control and DKO hearts.	1-6	Completed
Milestone(s) Achieved: Uncover the full array of cardiovascular phenotypes in DKO hearts that are shared with human HLHS patients at birth.	6	Completed
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
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
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
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
Milestone(s) Achieved: Implicate the human variants that segregate with HLHS in newborns as causal for disease pathogenesis.	18	Completed
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

Subtask 2: Quantify the number of ventricular cardiomyocytes at 72 hpf in control and DKO hearts.	3-8	Completed
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
Milestone(s) Achieved: Define the cellular mechanism underlying the rbfox-mediated ventricular hypoplasia.	12	Completed
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
Subtask 2: Measure aortic diameter in wildtype and *silent heart* mutants	18-24	18-24
Subtask 3: Generate two transgenes where the zebrafish *rbfox2* cDNA is driven off of either the *myl7* or *kdrl* promoter.	6-12	We used *rbfox1l* instead of *rbfox2*. We generated the *myl7:rbfox1l* transgene. We do not need to generate the *kdrl:rbfox1l* line.
Subtask 3: Inject each transgene into embryos from *rbfox1l+/-* incrosses, raise the babies to adulthood, and screen animals for germline transmission.	6-12	Completed.
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 for *myl7:rbfox1l.*
Subtask 4: Evaluate ventricular chamber area and aortic width in DKO and DKO;transgenic embryos.	24-36	We quantified ventricular function and aortic width instead of ventricular area (complete).
Milestone(s) Achieved: Distinguish primary from secondary phenotypes caused by Rbfox mutations.	36	Complete
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	Change: 18-24 months
Subtask 2: Stain histological sections from control and *rbfox2-/-* hearts and image.	6-12	Change: 18-24 months
Milestone(s) Achieved: Identification of cardiomyopathy subtype (hypertrophic, dilated, fibrotic) present in rbfox2-/- adults.	12	Change: 18-24 months
Major Task 2: Cellular/Subcellular assessment of *rbfox2-/-* adult ventricles		
Subtask 1: Quantify cardiomyocyte length, width, and area from single cells	18-24	18-24
Subtask 2: Quantify sarcomere integrity in cardiomyocytes from control and *rbfox2-/-* hearts.	18-24	18-24
Subtask 3: Determine cardiomyocyte nucleation and ploidy from control and *rbfox2-/-* hearts.	18-24	18-24

Milestone(s) Achieved: Document whether alterations in cell size, sarcomeric structure, ploidy, or nucleation accompany heart failure in rbfox2-/- adults.	24	24
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	Complete – we have a swim tunnel that we can use so we do not have to purchase the Loligo
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	Change: 18-24 months
Subtask3: Record animal numbers bi-weekly for 3 months to generate a Kaplan-Meier survival graph.	12-24	Change: 18-24 months
Subtask 4: Perform cardiac stress tests on each *Rbfox2* genotypic cohort at 2 months of age and quantify Ucrit values.	15-30	15-30
Milestone(s) Achieved: Identification of the cellular mechanism underlying the rbfox2-mediated cardiomyopathy	30	30
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	Complete
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	Complete
Subtask 3: Bioinformatics analysis of gene expression changes and alternatively spliced transcripts.	6-12	Complete
Milestone(s) Achieved: Identification of the gene expression changes and alternatively spliced transcripts in rbfox DKO embryonic hearts.	12	Complete
Major Task 2: RIPsequencing from embryonic hearts		
Subtask 1: Isolate hearts from 200 wildtype embryos at 48 hpf.	3-6	Change: 24-36
Subtask 2: Perform RIP experiments, isolate RNA, and send to the Harvard Biopolymers Core for quality control, library construction and sequencing.	6-12	Change: 24-36
Subtask 3: Bioinformatics analysis of RNA immunoprecipitating with Rbfox1l and Rbfox2.	12-18	Change: 24-36
Milestone(s) Achieved: Identification of cardiac RNA targets bound to Rbfox proteins in vivo.	18	Change: 36
Major Task 3: IP/MassSpec from embryonic hearts		
Subtask 1: Isolate hearts from 200 wildtype embryos at 48 hpf.	3-6	Change: 24-36
Subtask 2: Perform IP experiments, run gel, and send unique bands to the MassSpec core for identification.	6-12	Change: 24-36
Milestone(s) Achieved: Identification of proteins bound to Rbfox proteins in the embryonic heart.	12	Change: 24-36
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	12-36: 30% complete
Subtask 2: Hypothesis-driven follow-up experiments will be performed in the adult based on data gathered in Subtask 1.	18-36	18-36
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 1 include completing Specific Aim 1 and part of Specific Aim 3 as proposed in the SOW (see section 3 below), training opportunities for 4 trainees, five invited talks (only 3 delivered due to COVID-19) on the zebrafish HLHS model, 1 review that is *in press*, and 1 manuscript that is in preparation for publication.

2. **Specific Objectives:** Our specific objectives remain unchanged from the original application. They include the following specific aims:

   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. (COMPLETE)

   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. (INCOMPLETE)

   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:**

   Specific Aim 1 has been completed.

   MAJOR TASK 1: We performed an organwide phenotypic analysis of *rbfox* DKO hearts at 72 hpf (the latest stage possible in this cohort due to morbidity) to uncover the full array of cardiovascular phenotypes shared with human HLHS patients at birth (Fig. 1). Specifically, we analyzed ventricular and atrial chamber area during diastole and systole and pump function of each chamber by % fractional area change (FAC). We also analyzed aortic diameter, the length of the myocardial outflow tract, and aorta and valve formation. First, short videos captured by live imaging of GFP-expressing hearts over successive cardiac cycles were used to extract still frames of control and DKO ventricular and atrial chambers during diastole and systole (Fig. 1A,B). These still frames were then used to quantify ventricular

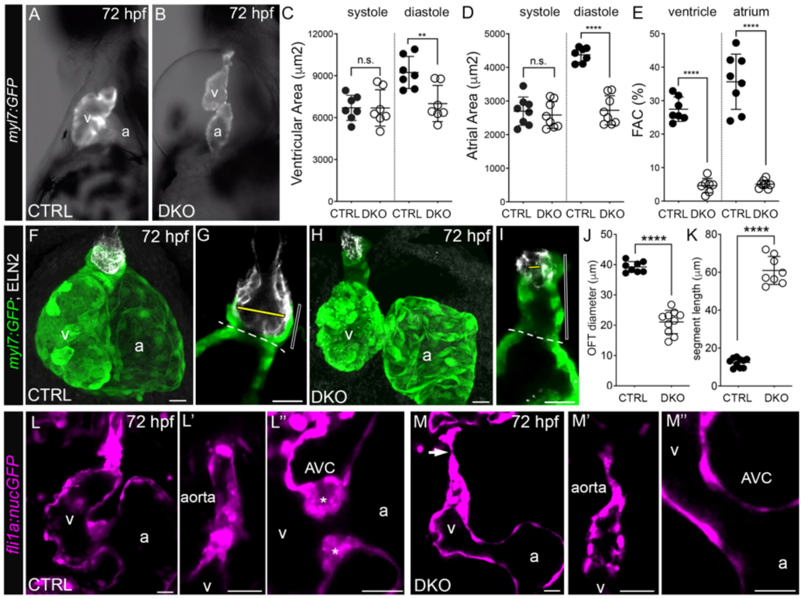

Figure 1. Zebrafish embryos deficient for Rbfox1l and Rbfox2 display cardiovascular phenotypes that mirror those seen in HLHS patients. See text for full description of panels. Abbr: CTRL, control; DKO, rbfox double knock out, AVC, atrioventricular canal.

and atrial areas. Although chamber areas are similar between cohorts during systole, a 24% reduction was observed in DKO hearts during diastole (Fig. 1C,D), demonstrating non-compliance of the chamber walls. To estimate contractile function, these area measurements were used to calculate %FAC, which approached 0 in DKO ventricular and atrial chambers (Fig. 1E). **Major Conclusion:** Rbfox proteins are required for cardiac pump function.

Next, max projections of confocal images of control and DKO hearts carrying the *Tg(myl7:GFP)* reporter following immunostaining for GFP and Elastin2b (ELN2), a marker of smooth muscle cells in the OFT, confirmed the presence of diminutive ventricles with abnormal myocardial segments that are 41% narrower and 80% longer in DKO compared to controls (Fig. 2F-K). The restriction of ELN2+ smooth muscle cells to the distal-most region of the myocardial segment (Fig. 2H,I) suggests that this relatively small portion represents the OFT while the adjacent section is the second heart field-derived ventricle that failed to balloon during chamber formation. Finally, single optical sections of hearts carrying the *Tg(fli1a:GFP)* endothelial reporter revealed that the aorta is stenotic in DKO embryos and often appears completely closed (Fig. 1L,L',M,M'). In addition, endocardial cushions, which are the precursors to the valves, form in the atrioventricular canal (AVC) of control hearts (Fig. 2L'' – see asterisks). However, these cushions are lacking in DKO (Fig. 1M''), demonstrating defective valve formation in the absence of Rbfox1l and 2. We did not observe a thickening of the endocardium that is indicative of endocardial fibroelastosis, suggesting that this is a relatively late manifestation of altered morphology in human HLHS hearts. **Major Conclusion:** Zebrafish *rbfox1l;rbfox2* DKO hearts show a similar array of cardiovascular phenotypes as observed in HLHS patients at birth.

MAJOR TASK 2: We established that *rbfox2* variants that segregate with HLHS in newborns fail to rescue the cardiovascular defects observed in *rbfox* DKO zebrafish. Specifically, we confirmed that injection of mRNA encoding human RBFOX2 is capable of rescuing ventricular fractional area change (pump function) in zebrafish DKO embryos (Fig. 2A-D). In addition, we created the same mutations observed in 3 HLHS probands as reported in Homsey et al. in the human *rbfox2* cDNA (Homsey et al., Science 2015). We then performed in vitro transcription, isolated the mRNA, and injected it into embryos. We found that %FAC was not changed or rescued in DKO embryos injected with the human variant *rbfox2* compared to uninjected DKO embryos (Fig. 2D). **Major Conclusion**: Our data suggest that the molecular targets of Rbfox proteins are conserved between zebrafish and human and that anything we learn in the fish will be relevant to human cardiovascular biology. Our data also

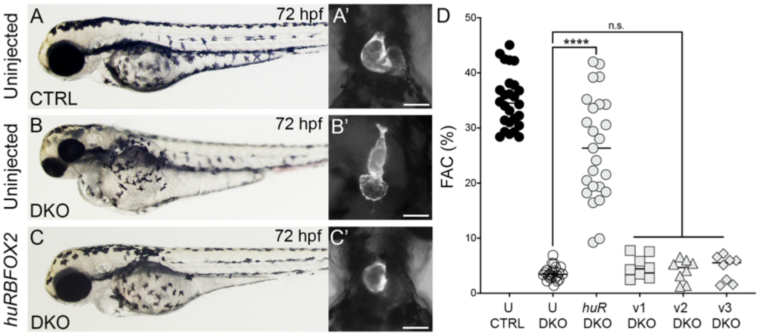

Figure 2. Human Rbfox2 rescues the cardiovascular defects observed in zebrafish rbfox mutants, while the human variants associated with HLHS do not. (A-C) Brightfield images of uninjected control, uninjected DKO, and human Rbfox2 injected DKO animals carrying the *myl7:GFP* myocardial reporter. (A',B',C') Ventral view of hearts expressing GFP are shown for each cohort. (D) Quantification of %FAC with "v1, v2, and v3" representing the 3 Rbfox2 variants reported by Homsey et al.

suggest that the three Rbfox2 variants reported by Homsey et al. are causal for HLHS pathogenesis.

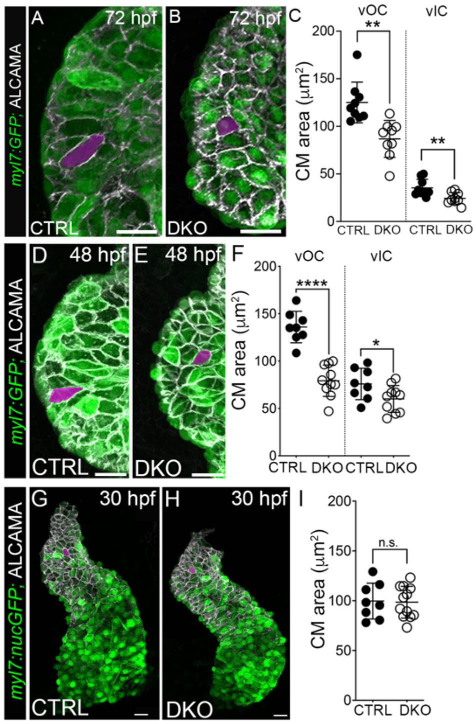

Figure. 3. Cardiomyocyte area is reduced in DKO animals. (A,B,D,E) Magnified view of the outer curvature of the ventricle. Representative cardiomyocytes are highlighted in pink. (C,F) Quantification of cardiomyocyte area in the vOC and vIC. (G,H) Confocal max projection of linear heart tubes at 30 hpf. Representative cardiomyocytes are highlighted in pink. (I) Quantification of cardiomyocyte area. Abbr: vOC, ventricular outer curvature; vIC, ventricular inner curvature.

<u>MAJOR TASK 3:</u> We determined the <u>mechanism(s) underlying the observed ventricular hypoplasia observed at 48 and 72 hpf in DKO embryos</u> (Fig. 1F,H). To quantify cardiomyocyte area, we first fixed animals at 30, 48, and 72 hpf and performed immunohistochemistry for GFP to mark cardiomyocytes and anti-ALCAMA (also called ZN-8) to mark cell borders and imaged the hearts by confocal microscopy. We used Fiji software to determine the area of individual cardiomyocytes on Z-stack merged projections. We found that cardiomyocytes in the outer and inner curvature of the 48 and 72 hpf ventricular chamber are significantly smaller in DKO than in control (Fig. 3A-F). However, no difference in cardiomyocyte area was observed at 30 hpf (Fig. 3G-I).

We showed in the original grant application that there is no change in cardiomyocyte number between control and DKO hearts at 30 and 48 hpf. We quantified cardiomyocyte numbers in the ventricular chamber of 72 hpf control and DKO embryos. Between 48 and 72 hpf, the heart grows by proliferation with an increase of ~30 cardiomyocytes on average in the ventricle. Although not statistically significant due to variability, there are 18 fewer cardiomyocytes on average in DKO ventricles compared to controls (Fig. 4A-C). To learn if this deficit is caused by less proliferation, we quantified the myocardial proliferative index using a standard 48 hpf BrdU pulse and 72 hpf chase. We fixed BrdU-pulsed control and DKO embryos carrying the *myl7:nucGFP* transgene at 72 hpf, performed immunohistochemistry for GFP and BrdU, and imaged the hearts by confocal microscopy. We quantified the myocardial proliferative index by determining the fraction of GFP+; BrdU+ double positive cells from the total GFP+ population in the ventricle. We found that DKO hearts fail to proliferate compared to controls (Fig. 4D-F). **Major Conclusion:** These data reveal that defective cardiomyocyte growth in

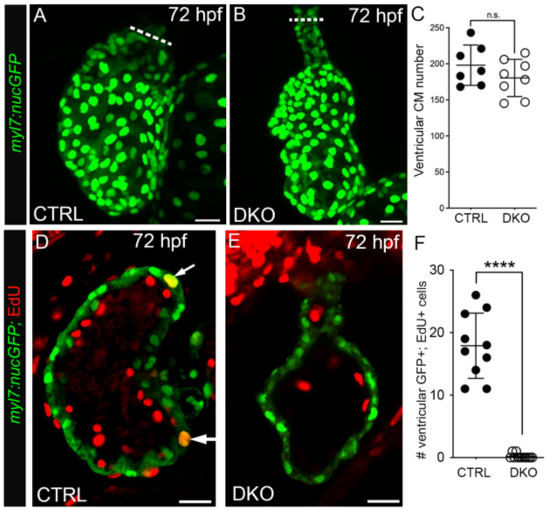

Figure 4. DKO ventricles have fewer cardiomyocytes and fail to proliferate. See text for explanation.

DKO animals initially accounts for the smaller ventricular chamber size at 48 hpf, a phenotype that is later exacerbated by a proliferation deficiency at 72 hpf.

MAJOR TASK 4: We discerned primary from secondary phenotypes caused by Rbfox mutations. First, we proposed a more comprehensive expression analysis of Rbfox1l and Rbfox2 in conjunction with cardiomyocyte and endocardial markers. To gain spatial resolution into the cardiac expression of *rbfox1l* and *rbfox2*, we immunostained double transgenic embryos harboring the myocardial *Tg(myl7:GFP)* and endothelial *Tg(kdrl:mCherry)* fluorescent reporters with previously described antibodies that recognize Rbfox1l and Rbfox2. While Rbfox1l is restricted to GFP+ myocardial nuclei at linear heart tube (30 hpf; Fig. 1A, A') and chamber stages (48 hpf; Fig. 1B,B'), Rbfox2 localizes to both myocardial and endocardial nuclei (Fig. 1D-E') that include the developing valve cushions in the atrioventricular canal (AVC; Fig. 1E, E'). At 72 hpf, Rbfox1l remains restricted to striated muscle (Fig. 1C-C'') while Rbfox2 is more broadly distributed (Fig. 1F-F''). To learn if Rbfox1l and 2 are sustained in the heart through adulthood, we immunostained cardiac sections from adults carrying the *Tg(myl7:nucGFP)* fluorescent reporter. Rbfox1 co-localizes with GFP in myocardial nuclei (Fig. 1G, Fig. S2A) with no expression in smooth muscle cells of the outflow tract (OFT; Fig. 1H), the zebrafish equivalent of the aortic root in mammals (reference). Although Rbfox2 is also observed in the adult heart, it is mostly expressed in presumptive endocardial cells, relatively fewer cardiomyocytes, and smooth muscle of the OFT (Fig. 1I-J, Fig. S2B). **Major Conclusion:** Rbfox1l and Rbfox2 expression is overlapping in the myocardial during zebrafish heart during development, and Rbfox2 is expressed more broadly in tissues that include the endocardium and valve primordia. Expression of both proteins is maintained in a similar pattern in the adult heart.

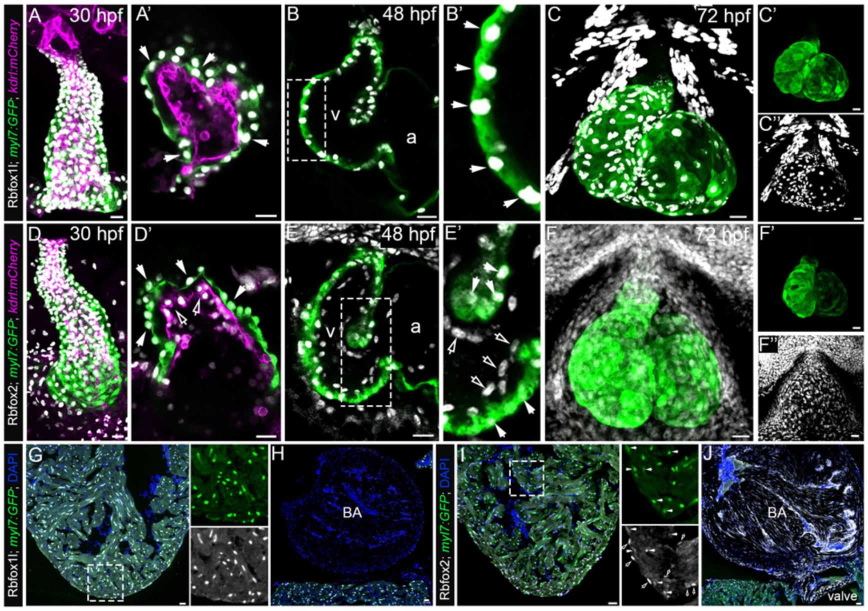

Figure 5. Rbfox1l and Rbfox2 expression overlap in the myocardium and are maintained through adulthood. (A,D) Max projection of linear heart tubes from 30 hpf Tg(myl7:GFP); Tg(kdrl:mCherry) embryos stained with anti-GFP, anti-mCherry, and anti-Rbfox1l or anti-Rbfox2. (A',D') Single slice of linear heart tubes shown in A and D. White arrowheads highlight myocardial Rbfox1l and Rbfox2 expression, while open arrowheads highlight endocardial Rbfox2 (D'). (B,B',E,E') Single slice of 48 hpf hearts with boxed regions in B and E shown in B' and E'. White arrowheads highlight myocardial Rbfox1l and Rbfox2 expression, while open arrowheads highlight endocardial Rbfox2. (C,F) Merged max confocal projections at 72 hpf. (C'C''F'F') Split channels from C and F. (G-J) Cardiac sections of myl7:nucGFP hearts stained with anti-GFP, anti-Rbfox1l or Rbfox2, and DAPI. Ventricular apex is shown in G and I and bulbous arterious (outflow tract) is shown in H and J. Boxed regions in G and I shown in split panels to the right.

We examined sarcomere organization in cardiomyocytes as these are the functional units of striated muscle that cause contraction. Max projections of control hearts following immunostaining for markers of both thick and thin filaments revealed a classic striated configuration with individual components arranged in a repeating pattern (Fig. 6A-D''). By contrast, max projections of DKO hearts revealed that while sarcomere components are expressed at appropriate levels, they fail to organize into highly ordered structures (Fig. 6E-H''). This phenotype is also apparent at 30 hpf in DKO hearts when sarcomere organization is first becoming apparent in control linear heart tubes (Fig. 6I-J'). **Major Conclusion:** Rbfox proteins are essential for cardiomyocyte sarcomere organization, suggesting an cell-autonomous function.

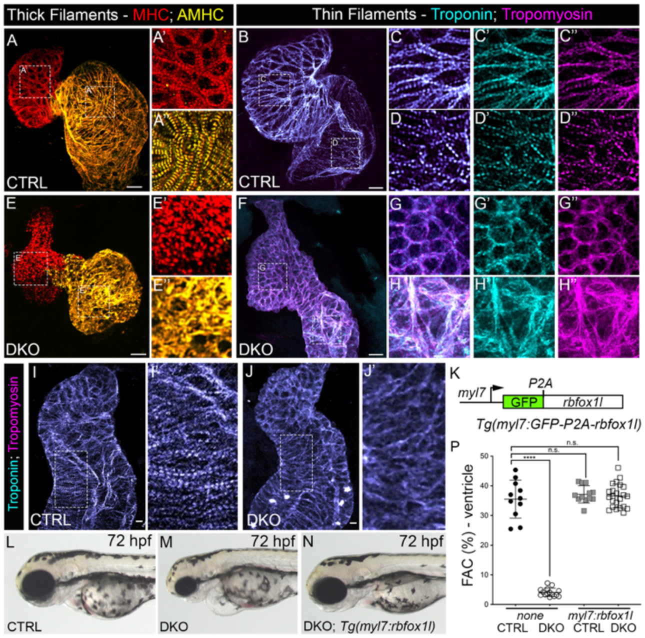

Figure 6. Rbfox is required cell-autonomously in cardiomyocytes for myofibril assembly and aorta formation. (A-H') Merged channel max confocal projections of 48 hpf hearts from control and DKO animals stained with antibodies that recognize Myosin Heavy Chain (MHC), Atrial Myosin Heavy Chain (AMHC), Troponin, and Tropomyosin as indicated. Boxed regions are shown in the ' panels that are either merged or split channels. (I-J') Merged channel max confocal projections of 30 hpf hearts from control and DKO animals stained with antibodies that recognize Troponin and Tropomyosin. Boxed regions are shown in I' and J'. (K) Schematic of the transgene injected to generate the stable *Tg(myl7:rbfox1l)* strain. (L-N) Brightfield views of 72 hpf embryos with indicated genotypes. Anterior right. (P) Quantification of percent fractional area change. Statistics: n.s., not significant; ****, p<0.001.

To test this hypothesis directly, we added back Rbfox1l specifically to the myocardium in DKO animals to learn whether heart function might be rescued. We chose to assess Rbfox1l as the RNA Recognition Motif (RRM) is more highly conserved in length and amino acid sequence with the RRM in human Rbfox2. To this end, we generated a stable transgenic line constitutively expressing GFP followed by a viral P2A sequence and Rbfox1l under control of the cardiomyocyte-specific *myl7* promoter (Fig. 6K). No evidence of cardiac edema was observed in DKO hearts carrying the transgene (Fig. 6L-N), indicating that function might also be restored. Short videos of GFP-expressing hearts were taken and used to calculate %FAC as previously described. FAC was not altered in wild-type embryos overexpressing Rbfox1l, suggesting that protein abundance is not detrimental. Moreover, FAC was completely rescued in DKO animals carrying the transgene (Fig. 6P). In addition, aortic diameter was also rescued in DKO animals back to control widths (not shown). **Major Conclusion:** Overall, these data demonstrate that Rbfox proteins are required specifically in cardiomyocytes to support sarcomere assembly and contractile function from the earliest developmental stages of cardiogenesis and that aortic and valve stenosis or atresia are secondary to defects in pump function.

The majority of Specific Aim 2 will be performed in Budget Year 2.

Specific Aim 3 is partially complete.

MAJOR TASK 1: We performed bulk RNAsequencing on 30-70 pooled embryonic hearts dissected from 48 hpf wildtype and DKO animals. We sequenced 10 replicates per cohort and performed hierarchical clustering and principal component analysis. We found that 8 of the 10 control replicates cluster together, while 8 of the 10 mutant replicates cluster together and away from controls. We analyzed differential gene expression between these select replicates and found that 421 genes are upregulated while 563 genes are downregulated (Fig. 7A). The biggest surprise came when we analyzed the gene ontology terms enriched in the downregulated subset. The top hits encode structural components of the mitochondrion including the ribosomal subunit, electron transport chain complexes, krebs cycle complexes, and inner membrane factors (Fig. 7B). We validated a handful of the genes by qPCR (Fig. 7C). We did not find that transcripts encoding sarcomere components were low.

Since Rbfox is known to play a role in splicing, we analyzed our data for alternative splicing defects in DKO hearts. We identified 213 significant alterations with 153 skipped exons (SE), 48 mutually exclusive exons (MXE), 8 alternative 3' splice sites (A3SS), 2 alternative 5' splice sites (A5SS), and 2 retained introns (RI) (Fig. 7D). We generated a preliminary volcano plot with the top hits shown along the ceiling (Fig. 7E). We found alterations in a known Rbfox target, *pdlim5b*, which encodes a cytoskeletal protein. We also found alternative isoforms in other transcripts encoding actin cytoskeletal components including *tropomyosin* 1 (*tpm1*), *tropomyosin 3 (tpm3), alcama,* and *tnnt3b*. In addition, we found that components of the MICOS complex, including Mic19 and Mic25, are misspliced.

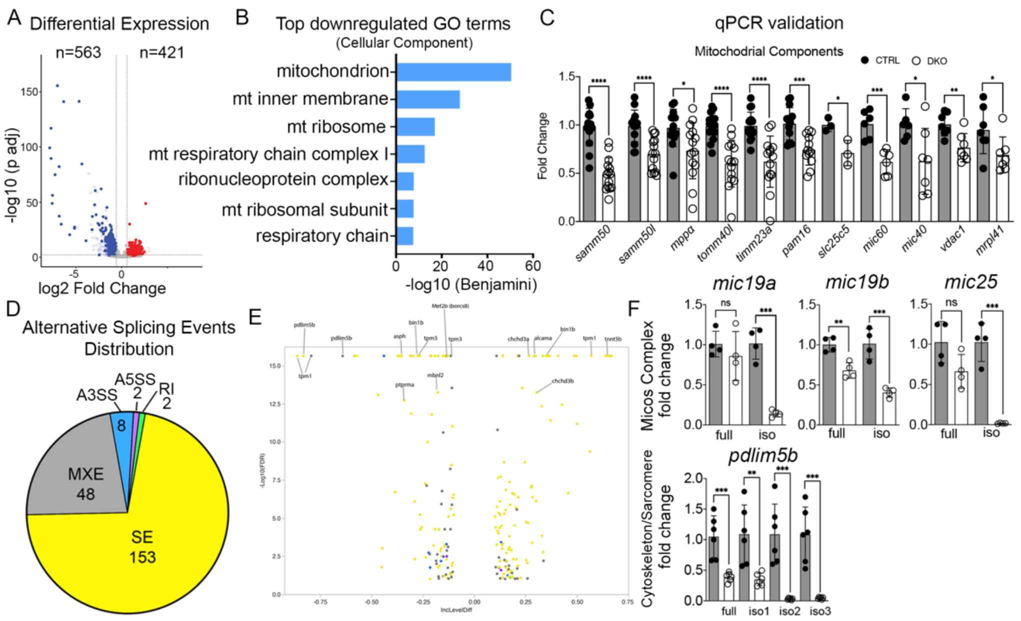

Figure 7. Rbfox proteins are required for mitochondrial biogenesis and regulated alternative splicing of transcripts encoding actin cytoskeletal and MICOS components. (A) Volcano plot of differentially expressed genes between CTRL and DKO hearts at 48 hpf. (B) Top GO terms of genes downegulated in DKO hearts. (C)qPCR validation of genes identified as downregulated in DKO hearts by RNAseq. (D) Pie chart showing the number of missplicing events in each category. (E) Volcano plot showing misspliced transcripts. (F) qPCR validation of RNAseq and isoform analysis. "Full" shows the level of expression of a full-length transcript while "iso" shows a specific isoform.

These are particularly interesting because the MICOS complex creates cristae junctions in mitochondrial which are required for inner membrane folding. The inner membrane is also where the electron transport chain components are found that generate ATP. **Major Conclusion**: From these data, we hypothesize that cardiomyocytes fail to assemble myofibers and contract because of a primary defect in mitochondrial biogenesis and ATP production.

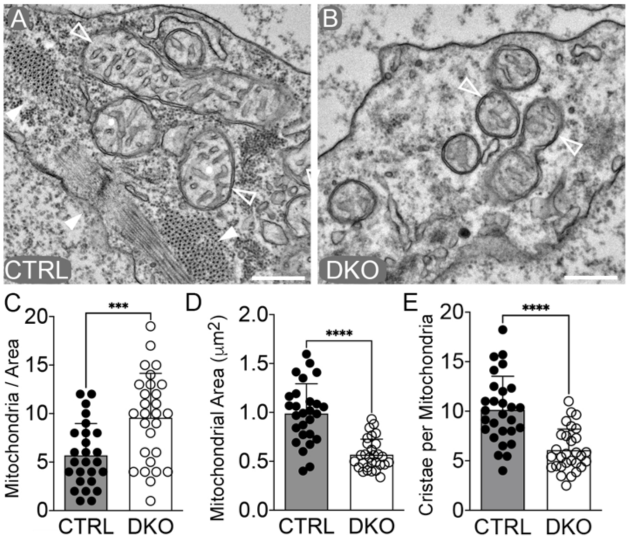

Figure 8. Mitochondria biogenesis is altered in DKO mutants. (A,B) Transmission electron microscopy of control and DKO cardiomyocytes in 48 hpf zebrafish hearts. White arrowheads show myofiber in cross-section or a Z-disc longitudinally. Open arrowheads highlight mitochondria. (C-E) Graphs quantifying the number of mitochondria per defined area (250 microns), mitochondrial area, and cristae (or referred to as inner membrane folds) per mitochondria. Sample size is 3 hearts per cohort. Statistics: ***, p<0.01; ****, p<0.001.

<u>MAJOR TASK 4:</u> Based on data from MAJOR TASK 1, we propose to test the hypothesis that Rbfox-mediated splicing of MICOS components is required for mitochondrial biogenesis and ATP production. In the absence of functional mitochondria, cardiomyocytes do not have adequate amounts of ATP for normal cell growth or myofibril assembly, which results in lowly contractile hearts with HLHS-like phenotypes. To begin testing this hypothesis, we performed transmission electron microscopy on 48 hpf control and DKO animals and imaged the heart in each cohort. In control cardiomyocytes, cross-sections of large bundles of myofibers can be seen, as well as longitudinal arrays of Z-lines with highly ordered thin and thick filmaments (Fig. 8A, white arrowheads). In addition, mitochondria appear healthy and contain visible folds of the inner membrane (Fig. 8A, open arrowheads). By contrast, DKO cardiomyocytes lack sarcomeres completely, and the mitochondrial appear small with abnormal or missing inner membrane folds. We quantified this mitochondrial defect and observed increased numbers in a defined area, smaller organelles that had less inner membrane folds (Fig. 8C-E). **MAJOR CONCLUSION**: Rbfox is required for mitochondrial biogenesis in cardiomyocytes.

4. Other Achievements: My laboratory published one review chapter that is *in press* at Cold Spring Harbor. While the title is "Innate Mechanisms of Heart Regeneration", the focus of the review is cardiomyocyte proliferation. As such, the topic is relevant to HLHS as cardiomyocytes in the left ventricle fail to proliferate. I anticipate submitting an original research manuscript revealing the data shown above for publication in budget year 2.

- Yin HM, Burns CG*, **Burns CE***. Innate Mechanisms of Heart Regeneration. Cold Spring Harbor perspectives. (2021) *in press*.