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

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

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

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

3. **ACCOMPLISHMENTS:**

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

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

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

Specific Aim 1: Single-cell transcriptomics for population mapping of FACS-sorted *Drosophila* cardioblasts and human Multipotent Cardiac Progenitors, to track emerging cell identities and how they are altered as a result of RPL13 knockdown.	**Timeline**	**Site 1**	**Progress (%)**
Major Task 1: Molecular Characterization of *Drosophila* cardioblasts by single cell-RNAseq	Months	SBP	
Subtask 1. **RNA-seq of Stage 16-17 embryonic cardioblasts.** To determine whether cardioblasts have undergone a transformation in molecular/cardiac identity following *RpL13* knockdown.	1-3	Dr. Schroeder	50%
Subtask 2. **Single-cell RNA-seq of Stage 16-17 embryonic cardioblasts.** Obtain molecular signatures of individual cells and determine whether subpopulations of cells respond differentially to the KD of *RpL13*.	2-5	Dr. Schroeder	100%
Subtask 3. **Computational Analysis of scRNA-seq data, generation of genetic map and verification of candidate genes by ISH and antibody staining.** Select differentially expressed genes as indicated by RNA-seq and visualize gene expression changes in the embryo.	5-9	Dr. Schroeder	90%
Milestone(s) Achieved: Identified pathways and genes that are altered in expression following *RPL13* KD in Drosophila cardioblasts.			
Major Task 2: Molecular characterization of cardiac progenitors in Multipotent Cardiac Progenitors by single cell-RNAseq			

Subtask 1. **Single-cell RNA-seq of Multipotent Cardiac Progenitors.** Time course following *RPL13* siRNA treatment to determine changes in the transcriptomic landscape of the heterogenous cell population.	6-10	Dr. Schroeder	75%
Subtask 2. **Computational Analysis of scRNA-seq data** and comparison of pathways affected by knockdown of *RPL13* between Drosophila and human MCPs.	10-14	Dr. Schroeder	0%
Milestone(s) Achieved: Identify various subpopulations of cells in MCP cultures and attach a molecular signature to each population. Identify key pathways altered by *RPL13* knockdown. Compare pathways between Drosophila and MCPs and look for parallels between species.			
Specific Aim 2: Polysome Profiling to identify direct and indirect translational targets of *RPL13* in human Multipotent Cardiac progenitors, leading to construction of a *RPL13*-centric genetic network driving cardiac differentiation.			
Major Task 3. Measure overall Protein Translation in MCP and *Drosophila* Cardioblasts following *RPL13* knockdown. Puromycin protein synthesis quantification assay to measure protein production following *RPL13* knockdown.	12	Dr. Schroeder	90%
Milestone Achieved: Determined how *RPL13* KD affected overall protein translation in MCPs and *Drosophila* cardioblasts.			
Major Task 4. **Ribosomal Protein Quantification in MCPs**			
Subtask 1. **Polysome isolation from MCP cells treated with RPL13 siRNA.** Optimize conditions and select appropriate markers/antibodies for FACS sorting.	13-14	Dr. Schroeder	0%
Subtask 2. **Quantification of ribosomal subunit levels and stoichiometry between controls and *RPL13* siRNA treated MCPs using liquid chromatography coupled to tandem mass-spectrometry LC-MS/MS).** Determine whether subunits are enriched in monosomes vs. polysome.	14-15	Dr. Schroeder	0%
Milestone(s) Achieved: Determined changes in the levels and stoichiometry of ribosomal proteins following *RPL13* knockdown. Determined enrichment of ribosomal proteins in the various polysome fractions.			
Major Task 5. **Polysome Profiling with RNA-seq in MCP cells** *NOTE: We have modified the methodology of this major task as described in a previous progress report. Accomplishments with new method not reflected in the percentages below. In this reporting period, we generated transgenic flies and validated them by PCR. We are recombining them to generate lines that we can cross with RpL13-RNAi, verify RpL13 KD, and to move forward*			

with RNAseq to capture the translatome (see details below).			
Subtask 1. Polysome Profiling to identify mRNAs targeted by ribosomes in MCPs and how the mRNA targets shift following RPL13 siRNA treatment. Timecourse following siRNA treatment. RNA-seq followed by data analysis and interpretation.	14-20	Dr. Schroeder	0%
Subtask 2. **Identify mRNA species that are directly bound by RPL13 loaded ribosomes.** Polysome fractionations will be subject to a pulldown using RPL13 antibody, to isolate ribosomes loaded with RPL13. Bound mRNA will be processed for RNA-seq to identify and analyze RPL13 targets.	18-22	Dr. Schroeder	0%
Subtask 3. **Gather RNA-seq data and perform Computation Analysis and gene network design.**	22-24		0%
Milestone(s) Achieved: Identified direct and indirect mRNA targets of *RPL13* in differentiating MCPs. Developed genetic maps by computational analysis that describe and predict *RPL13* involvement in cardiac differentiation pathways.			

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

In our previous reports, we described completing a single-cell (sc) RNAseq experiment using *Drosophila* cardioblasts to examine transcriptomic changes caused by knockdown (KD) of the ribosomal protein RpL13 (Major Task 1). In our analysis, we found a block/delay in cardioblast differentiation and through ontology analysis we identified biological pathways that were altered following *RpL13* KD (**Fig. 2 previous progress report**). We have continued to refine the analysis of the data. We also performed additional deeper sequencing of the library preps to acquire more genes. Through this refinement there have been some shifts in the genes that were significantly changed in expression, but importantly most of the pathways we identified initially have remained the same, particularly cardiogenic factors and basement membrane formation (**Fig.1**). We included a new category of genes involved in splicing that could mediate overall changes in the translational landscape of the heart. To validate the results, we are planning to replicate the fly cardioblast scRNAseq experiment in the next few months which will help provide additional power to our data to guide which interacting pathways are most promising.

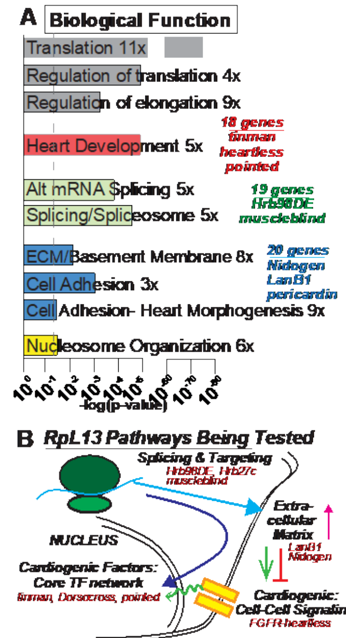

*Fig. 1. Gene Ontology analysis of RpL13 KD sc-RNAseq data. **A.** Select biological functions altered by RpL13 KD. Top pathways to be explored. **B,** Model on how combined dysregulation of pathways by RpL13 KD lead to altered cardiac differentiation. Alterations in splicing can increase deposition of ECM (as suggested by sc-RNAseq results and staining) or affect cardiogenic factors directly. Changes in ECM can disrupt cell signaling that feeds back to alter the cardiogenic network and redirect differentiation.*

To test whether genes from enriched biological categories identified from sc-RNAseq data genetically interact with *RpL13* and contribute to the development of cardiac defects, we created a *RpL13*-sensitize line (*s-RpL13-RNAi*) described in the previous progress report that produces an intermediate cardiac phenotype whereby approximately 20-25% of hearts displayed severe cardiac phenotypes by phalloidin staining while the remaining hearts were largely normal (**Fig. 2A and Table 1**). By crossing the s-*RpL13-RNAi* line with RNAi's or heterozygous mutants of candidate genes, we can determine if the genetic combinations improve or exacerbate cardiac phenotypes. We have tested several genes from the three categories (**Table 1**) including heterozygous mutants of core cardiogenic factors *Dorsocross A/Tbx20*, *tinman/Nkx2-5* (**Fig 2B**), and *pannier/GATA4.* Both *Dorsocross* and *tinman* combinations with s-*RpL13-RNAi* exacerbated the cardiac phenotype, whereas *pannier* did not, suggesting that *RpL13* may target specific Transcription Factors (TFs) /mechanisms to redirect cardiac differentiation, rather than generically. We found that *s-RpL13-RNAi* combination with cardiogenic cell signaling factor *heartless/FGFR* also exacerbated the phenotype (**Fig 2B and Table 1**). Combinations of s-*RpL13-RNAi* with cardiogenic TF *pointed/ETS1* and either a *pointed-RNAi* KD or a *pointed*-heterozygous mutation led to opposing effects. Combined *pointed-RNAi* KD specifically in the heart improved the cardiac phenotype whereas combinations with the *pointed* het mutant led to an exacerbation (**Table 1**). Mutants have reduced *pointed* expression earlier in development that is widespread whereas KD of *pnt* is targeted to cardiogenic mesoderm at a relatively later developmental stage, suggesting that *RpL13* and *pointed* interaction is dependent on 1.) timing during development, and/or 2.) their spatial expression and site of interaction. We have also tested a number of splicing factors and so far found combinations with splicing factors *Hrb98DE-RNAi* and *muscleblind-RNAi* to exacerbate cardiac phenotypes (**Fig 2C and Table 1**). Importantly, both genes have been shown to be involved in development and patterning. Interestingly *Hrb98DE* is involved in IRES-dependent translation,

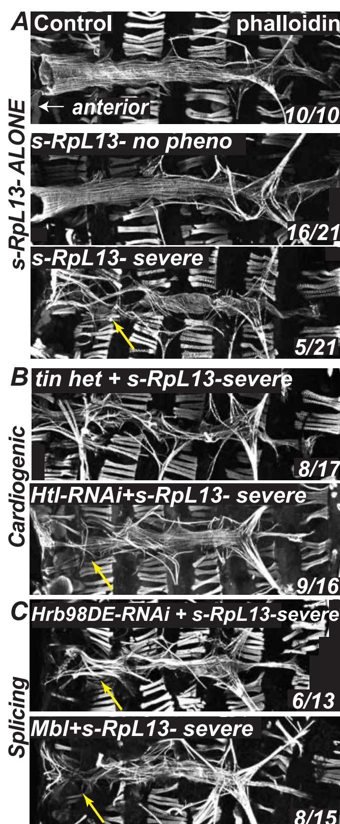

*Fig. 2. Genetic Interactions. **A** s-RpL13 alone led to ~75% of flies with normal hearts while 25% displayed severe defects. **B** s-RpL13 and cardiogenic factor tinman/Nkx2.5 and FGF receptor heartless/htl KD genetically interacted resulting in 47% and 56% of flies with severe heart defects, respectively. **C** s-RpL13 and splicing factors Hrb98DE and muscleblind/ mbl KD interacted resulting in 46% and 53% of flies with severe cardiac defects. Numbers indicate the proportion of flies with specified phenotypes. Yellow arrows points to cardiac defects that were more severe in the anterior region of the heart.*

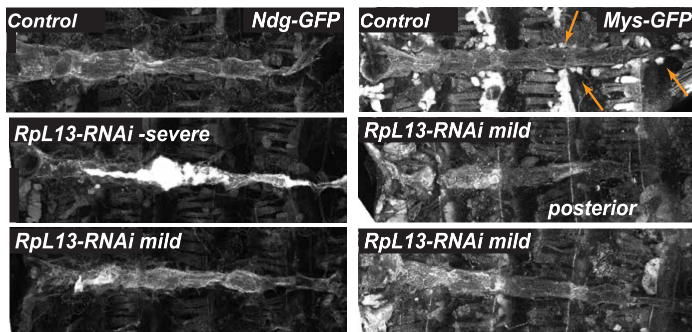

Fig 3. ***Left,*** *Severe and mild s-RpL13 hearts showed increased Nidogen/NDG expression suggesting participation in cardiac pathogenesis.* ***Right,*** *Mild cardiac phenotypes produced by s-RpL13, resulted in regional changes in myospheroid expression, which was reduced in the posterior region of s-RpL13 hearts. Additionally, pericardial cells expressing myospheroid (orange arrows) seen in controls were absent in s-RpL13 hearts.*

which *RpL13* has been shown to regulate in viruses (Han S, et al. 2020 *J Virol*). We have written a grant to look more closely at changes in splice variants following *RpL13* KD in the heart. sc-RNAseq results showed an increase in a number of Extracellular Matrix (ECM) components. We tested interactions between *s-RpL13-RNAi* with several ECM structural components including *Nidogen*, *LamininB1, pericardin,* and *integrin1*α*.* While co-KD of pericardin and *integrin1*α with *s-RpL13* exacerbated the cardiac phenotype slightly, combinations with *Nidogen/Ndg* (cell adhesion glycoprotein) and *Laminin B1-RNAi* led to an improvement of phenotype and a considerable reduction in the incidence of severe cardiac phenotypes almost down to zero (**Table 1**), indicating a rescue of *RpL13-RNAi* induced phenotype. We therefore explored the expression of some ECM factors including *Nidogen* and *myospheroid* (*mys*, β-subunit of the integrin dimer) using GFP fusion constructs (**Fig 3**). In *s-RpL13-RNAi* hearts with severe phenotypes, there was an increased in *NDG* deposition in adult hearts, whereas those hearts that were normal did not show an increase, suggesting that *Ndg* may be involved in the pathogenesis of the heart. In controls, *MYS* expression was found throughout the heart and in pericardial cells surrounding the heart (**Fig 3, arrows).** Interestingly, in *s-RpL13* hearts *MYS* expression was present in the anterior region of *s-RpL13-RNAi* hearts but much reduced in the posterior region. Additionally, we did not see *MYO* labelled pericardial cells in *s-RpL13-RNAi* line, suggesting a signaling or non-cell autonomous mechanism contributing to the cardiac phenotype. The regional differences in expression is interesting because in looking closely at the severe phenotypes produced by *s-RpL13-RNAi* (**Fig 2,** yellow arrows), we see that a vast majority of the defects are localized to the anterior region of the heart. This suggests that *RpL13* may have targeted and weighted function in certain segments of the heart, particularly the anterior conical chamber/outflow tract. Because *Hox* genes are known to regulate anterior-posterior differentiation/patterning of cardiac segments, we will test whether overexpression of the posterior heart expressing *Hox* genes (*Ubx, Abd-A)* throughout the heart could perhaps rescue and override the anterior defects induced by *s-RpL13-RNAi.* Conversely, we will also test whether expression of the anterior heart expressing *Hox* gene (*Antp*) throughout the heart could exacerbate the phenotype in *s-RpL13-RNAi* lines. The ECM remodeler *Timp* (Tissue inhibitor of metalloproteases) was upregulated in s-*RpL13-RNAi* hearts based on the sc-RNAseq results, therefore we will modulate expression of *Timp* and other ECM remodelers in s-*RpL-RNAi* hearts to see if they are able to modulate/rescue the cardiac phenotype. These interaction studies are helping us build a pathway/network and could indicate that misregulation of ECM remodelers may be the cause of ECM misexpression. Previous research has shown that ECM components can modulate binding of ligands onto receptors (including *Heartless* FGF receptor signaling) to drive differentiation and can be modulated by downstream effectors *pointed* and *TOR* growth signaling pathway in blood cells (Dragojlovic-Munther *et al.* 2013, *Dev Biol*). Many of components of this signaling pathway are also altered in our sc-RNAseq results and therefore we will test in *s-RpL13-RNAi* fly hearts. With the ability to modulate and identify phenotypes using *s-RpL13* line*,* we have now just created another *s-RpL13* line that incorporates a heart-specific fluorescent marker (tdtk; Klassen MP, et al 2017, *Elife*; Schroeder AM, *et al.* 2022, *PLoS Genetics*), which will speed up our evaluation of ~57 genes in our intitial candidate list.

Genotype	no-pheno/ mild	severe pheno	% severe pheno
Control	6/6	0/6	**0**
s-RpL13	16/21	5/21	**23.8**
Cardiogenic- Transcription Factors			
Dorsocross A het	10/10	0/10	**0**
+ s-RpL13	4/15	11/15	**73.3**
tinman het	6/6	0/6	**0**
+ s-RpL13	9/17	8/17	**47.1**
pannier het	7/7	0/7	**0**
+ s-RpL13	13/18	5/18	**27.8**
Cardiogenic- Cell signaling			
heartless-RNAi	10/10	0/10	**0**
+ s-RpL13	7/16	9/16	**56.2**
pointed-RNAi	10/10	0/10	**0**
+ s-RpL13	14/16	2/16	**12.5**
pointed-het	7/7	0/7	**0**
+ s-RpL13	5/9	4/9	**44.4**
Splicing Factors			
Hrb98DE-RNAi	10/10	0/10	**0**
+ s-RpL13	7/13	6/13	**46.2**
muscleblind-RNAi	10/10	0/10	**0**
+ s-RpL13	7/15	8/15	**53.3**
Extracellular Matrix			
Nidogen-Rnai	10/10	0/10	**0**
+ s-RpL13	15/15	0/15	**0**
Laminin B1-RNAi	10/10	0/10	**0**
+ s-RpL13	15/16	1/16	**6.3**
pericardin-RNAi	10/10	0/10	**0**
+ s-RpL13	10/16	6/16	**37.5**
integrin1-α-RNAi			
+ s-RpL13	7/11	4/11	**36.4**
Nucleolar Stress			
Nucleostemin1-RNAi	11/11	0/11	**0**
+ s-RpL13	4/11	7/11	**63.6**

Table 1. *s-RpL13 alone produced 25% of progeny with heart defects. Mutant hets or RNAi lines alone did not produce defects. Red indicates increase in proportion of severe phenotypes while blue indicates a decrease when s-RpL13 was combined with gene candidates indicating a genetic interaction.*

Furthermore, our automated cardiac analysis tool will allow us to analyze fluorescent hearts for cardiac function, allowing us to detect more subtle functional effects on heart function.

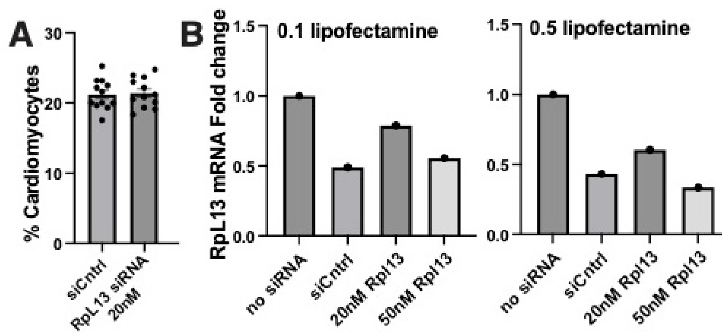

Fig 4. Control experiments testing RpL13 siRNA treatment conditions. A, 20uM of RPL13 siRNA did not elicit a robust reduction of cardiomyocytes as stained by cardiac marker ACTC1. B, Quantitative RT-PCR testing 2 concentrations of RPL13 siRNA with two different lipofectamine concentrations. siRNA control and RPL13siRNA elicited a similar down regulation of RPL13 mRNA compared to no siRNA treatment.

Since our last progress report, we have continued to troubleshoot our cell-culture experiments in order to run a sc-RNAseq on Multipotent Cardiac Progenitors (MCPs; Major Task 2). We ran an additional control experiment to again verify our conditions, however in this run, we did not see changes in the proportion of cardiac (**Fig 4A**) and fibroblast cells. We again tested *RpL13* expression levels by quantitative PCR using various concentrations of *RPL13* siRNA, control siRNA and included a no siRNA control, and incubated in two concentrations of lipofectamine. Our control siRNA was producing a similar KD in *RPL13* levels as our *RPL13* siRNA when compared to no siRNA control (**Fig 4B**). siRNA control may be having non-specific effects on ribosomes in general or *RPL13* in particular. We will test other siRNA control siRNAs to induce a more robust *RPL13* KD compared to our controls. Once we trouble shoot our conditions for *RPL13* KD in MCP cells we will perform single-cell RNAseq. This will then allow us to complete proteomic experiments described in Major Task 4, which requires protein samples from MCP cultures. We also set up a collaboration with Dr. Karen Ocorr at SBP to pursue studies in zebrafish as a potential vertebrate model other than MCPs to study *rpL13* in the heart. Preliminary data on the initial characterization of the zebrafish heart found that *rpL13* morpholino (mo-*rpL13*) KD caused significant edema (**Fig 5 A,B**) and defects in cardiac looping (**Fig 5C,D**). There was also a significant decrease in cardiac cell numbers (**Fig. 5E**) and Fractional Area Change (**Fig. 5F**) in both the atria and ventricle following *rpL13* KD. There was a slight increase in Heart Period (**Fig. 5G**), possibly compensating for the reduced cardiac output. A genetic interaction was detected between *rpL13* and cardiogenic TF *nkx2.7*. KD of *rpL13* and *nkx2.7* individually, using low morpholino concentrations, led to no changes in heart parameters including Diastolic Surface Area of the ventricle (**Fig. 5H**). However combined *rpl13* and *nkx2.7* morpholinos led to a significant decrease, thus indicative of an interaction, similar to that observed in *Drosophila* (**Fig. 2B and Table 1**). This collaboration now provides an opportunity to include zebrafish as an alternative vertebrate model to MCPs to test the most promising candidates that stem from the *Drosophila* genetic interaction testing, that will provide additional insights in development including looping, hemodynamics, and chamber formation. We plan to use *CRISPR* transformants alongside morpholinos to KD *RpL13* and further study its role and identify genetic interactors in the vertebrate heart.

In our previous progress report, we described a method called Ribo-STAMP, as an alternative method for MAJOR Task 5 to probe the translatome/proteome. We sent our constructs for injection and received various transgenic lines for each of our three constructs (UAS-APOBEC1; UAS-*RpS2-APOBEC1*; UAS-*RpL13-APOBEC1*). We performed PCR and verified the insertion of APOBEC1 (APO1) in the various fly lines. Because APO1 modifies *mRNA* sequences and we intend to use RNAi to KD expression, we are testing whether RNAi mediated KD of *RpL13* will produce sufficient *RpL13* KD in the presence of *APO1*. Based on communication with our collaborators (Yeo Lab, UCSD), we can overcome this potential caveat by titering the expression of *APO1* and selecting certain *RpL13*-RNAi sequences that target regions in *RpL13 mRNA* less likely to be modified by *APO1*. In this effort, we are currently crossing fly lines to recombine APO1-constructs with various cardiac and mesodermal drivers (Hand4.2-GAL4,tdtk; Hand-GMR-GAL4,tdtk; TwistGAL4;24B-GAL4). We will then use these recombined Driver-APO1 lines and cross with various *RpL13*-RNAi lines to test for their ability to induce *RpL13* KD in the hearts by quantitative RT-PCR. As a first pass, Hand4.2-GAL4,tdtk crossed to the three transgenic lines led to viable adult flies, which we will be imaging shortly to determine heart phenotypes by fluorescent imaging. While Hand4.2-GAL4 alone leads to loss of the adult heart, we expect that in combination with *APO1* constructs, RpL13-RNAi induction may lead to less severe phenotypes due to dilution of GAL4 onto two UAS sites. We will test and select the appropriate drivers and RNAi lines that produce robust *RpL13* KD. We will then test to determine whether APO1 is modifying *mRNA* in embryos and/or adult hearts by bulk RNAseq initially, and single-cell RNAseq in embryos (See **Fig 8** in previous progress report). Alternatively, if RNAi does not work, we could test heterozygous mutants or deficiency lines in combination with APO1. We will continue to pursue the experiments as described in the previous progress report.

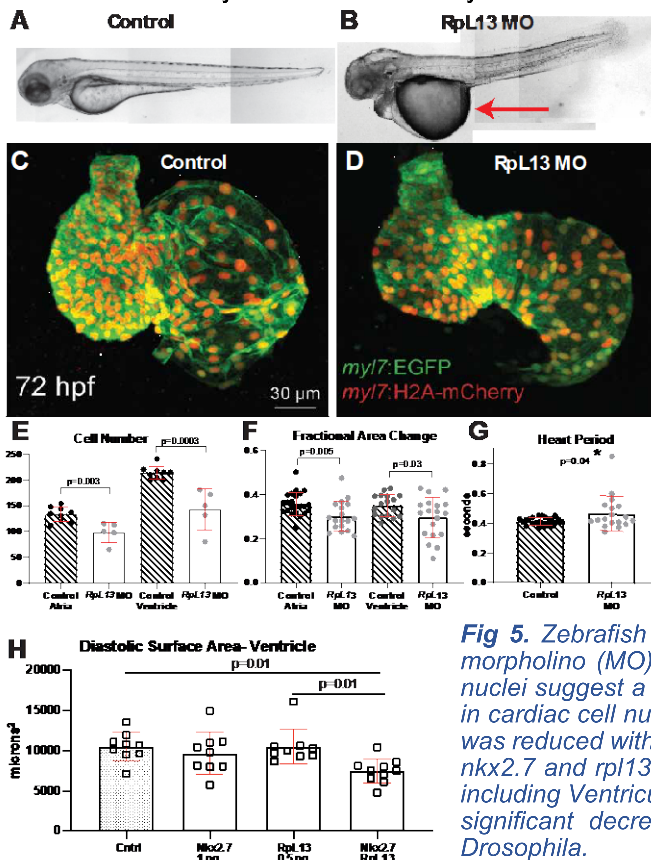

Fig 5. *Zebrafish phenotypes 72 hours post fertilization (hpf).* ***A,B*** *KD of rpl13 by morpholino (MO) led to cardiac edema (arrow).* ***C,D*** *Staining of cardiac cells and nuclei suggest a looping defect following rpl13 KD.* ***E,*** *we also detected a decrease in cardiac cell number in the atria and heart.* ***F,*** *Fractional Area Change/contractility was reduced with rpl13 MO, while Heart Period increased (****G****).* ***H,*** *While low doses of nkx2.7 and rpl13 MO did not produce significant differences in the zebrafish heart, including Ventricular Diastolic Surface Area, combined KD rpl13 and nkx 2.7 led to a significant decrease in area, indicating a genetic interaction also observed in Drosophila.*

We have also performed Puromycin (Puro) staining in fly embryos (Major Task 3) to test overall protein translation. Our initial experiments suggest a decrease in overall translation caused by *RpL13* KD. We collected embryos of control and *RpL13*-RNAi crossed to mesodermal driver Twist-GAL4. We incubated the embryos in Puro under two conditions (2μM Puro for 30 mins vs 6μM Puro for 15 mins) as an initial test (**Fig. 6**). We found that 2μM for 30 mins showed higher puromycin signal in control cardiomyocytes compared to *RpL13-RNAi*, suggesting higher levels of Puro incorporation into translating *mRNA,* thus higher levels of translation in controls compared to *RpL13* KD. In the other condition (6μM for 15 mins) we did not see a significant difference. We will repeat the embryo experiment to verify the result, and will try other conditions with higher puromycin concentrations for longer durations to see if we get a better separation of signal between the two conditions. Because we use whole embryos, we will likely need a longer time period of incubation to allow the drug to penetrate into the embryo. We will also test puromycin incorporation in dissected adult hearts. By inducing *RpL13*-RNAi only in adult

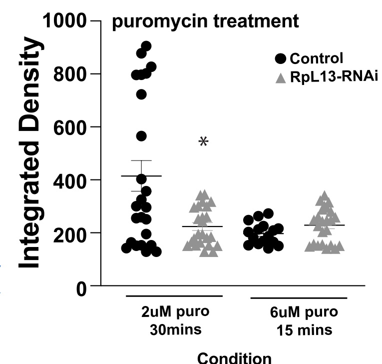

Fig 6. *Puromycin incorporation experiment using various drug concentrations and durations in fly embryos to test for overall protein translation. Preliminary results suggested reduced puromycin staining in RpL13-RNAi embryos compared to controls. We will repeat the experiment with higher puromycin concentrations for longer durations.*

hearts using a temperature sensitive driver and then treating with puromycin 24 hours later, we can determine the short-term effects of *RpL13* KD on translation in the heart. We will also be able to validate the embryo puromycin experiments because this preparation exposes the heart directly to puromycin (instead of an entire embryo), and may have better puromycin staining and signal. These experiments are currently ongoing.

○ **What opportunities for training and professional development has the project provided?**
Nothing to report.

○ **How were the results disseminated to communities of interest?**
Nothing to report.

○ **What do you plan to do during the next reporting period to accomplish the goals?**
Nothing to Report (end of grant).

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.**

○ **Actual or anticipated problems or delays and actions or plans to resolve them.**
Because of a labor shortage, we had to establish a new source of MCPs from another collaborator and set up our own cell-culture equipment, reagents and tools in our own lab. We performed several control experiments, which unfortunately have resulted in inconsistent cardiac phenotypes or responses following RpL13 knockdown protocols. We therefore are continuing to troubleshoot and determine the conditions to generate an appropriate response to RpL13 knockdown we observed in the original MCP cell line. After this, we will proceed with the scRNAseq experiment (MAJOR TASK 2), using nuclei instead of whole-cell due to cardiac cell size and analyze the results (as in AIM1). We will look for trajectories in gene expression that would explain changes in cell-fate choices among the MCP cells.

Establishing our MCP culture conditions will then allow us to extract sufficient protein to perform mass spec and measure changes in ribosome subunit composition (MAJOR TASK 4).

○ **Changes that had a significant impact on expenditures**.
Nothing to report.

○ **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**.
Nothing to report.

○ **Significant changes in use or care of human subjects**.
Nothing to report.

○ **Significant changes in use or care of vertebrate animals.**
Nothing to report.

○ **Significant changes in use of biohazards and/or select agents**.
Nothing to report.

6. PRODUCTS:

○ **Publications, conference papers, and presentations**

- **Journal publications.**

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

Nothing to Report

- **Other publications, conference papers, and presentations.**

- Ribosome Heterogeneity and Specialization. Presentation and Poster.
The Royal Society, London England. Nov. 6-7 2023
Title: Ribosomal Proteins are a novel class of cardiac disease candidates to regulate heart development by interacting with cardiogenic, ECM, splicing and nucleolar stress genes
Authors: Schroeder A, Nielsen T, Kervadec A, Theis J, Colas, Vogler G, Ocorr K, Bodmer R

-Publication on work examining other translational genes in cardiac development performed alongside and informing RpL13 studies
Schroeder AM, Nielsen T, Lynott M, Vogler G, Colas AR, Bodmer R. Nascent polypeptide-Associated Complex and Signal Recognition Particle have cardiac-specific roles in heart development and remodeling. 2022. *PLoS Genetics.* 18(10):e1010448.

○ **Website(s) or other Internet site(s)**
Nothing to Report

○ **Technologies or techniques**
Nothing to Report

○ **Inventions, patent applications, and/or licenses**
Nothing to Report

○ **Other Products**
Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

○ **What individuals have worked on the project?**

Name:	Analyne Schroeder
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-7537-458X
Nearest Person Month Worked:	8.7
Contribution to Project:	Dr. Schroeder has designed and performed the experiments, obtained necessary fly stocks and performed fly husbandry. Collected embryos, troubleshoot scRNA-seq experiments, acquired data and performed data analysis of immunostaining.
Funding Support:	N/A

Name:	Marco Tamayo
Project Role:	Lab Coordinator

Researcher Identifier (e.g. ORCID ID):	0000-0001-9891-0755
Nearest Person Month Worked:	1.9
Contribution to Project:	Assisted with the maintenance of fly stocks, embryo collections, preparation of reagents and constructs.
Funding Support:	N/A

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

CHANGES IN ACTIVE OTHER SUPPORT

Name of Individual: Schroeder, Analyne
Current Appointments: 03/2023 Staff Scientist

CURRENT
THIS AWARD- CLOSED

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

Major Goals: The main goal is to identify new CHD candidate genes and uncover pathways and mechanisms involved in CHD pathogenesis by focusing on an unconventional gene candidate involved in protein translation, the large ribosomal protein RPL13.

Specific Aims:

1. Single-cell transcriptomics for population mapping of FACS-sorted Drosophila cardioblasts and human Multipotent Cardiac Progenitors, to track emerging cell identities and how they are altered as a result of RPL13 knockdown.
2. Polysome Profiling to identify direct and indirect translational targets of RPL13 in human Multipotent Cardiac progenitors, leading to construction of a RPL13-centric genetic network driving cardiac differentiation.

Project Number: W81XWH-21-1-0159
Name of PD/PI: Schroeder, Analyne
Source of Support: Department of the Army
Project Performance Period: 03/01/2021 – 08/31/2023
Total Award Amount (including Indirect Costs):
Time Commitment per Budget Period:

YEAR (YYYY)	Person Months (##.##)
1. 2022	6.40 Calendar Months
2. 2023	6.40 Calendar Months

Grants Management Officer: Abigail Strock
Overlap: None

- **What other organizations were involved as partners?**

Nothing to Report

- **Organization Name:**

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report

9. APPENDICES

See Transition Plan attached.

Transition Plan Questionnaire

Directions: Please answer all questions that apply for each product under development. Please fill out one document per product. ***This is not an application for funding; however, answers will help us understand the outcomes and products from your award.***

1. After the award closes, would you be willing to periodically provide voluntary information (via email) regarding the project status (i.e. where the research is headed)? **Yes** ✔ or **No** ☐

These responses will help CDMRP demonstrate the return on its investments and will help demonstrate that the CDMRP is a responsible and successful steward of federal research funding.

2. What **conclusion(s)** does your final data support?

1) The large Ribosomal subunit Rpl13 participates in cardiac differentiation as its knockdown (KD) led to a delay/block in the maturation of the cardiac transcriptome in Drosophila
2.) RpL13 KD disrupted specific genes from various biological pathways to redirect cardiac differentiation in flies: cardiogenic factors, splicing, and Extracellular Matrix (ECM) Organization.
3.) Through genetic interaction studies, we are generating networks and pathways involved in RpL13 KD mediated cardiac defects e.g. RpL13 KD disrupts ECM factors and remodelers to disrupt ECM deposition in the heart. RpL13 genetically interacts with several genes involved in regulating splicing, particularly during development.
4) RpL13 KD led to a reduction in overall protein translation in fly cardioblasts. A reduction in overall translaton may contribute to the change in translational targets and proteome resulting in redirection of cardiac differentiation.

3. Will you/have you applied for/obtained follow-on-funding for this project? **If yes**, please list (a) funding organization, (b) total budget requested/obtained, and (c) title of the funded proposal. *This information will be recorded as an outcome to this award*.

Yes. We submitted an R01 application to the National Institutes of health.
Total Budget requested: $ 3,889,585
Title of application: Investigating Ribosomal Protein Genes in Cardiac Programming using Model Systems

4. What will be **the next step(s)** for this project?

1.) Determine whether RpL13 KD leads to changes in splicing. We will perform long-read scRNAseq to identify the different splice isoforms with single-cell resolution. Changes in splice variants can shift the proteomic landscape to redirect cardiac differentiation.
2.) Test various oligogenic combinations of candidate genes from our networks, to determine the critical genes/pathways that lead to CHD. This could help in developing much needed mammalian models of CHD.
3.) Work with human geneticists to look for potential oligogenic combinations in patient genomes that include RpL13 and/or other ribosomal subunits, with genes/pathways identified from our results.
4.) Compare RpL13 KD with KD of other ribosomal subunits to determine whether ribosomal subunits play specialized roles in the heart or whether there is a generic response to any disruption in ribosomal subunits.

5. How would you classify your **lead candidate product**? e

(a) Therapeutic (Small Molecule, Biologic, Cell/Gene Therapy): Please choose, if applicable
(b) Diagnostic
(c) Device
(d) Research Tool to Address a Research Bottleneck
(e) Knowledge Product (Non-material product such as a compound library, database, something that improves clinical practice, education, etc.)
(f) Other - Please Specify:

6. How does your candidate product aid the Warfighter, Veteran, Beneficiary, and/or General Population?

There is a high incidence of Congenital Heart Disease (CHD) in the general population, which many times goes undiagnosed. CHD, whether mild or more severe, can be exacerbated with environmental stress induced by general physical/psychological/mental exertion such as military training or deployment, that may require immediate and/or long-term treatment. Individuals with CHD conditions suffer adverse cardiovascular outcomes in later life that incur substantial costs to the military and general public. Our work uses the large Ribosomal Subunit RpL13 as a central candidate in driving CHD, that helped identify novel downstream genes and pathways driving cardiac defects. Our work is helping to understand the genetic etiology of CHD, This helps inform clinicians allowing them to make more precise diagnosis and identify those patients requiring preventive measures to minimize exacerbation of the condition. Additionally, our work has identified genetic combinations that improve cardiac phenotypes, that could help direct development of interventions.

7. Therapy / Product Development, Transition Strategies, and Intellectual Property

Describe the steps and relevant strategies required to move the candidate product (knowledge or tangible) to the next phase of development and/or commercialization. Please address any issues with intellectual property.

PIs are encouraged to explore the technical requirements and the current regulatory strategies involved in product development as well as to work with their organization's Technology Transfer Office (or equivalent regulatory/legal office), federal/international regulatory experts, to develop the transition plan and to explore developing relationships with industry, DoD advanced developers (e.g. USAMMDA), and/or other funding agencies to facilitate moving the product into the next phase.

The development of Ribo-STAMP will be a valuable tool for translation biology and the fly community to explore changes in the translatome with single-cell resolution in an in vivo model. Upon our validation of APOBEC1 activity in flies and identifying RpL13 mRNA targets, we will make the flies available to the community for their research needs.

We will work with human geneticists to evaluate the RpL13-centric genetic network driving CHD we generated in CHD patient genomes. We will collaborate with a group from Mayo Clinic working with Hypoplastic Left Heart Syndrome patients, as well as a physician-scientist at Rady's Children Hospital who works with CHD patients, to perform this computational study.