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Congenital Heart Defects and the Expression of Ccdc141

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CONGENITAL HEART DEFECTS AND THE EXPRESSION OF *CCDC141*

by
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A thesis submitted to the faculty of the University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

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ABSTRACT

Congenital heart defects (CHDs) are one of the most prevalent types of birth defects in the United States. Both environmental and genetic components are known to contribute to the development of CHDs. One of the ways toxins present in the environment cause CHDs is by disrupting the expression of genes known to be vital in normal cardiac morphogenesis. Thus, the identification of both toxins that cause birth defects in cardiac development and genes expressed during heart development is crucial in order to fully understand the relationship between environment and genetics as they relate to CHD. Due to their external development, high fecundity, and knowledge of their genome sequence, zebrafish are an excellent model organism in which to study both gene expression and the role of toxins in cardiac development. Further, many zebrafish genes are orthologous to those found in humans, and thus allow genetic findings in zebrafish to be extrapolated to research on human disease and physiology. In order to identify genes expressed in the heart during development a single-cell RNA sequencing (scRNAseq) study was performed by the Bloomekatz laboratory, which preliminarily identified a novel gene *ccdc141* (coiled-coil domain containing 141) as having a role in cardiac development and possibly CHDs. Here, we have sought to validate these preliminary studies by determining the spatial and temporal gene expression of *ccdc141* throughout the early stages of zebrafish development. *In situ hybridization* (ISH) procedures utilized for this purpose are ideal for visualizing gene expression as the entire organism can be viewed at once. Our hypothesis is that *ccdc141* will be expressed in the heart of developing zebrafish. Results obtained through ISH

show that *ccdc141* is expressed in the heart and in cardiac precursors, as well as in somites and the head. These data will aid in further studies to determine the function of *ccdc141* in development. In addition to our work on identifying the gene expression of *ccdc141*, we created an extensive table of environmental toxins that disrupt cardiac development in zebrafish and may contribute to the pathogenesis of CHD in humans.

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LIST OF ABBREVIATIONS

CHD: congenital heart defect

scRNAseq: single-cell RNA sequencing

ccdc141: coiled-coil domain containing 141

myl7: myosin light chain 7

ISH: *in situ* hybridization

hpf: hours post fertilization

ALPM: anterior lateral plate mesoderm

ECG: electrocardiogram

YSL: yolk syncytial layer

SHF: second heart field

AV valve: atrioventricular valve

AVC: atrioventricular canal

AHR: aryl hydrocarbon receptor

ARNT: aryl hydrocarbon receptor nuclear translocator

CCGC: cell cycle gene cluster

PAH: polycyclic aromatic hydrocarbon

SV-BA: sinus venosus-bulbous arteriosus

SV: stroke volume

hERG: human ether-á-go-go gene

INTRODUCTION

Congenital heart defects (CHDs) are one of the most common forms of birth defects, affecting approximately 1% of live births and are a leading cause of infant mortality (5). Defects in the heart valves and septa formation, aortic and pulmonary stenosis, and Tetralogy of Fallot are a few examples of the different types of congenital heart defects, all of which can cause lifelong problems for patients (5). Oftentimes, if the birth defect is not severe enough to cause immediate problems at birth, it is not discovered until later in the child's life when further medical problems arise (6). Typically, patients experience pain or breathlessness during exercise, abnormal heart sounds, and fatigue, but all of these symptoms are nonspecific and could be the result of any number of other diseases, making CHD difficult to diagnose (7). This study seeks to analyze gene expression and exposure to environmental toxins in zebrafish as a first step towards a better understanding of the causes and mechanisms of CHDs in humans.

In order to determine the etiology of CHD, it is necessary to analyze the expression of genes that play a role in cardiogenesis. Gene expression is the foundation of development and cellular differentiation as different genes are regulated (turned on or off) based on the needs of the cell during different stages of development or in response to environmental factors, including environmental toxicants. Thus, the study of gene expression is vital in determining the etiology of diseases, including CHDs. Results collected from studying how and when a gene is expressed in normal development can be used as a control when looking at gene expression and function in diseased cells (8). By comparing the temporal and spatial gene expression patterns of a diseased

cell to that of a developmentally normal cell, we can identify which specific gene(s) of the diseased cell are incorrectly regulated and thus causing disease – an important step toward determining the function of the gene and ultimately new treatments and cures, like genetic therapies (9, 10).

I. Zebrafish as Model Organism

A model organism is one that can be used to study a wide range of biological disciplines (11). They are well studied organisms that are easily manipulated and maintained in a laboratory setting and offer the benefit of having gene orthologs in other species (12). In particular, model organisms are good for studying the molecular underpinnings of human physiology that cannot be sufficiently studied due to ethical or practical considerations. Model organisms are essential in biological research and offer a wealth of information, from which experimental results can be extrapolated to other species.

Zebrafish (*Danio rerio)* are a particularly valuable model organism for studying a variety of subjects, from fundamental biological processes such as gene expression, to modeling clinically relevant human diseases. Importantly, the entire genome of zebrafish has been sequenced, facilitating the study and identification of genes and genetic mutations. Zebrafish are orthologous to a variety of other organisms, including humans, in which around 70% of genes have a zebrafish ortholog (12). *ccdc141*, the gene of interest in this experiment, is one of those orthologs. Thus, the function of particular genes whose function in other organisms is unclear can be studied in zebrafish (13). Orthologous genes can be manipulated and mutated to observe how physiological, developmental and cellular processes in zebrafish are affected; these results can then be extrapolated to humans. This is particularly important in this study, as the main goal

is to determine the expression of *ccdc141* in zebrafish, a crucial first step in determining its function(s) in human development.

Zebrafish embryos have other unique characteristics that make them ideal model organisms. Principally, zebrafish embryos are transparent and undergo external development, allowing tissue structures, gene expression, and other processes to be easily observed (14). It also allows the researcher to easily access the embryo for genome manipulation and for live imaging. External development allows for the study of development immediately following fertilization and throughout development, since development occurs outside of the mother, a significant aspect not afforded by other species. Further, zebrafish have high fecundity, and thus are able to produce large numbers of embryos at once, providing more material for study, as opposed to an organism that produces only one or two embryos at a time (14). Hundreds to thousands of zebrafish can be stored in one room, saving both space and money. Lastly, zebrafish have a very quick maturation period, allowing us to observe embryonic development in its entirety much more quickly than could be done with organisms that take weeks or months to fully develop, again saving both time and money (14).

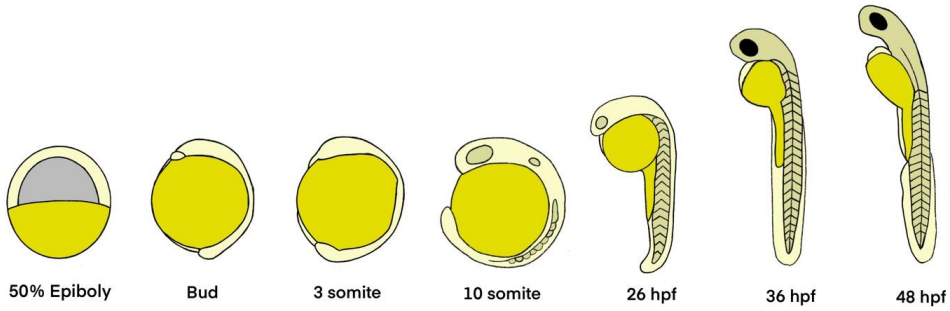


Figure 1. Stages of zebrafish development highlighted in this study. At 50% epiboly, the animal and vegetal poles are 50% covered by the blastoderm, which is uniform in thickness. Epiboly, the movement of blastoderm cells as they surround the yolk and enclose the blastopore then occurs, completely covering the yolk by the bud stage (as indicated by the prominence of the tailbud.) At 3 somites (s) stage, the somitic furrow has formed and the polster, the hatching gland rudiment underlying the forebrain, is prominent. In the 10s stage, the otic placode and trunk somites are visible. Linear heart tube elongation and initiation of a heartbeat are characteristics of 26 hours post fertilization (hpf), as well as pigmentation of the skin and retina. Motility begins at 36 hpf, and the atria and ventricle become distinct; cardiac looping is also occurring. By 48 hpf, the pectoral buds are elongated, heart looping is complete, and coordinated contraction of the two heart chambers begins. Adapted from (4).

II. *ccdc141*

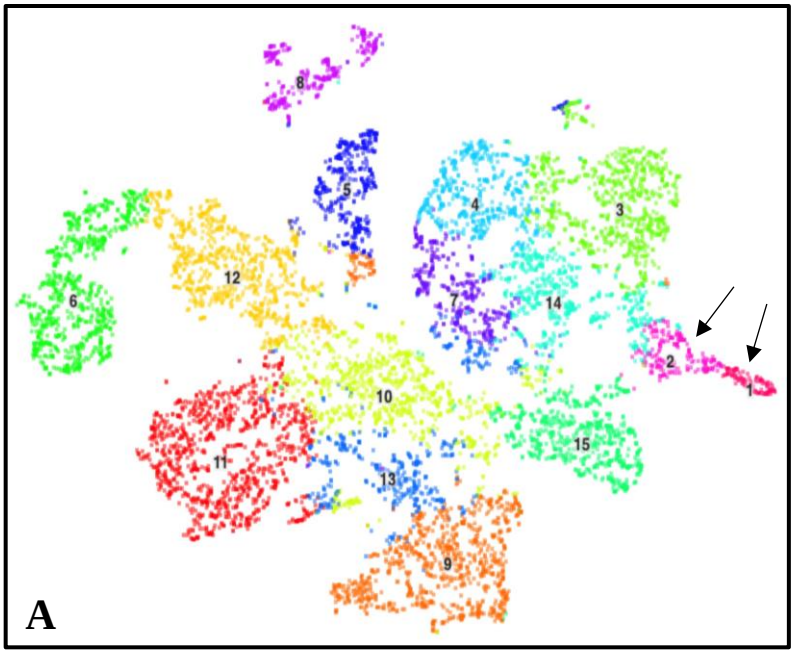
Congenital heart diseases have a well-known genetic component – babies are more likely to be born with a heart defect if the mother or another member of its immediate family also suffers from a CHD (7). *ccdc141* (coiled-coil domain containing 141), a protein coding gene located on chromosome 9 of zebrafish, could be one of the genes involved in the development of CHDs. *ccdc141* was identified through the single cell RNA sequencing (scRNA-seq) of mouse mesoderm cells, including progenitors and differentiated cardiomyocytes. A t-distributed stochastic neighbor embedding (t-SNE) visualization of this data clusters cells with similar gene expression patterns closer to one another and farther away from cells with varying gene expression (Figure 2A). Clusters 1 and 2 are the clusters of cardiomyocyte cells. Analysis of genes expressed in these clusters identified *ccdc141* as a gene highly expressed in these clusters and not in the other clusters.

ccdc141 has previously been implicated in the regulation of heart rate (15, 16). A genome wide association study identified five previously unknown loci associated with heart rate regulation including *ccdc141* (15). Observed via electrocardiogram (ECG), results showed that the associated SNP allele 'A' close to *ccdc141* is associated with a prolonged QRS duration, which slows the spread of ventricular depolarization, and increased heart rate. In fact, individuals with the associated SNP allele showed the highest increase in heart rate of all alleles identified (15).

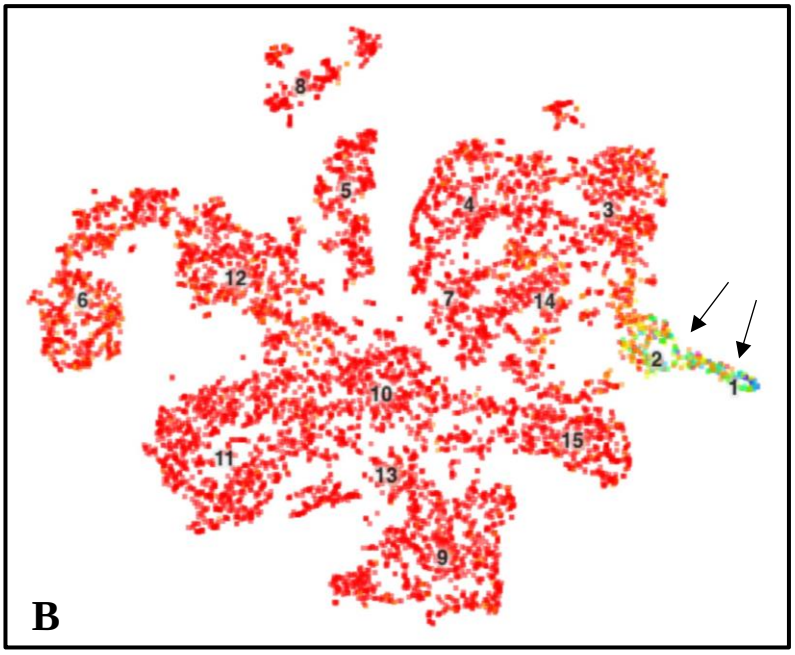
Additionally, *ccdc141* is has been shown to be involved in cellular migration in the nervous system, where the gene is heavily expressed. Due to this expression pattern, several studies have been conducted to determine the role of *ccdc141* in development and disease of the nervous system in humans. Mutations in *cdc141* have been identified in the development of

hypogonadotropic hypogonadism, a disease characterized by the dysfunction of the gonads (17). In this disease, *ccdc141* reduces the movement of migrating GnRH neurons, impairing gonadotropic releasing hormone (18).

The preliminary data described above regarding the role of *ccdc141* in the heart indicates that *ccdc141* variants/mutations could be involved in the development of CHDs. Based on these data, we will examine the dynamics of *ccdc141* expression in an attempt to shed further light on its function in the heart and its possible role in CHD. Use of the zebrafish *ccdc141* gene is ideal in this study, as it is orthologous to the human *ccdc141* gene; therefore, data collected regarding the function of *ccdc141* in zebrafish will likely be applicable to humans.



- Cardiomyocytes
- Developing cardiomyocytes
- Cranial pharyngeal mesoderm
- Somite mesoderm
- Endothelium
- Blood
- Pre-somitic mesoderm
- Epithelium
- Allantois
- Early extraembryonic mesoderm
- Nascent mesoderm
- Hemogenic mesoderm
- Caudal mesoderm
- Lateral plate mesoderm
- Late extraembryonic mesoderm



Range of Read/UMI	Frequency
0	89.5%
1-3	8.2%
3-5	0.80%
5-6	0.35%
6-8	0.46%
8-10	0.28%
10-12	0.23%
12-14	0.08%
14-15	0.10%
15-17	0.06%
17-26	0.01%

Figure 2. t-distributed stochastic neighbor embedding (t-SNE) plot of *ccdc141*. t-SNE is a dimensionality reduction algorithm mapped in two dimensions on a scatter plot. It is used in single cell RNA-seq to visualize similar groups of cells. Each dot represents a single cell, and cells are grouped together based on their transcriptional profiles. Cells with similar profiles, or similar gene expression, are grouped together into clusters, and the closer the clusters are to one another, the more similar their transcriptional profiles as well. **A)** Clusters of all cell types in mouse. Clusters are differentiated by number and color as noted in the legend. **B)** Range of expression of *ccdc141* in the clusters. As the cell colors move from red to magenta (range 0-26) as shown in the figure legend, the expression of *ccdc141* in those cells increases. It is highly expressed in clusters 1 and 2, corresponding to cells of the heart, indicated by arrows. Adapted from (2, 3).

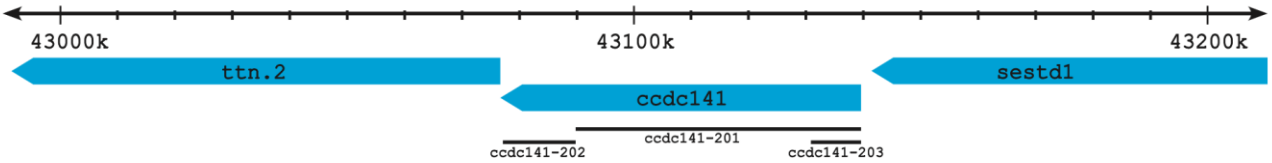


Figure 3. Location of *ccdc141* on chromosome 9 of zebrafish. *ccdc141* is located between 43,074,531 and 43,142,636 bp on chromosome 9, immediately downstream of *sestd1* and upstream of *ttn.2*. The direction of the arrows indicates the direction of the genes on the reverse DNA strand. 3 isoforms are indicated for *ccdc141*: ccdc141-201, ccdc141-202, ccdc141-203 with 25, 4, and 2 coding exons, respectively. Adapted from https://zfin.org/action/mapping/detail/ZDB-GENE-081104-472

III. Toxicology

Maternal exposure to environmental chemicals while pregnant, particularly in the first trimester, has been shown to increase the risk of CHDs in their children (7). Thus, both genetics and environmental factors contribute to cardiac developmental disruptions and CHDs. Chemicals and toxins present in the environment can affect gene expression by interrupting biological processes like DNA methylation, leading to defects in heart formation (19). Man-made contaminants are ubiquitous within our environment, deriving from a variety of origins including the burning of fossil fuels and organic matter, incomplete combustion of crude oil and coal, and insecticides and pesticides (20-24). Furthermore, they are often composed of multiple different components. For example, pesticides, insecticides, and herbicides are composed of compounds like carbaryl, glyphosate, and 2,4-dichlorophenoxyacetic acid (25, 26); emissions from automobiles and the burning of fossil fuels are composed of huge amounts of CO_2 and other compounds with multiple 6-membered carbon rings such as benzo-a-pyrene and phenanthrene (23, 27). However, the role of these chemicals in affecting cardiac development in both fish populations and in the human population is unknown. Similarly, it is unknown whether particular chemicals within a mixture disrupt cardiac development or whether disruption is a result of a combination of chemicals. Due to its high genetic and physiological conservation with humans and its external fertilization and development, zebrafish are a good model in which to investigate these questions. The ability to manipulate the zebrafish genome also make it a good model in which to determine the molecular and cellular processes disrupted by chemicals, which lead to these physiological defects. As an initial step towards the elucidation of these molecular pathogenic pathways, I conducted a literature search compiling a table (Table 1) of chemicals and toxins which cause cardiac defects in zebrafish embryos.

METHODS

I. In situ hybridization

Ia. Collection and Preparation of Embryos

Prior to beginning in situ hybridization, wild type (wt) embryos at the developmental stages 50% epiboly, bud, 3s, 10s, 26 hpf, and 36 hpf were collected. Two wt parents of the same line and equal size were transferred to a breeding tank with a divider placed between the fish. The tanks were then left overnight, and the divider was pulled the following morning at "daylight," around 8 am. To ensure time and stage of development, embryos were collected 1 hour later, placed in a petri dish with blue water, and left in the incubator at 28ºC to continue development.

When the embryos reached the desired stage of development, the chorions of the embryos at stages 24 hpf+ were removed (embryos <24 hpf were dechorinated post fixation in paraformaldehyde (PFA)) and embryos were placed in 1.5 mL centrifuge tubes. Next, the embryos were fixed in 4% fresh PFA overnight at 4ºC. The following day the embryos were dehydrated by washing sequentially in 25%, 50%, 75%, and two times in 100% MeOH/phosphate buffered solution/.1% tween (PBT) solutions for 10 minutes per solution before being placed in a 1.5 mL tube containing 100% MeOH and stored at -20ºC until needed.

Ib. Permeabilization

In preparation for ISH, two tubes of approximately 10 embryos per developmental stage, one for the control probe and one for the experimental, were rehydrated sequentially in 75%, 50%, 25% MeOH/PBT for 5 minutes per solution then washed 4x in PBT for 5 minutes at room temperature. Next, proteinase K (PK) was added to the embryos to digest the embryos so that the

RNA probe can enter the tissue and bind to *ccdc141*. PK at 10ug/ml was added to embryos at stages 24 hpf+. Embryos were left in PK for different times based on the stage of development: 5 minutes for 24 hpf embryos or 10 minutes for 36 hpf embryos. Embryos <24 hpf were incubated in 5 ug/ml PK for 4 minutes. After the addition of PK, embryos were rinsed twice in PBT to remove excess PK and fixed in 4% PFA for 20 minutes at RT. Then they were again rinsed with PBT 5x for 5 minutes per wash at RT.

Ic. Hybridization

After permeabilization by PK, embryos underwent prehybridization for 3 hours at 70ºC in 65% hybridization solution/PBT (hyb). The hybridization solution contains 50% formamide, 5x SSC, 500µg/ml yeast RNA, 50 µg/ml heparin, 0.1% Tween, and 9 mM citric acid. Following that, 7 µL of both the *ccdc141* and *myl7* probes were added separately to 200 µL 100% hyb and incubated for 20 minutes at 70ºC; the *ccdc141* probe was then added to one tube of embryos and the *myl7* probe was added to the other. Next, the embryos were hybridized overnight 16-24 hours at 70ºC.

Id. Probe Detection [blocking/antibody binding]

In preparation for blocking and antibody incubation, the hybridized embryos underwent a series of washes in the following order at 70ºC for 15 minutes each: 75% hyb/25% 2x saline-sodium citrate (SSC); 50% hyb/50% 2x SSC; 25% hyb/75% 2x SSC; and 2x SSC. Next, the embryos were washed twice for 30 minutes in 0.2x SSC at 70ºC. Following that was another series of washes performed in the following order for 5 minutes each at RT: 75% 0.2x SSC/25% PBT; 50% 0.2x SSC/50% PBT; 25% 0.2x SSC/75% PBT, 100% PBT. Embryos were then

blocked in a blocking buffer, which contains 5 mL PBT, 2mg/ml BSA, and 5% (0.25 mL) sheep serum, for 1 hour at RT. Next, 100 µL of the antibody anti-DIG-fab at a 1:5000 concentration was incubated in blocking buffer overnight at 4ºC. Digoxigenin and anti-DIG-fab were used as the label and antibody, respectively, because these have high specificity and reduce background, making them ideal for in situ hybridization (sigma).

Ie. Staining and Imaging

For the final stages of ISH, embryos were washed 6x for 15 minutes in PBT/BSA and then washed 3x with alkaline phosphatase buffer (NTMT) for 5 minutes. NTMT (an acronym of the first letter of the 4 ingredients) is a staining buffer containing 0.1M NaCl, 0.1M Tris-Cl, 0.05M $MgCl_2$, and 0.1% Tween. Embryos were transferred to clean staining dishes in NTMT and a staining solution was added to each well, followed by incubation in the dark at RT. Staining was checked every hour. Once the staining had developed and darkened, the embryos were then stopped via rinsing in NTMT and fixed in 4% PFA overnight at 4ºC. The next day, embryos were cleared by washing 5x in PBT/BSA for 5 minutes and then a 2x wash in MeOH for 5 minutes. After the washes, the embryos were incubated in MeOH at RT for 1-several hours. Lastly, Benzyl Benzoate:Benzyl Alcohol (BB:BA) clearing solution was added to the embryos for imaging via bright field microscopy.

GGTGACTTCCATCCTGACCATACATCAAGGTCAGTGCCGTGGTGACTTCCATCCTGACCA
TCTCACAGAGGAATCATTCTCCAATGACGAATACGAATGCACTTCTCCAGATGACATCT
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TAAGATTTTGGGGTCATTGAAAATGTGTATTTATTTGTATTTTAATAGAAATTTTGAAAT
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CTGTGGTGTGATTGTGTGACATAATAATCCACTCTTTGTTTAGGTTCAAGAACGGACACA
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ATTCACAGCTCTGCAGTGAGAGACTCTGGCCAATATGTGGTTACTGCCTCAAACTCAGC
CGGCACAGTGTCGTCCAGCTCCATGCTGCAGGTCAAAGGTAACGGCACTGACCTTGATG
TA

Figure 4: cDNA template probe sequence. Depicted is the complete template probe sequence of *ccdc141*. The probe is complementary to the sequence of *ccdc141* and will hybridize with the DNA. After probe hybridization, staining of the antibody bound to the probe will show the location(s) of gene expression. Sequence from UCSC Genome Browser.

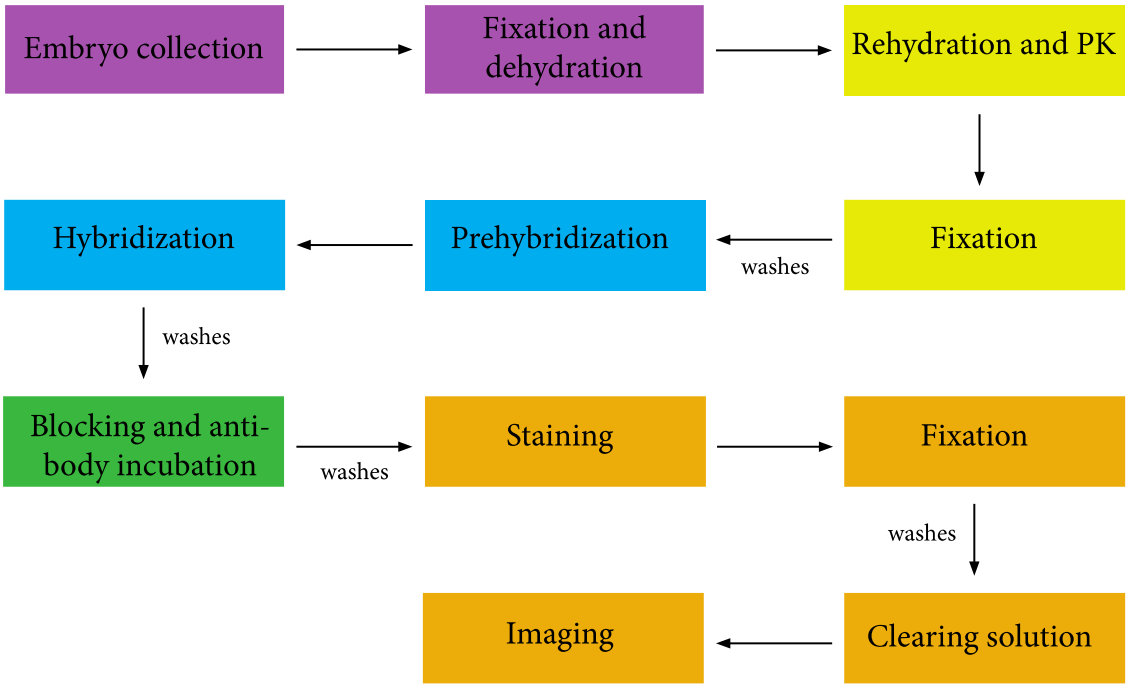


Figure 5. Flow chart of in situ protocol. Purple boxes indicate collection and preparation steps (IIa). Yellow boxes indicate denaturation steps (IIb). Blue boxes indicate hybridization steps (IIc). Green box indicates blocking and antibody detection step (IId). Orange boxes indicate staining and imaging steps (IIe). Figure adapted from (1).

II. Literature Search for Toxicants

For the literature search on environmental toxicants related to CHD, I used the PubMed/NCBI database for researching, the Mendeley program for compiling the articles and creating citations, and the ChemDraw software for creating the chemical structures shown in Table 1 (28-30). Keywords used for the search are as follows: environmental toxins, congenital heart defects, zebrafish toxins, embryonic developmental toxicity, and zebrafish environmental contaminants.

RESULTS

Part I: *ccdc141* is a gene with little known function, but knowing the specific location(s) and developmental stages during which it is expressed can help to elucidate its function. *ccdc141* was found to be expressed in zebrafish tissues that form and derive from the mesoderm germ layer, including those ultimately giving rise to the fully formed heart and the heart itself.

Part II: We have compiled the published findings of environmental toxicants which disrupt cardiac function in zebrafish.

Part I. *In situ* hybridization reveals location of *ccdc141* expression in zebrafish embryos.

In situ hybridization (ISH) allows for the visualization of gene expression. Thus, we have sought to perform *in situ* hybridization of *ccdc141* at different stages of zebrafish development, in order to aid in the effort of determining the functional relationship of the gene to zebrafish heart development. We sought to analyze *ccdc141* expression at the stages: 50% epiboly; bud; 3 and 10 somite stages; prim-5 and prim-25 stages; and long-pec stage of zebrafish. These stages were chosen because they are representative of the gastrula, segmentation, and pharyngula periods of zebrafish development. They are important periods in regard to heart development, as the heart begins formation in the gastrula period and nearly completes formation by the end of the pharyngula period. Cardiac progenitor cells first become evident in the 50% epiboly stage, located on either side of the lateral marginal zone of the embryo (13). In the bud and 3s stages, cardiac progenitor cells move dorsally toward the midline to reach the anterior lateral plate mesoderm (ALPM) and arrive at the embryonic axis by the 10s stage (31). The heartbeat and vascular circulation occur by the 26 hpf stage, after the fusion of the two tubular cardiac

primordia form the definitive heart tube (31). By 36 hpf, heart looping is complete, and the atrial and ventricular boundaries are clearly distinguished (31).

Ia. *myl7* expressed in myocardial precursors and myocardium.

ISH of *myl7* was used as a control because it has a well-known expression pattern in the heart. By comparing *myl7* expression to that of *ccdc141*, we can determine if the ISH was performed correctly (if *myl7* is expressed) or if the experiment was not successful (if there is no expression of *myl7*). ISH of *myl7* during cardiac looping in the 24 hpf and 36 hpf stages revealed *myl7* expression in the atrial and ventricular myocardium of the heart tube (Figure 7).

Ib. *ccdc141* is expressed in tissues related to cardiogenesis as well as other tissues.

Beginning with the gastrula period, embryos at 50% epiboly exposed to the *ccdc141* experimental probe exhibited expression of the gene in the yolk syncytial layer (YSL) (Figure 8A-C). The YSL is an important multinucleated extra-embryonic tissue located beneath the cellular blastoderm. It plays a vital role in zebrafish development through its involvement in coordinated cell movement, morphogenesis of the germ layers, specification of cell identity, and cardiac morphogenesis (32, 33).

At the bud stage, *ccdc141* appears to be expressed in the mesoderm of the embryo (Figure 8D-F). The mesoderm is one of the three germ layers that gives rise to many structures and tissues in the developing zebrafish, including the heart muscle.

In the 30 minutes of development after the bud stage, embryos enter the 3s stage in which *ccdc141* was found to be expressed in the polster and the paraxial, or presomitic, mesoderm (Figure 8G-I). One of the three domains of mesoderm, the paraxial mesoderm differentiates into

the somites, which are blocks of mesoderm that eventually give rise to a variety of vertebrate body structures, including the dermis, skeletal muscle, cartilage, tendons, and vertebrae (34, 35).

Expression of *ccdc141* in 10s embryos also was found to occur in specific types of mesoderm: the anterior lateral plate mesoderm (ALPM) and the somites (Figure 8J-L). The ALPM is a derivative of another of the three domains of mesoderm, the lateral plate mesoderm, which forms progenitor cells for several tissue types, including the heart and cardiovascular system (36).

By 22 hours post-fertilization (hpf), the linear heart tube of the zebrafish has formed and at 26 hpf, *ccdc141* expression was visualized in the somites and the atrial, ventricular, and possibly endocardial cells of the heart tube, though it is difficult to differentiate atrial/ventricular cells from endocardial cells at the magnifications used in this study (Figure 8M-P) (37). At this point, the peristaltic contraction of the heart tube occurs, allowing for blood circulation. Furthermore, this stage marks the beginning of cardiac looping, during which the linear heart tube begins to form into an S-shaped loop. Expression in the ventricular and atrial cardiomyocytes, somites, and head was present at 36 hpf; cardiac looping continues, and the atrium and ventricle become distinguishable at this stage (Figure 8Q-R). (Images presented in Figure 8A-P are unpublished data from the Bloomekatz lab, not my own, as I was unable to image these embryos personally due to the COVID-19 pandemic. However, figures 1-7, figure 8Q and R, and table 1 are my own).

Determining the tissues in which *ccdc141* is expressed at each stage was done by comparing the shape and position of the *ccdc141* expression pattern with the known shape of those tissues as observed through brightfield microscopy or via the expression of other genes known to be expressed in those tissues, such as *myl7*, which is known to be expressed in the

cardiac progenitors within the ALPM (10s) and in the heart (22s-36 hpf). However, further analysis such as the double fluorescent ISH is needed to definitely assign the identity of the tissues in which *ccdc141* is expressed.

By following the expression of *ccdc141* from the 50% epiboly stage to 36 hpf (Figures 6, 8), we see a clear and consistent pattern of *ccdc141* expression in tissues involved in cardiac morphogenesis; thus, it follows that *ccdc141* may play a role in the development of the heart in some capacity, although further study is needed to determine its exact function.

The information on *ccdc141* expression gathered from the study of these stages in zebrafish can then be used to facilitate further research into determining the role of *ccdc141* in normal heart development and in the development of CHD in humans.

	YSL	Mesoderm	Paraxial Mesoderm	ALPM	Somites	Ventricular CM	Atrial CM	Endocardial Cells	Head	Polster
50% Epiboly										
Bud										
3 somite										
10 somite										
26 hpf										
36 hpf										

Figure 6. Chart of *ccdc141* expression in various tissues. Blue color filled cells represent *ccdc141* expression in the corresponding tissue and developmental stage.

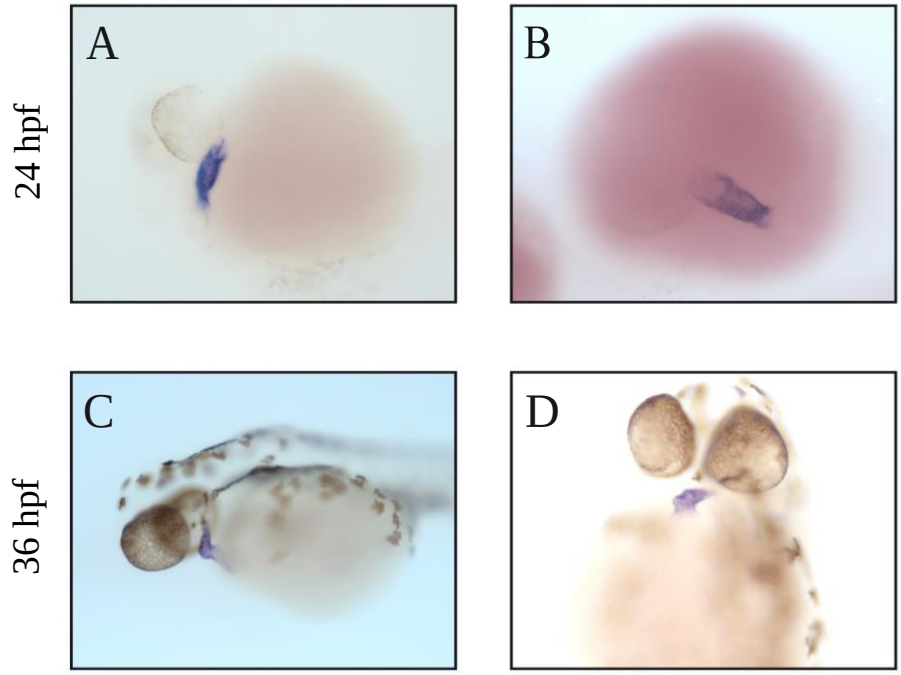


Figure 7. Images of *myl7* expression in studied stages of zebrafish development. **(A-B)** Expression in the atrial and ventricular portions of the heart, lateral view (A) and anterior view (B). **(C-D)** Expression in the atrial and ventricular portions of the heart, lateral view (C) and ventral view (D).

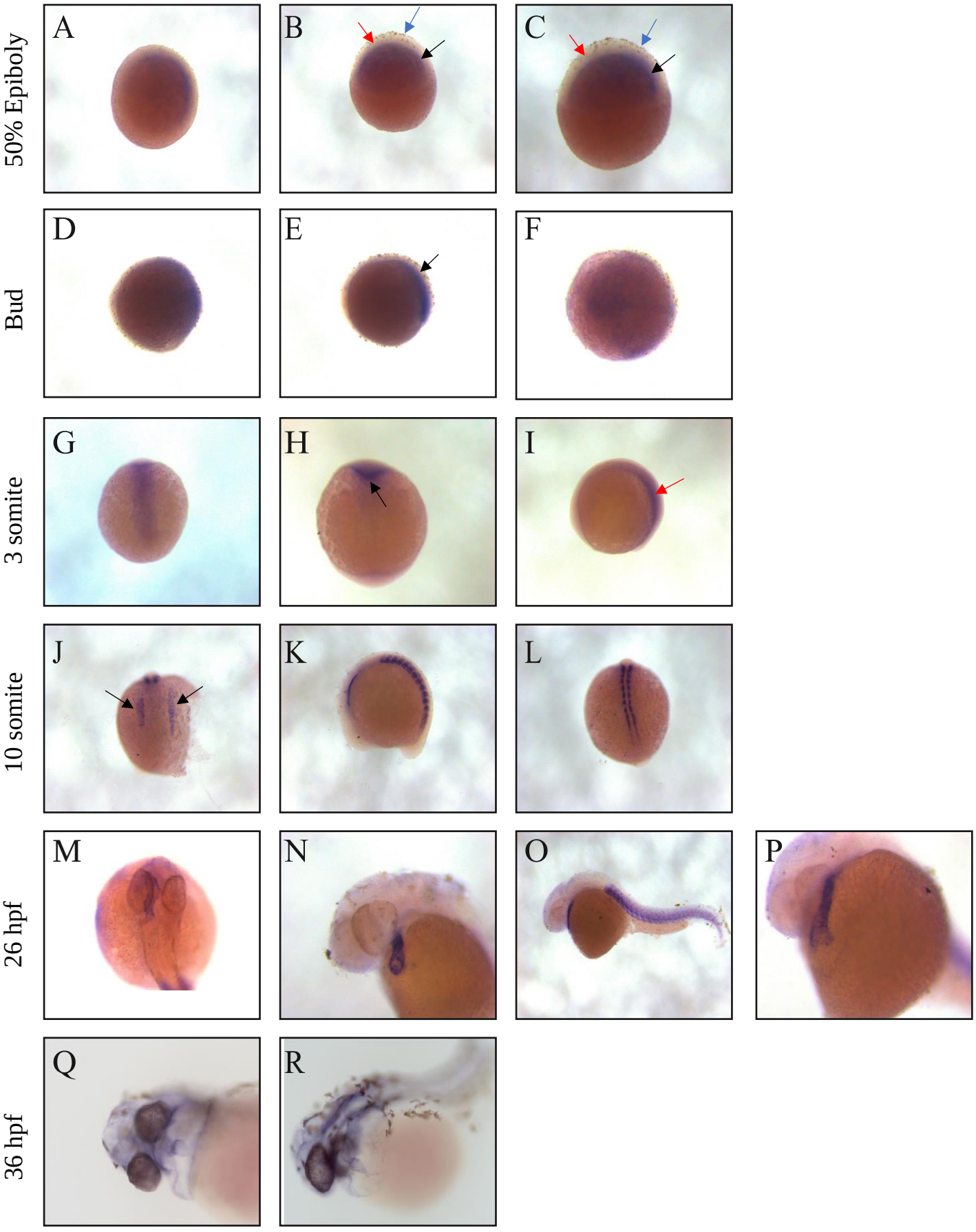

50% Epiboly
Bud
3 somite
10 somite
26 hpf
36 hpf
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R

Figure 8. Images of *ccdc141* expression during zebrafish development. (A-C) Expression in the yolk syncytial layer (YSL), animal pole (A) and lateral (B, C). YSL is visible through the enveloping layer (EVL) and blastoderm, which can be clearly distinguished (B, C). YSL, EVL, and blastoderm are designated by black, blue, and red arrows, respectively. **(D-F)** Expression in the mesoderm, anterior (D), lateral (E), and posterior views (F). The blastoderm is thicker on the dorsal side of the embryo at this stage, indicated by the arrow (D, E). **(G-I)** Expression in the polster (indicated by black arrow) and paraxial mesoderm (red arrow), anterior (G), dorsal (H), lateral (I) views. **(J-L)** Somites and ALPM expression, dorsal (J), lateral (K), posterior (L) views. Bilateral ALPM expression is distinct in (J), indicated by arrows. ALPM and somite expression are both visible in (K), and sequential blocks of somite expression are visible in (L). **(M-P)** Expression in atrial and ventricular regions of the heart, dorsal (M), lateral (N-P) views. Heart tube is visible just to the left of the midline (M). Somites and heart tube visible (O). **(Q-R)** Expression in the head, somites, and atrial and ventricular cardiomyocytes, ventral (Q), lateral (R) views. Images A-M, O were all taken at 300x magnification. Images N, P Q, R were taken at higher magnification. Images of A-P are Courtesy of Bloomekatz Lab, as the COVID-19 pandemic prevented the completion of this project. Images Q, R were taken by me.

Part II. Toxicology

The genetic and environmental components of CHD are highly interrelated and work in concert during the progression of heart defects. Duration of toxin exposure, concentration of exposure, and stage of development in which exposure occurred all affect the severity of the cardiac phenotype. Observed abnormal phenotypes vary among the toxins, but abnormal heart looping, ventricular abnormalities, and misplaced heart chambers are among the most common phenotypes observed in zebrafish embryos. Here we have compiled a list of toxins identified causing cardiac phenotypes in zebrafish embryos and we have highlighted a few more for in-depth description. Although the presented toxins are known to contribute to CHD, the complete etiology of their contribution to disease pathogenesis is still not well known and requires further study.

IIa. Ethanol

Changes in development caused by ethanol exposure are characterized by changes in gene expression, cell localization, and cellular adhesion, resulting in the disruption of several stages of cardiogenesis in zebrafish, including, but not limited to, myocardial migration, heart looping, and endocardial cushion formation (38). Both duration and concentration of exposure to ethanol is important, as increased concentrations and increased time of exposure produce more severe abnormalities. Short term exposure prior to cardiac specification or during myocardial midline migration does not produce persistent or severe morphological defects. However, once cardiac specification or myocardial midline migration is complete, short term exposure may cause atypical heart development. Exposure over the entire course of cardiac development results

in the most severe abnormal phenotypes, including defects of the atrioventricular (AV) valves and abnormal heart looping (Table 1: Row 22) (38, 39).

Disruption of the expression of genes known to be crucial in cardiac development may be the primary factor in producing these defects. Several genes, including *hand2*, *gata5*, *fgf8a*, *myl7*, and *vmhc* are affected, as are the Bmp and Notch signaling gradients. In control embryos, localization of these signaling cells is limited to the atrioventricular canal (AVC), whereas in embryos exposed to ethanol, Notch and Bmp signaling cells were present throughout the entire ventricle (39, 40).

IIb. Tetrachlorodibenzo-para-doxin (TCDD)

TCDD is an aryl hydrocarbon receptor (AHR) agonist. AHR mediates toxicity by regulating the expression of genes with functions ranging from olfactory receptor to lipoprotein lipase. Without a bound ligand, AHR resides in the cytoplasm in a complex with heat shock protein 90, but upon binding of a ligand, like TCDD, the AHR complex undergoes a conformational change, allowing it to translocate to the nucleus. Once in the nucleus, the AHR complex dimerizes with aryl hydrocarbon receptor nuclear translocator (ARNT). The ARNT heterodimer then binds to dioxin response elements (DREs) in the promoter region upstream of AHR-responsive genes and directs their transcription (41, 42). Binding of TCDD to AHR results in the downregulation of a group of genes responsible for cell division and growth, DNA repair, and cell cycle regulation, known as cell cycle gene cluster (CCGC). This downregulation results in a reduction in the number of cardiomyocytes, as well as increased expression of *bmp4* and *notch1b*, two genes necessary for proper heart valve formation (43, 44). Heart abnormalities caused by TCDD are observable at 72 hpf. Abnormal heart looping, reduced ventricle size, and

thin, elongated atrium are cardiac morphologies characteristic of TCDD exposure (Table 1: Row 38) (43).

IIc. Phenanthrene

Phenanthrene is an AHR agonist that is ubiquitous in the aquatic environment with well-known cardiotoxic effects, including arrhythmias and disruption of the later stages of cardiac morphogenesis by inhibiting cardiac conduction (45, 46). Phenanthrene exposure disrupts expression of genes involved in cardiac morphology, function, and specification such as matrix metalloproteinase-9 (MMP-9) whose expression is increased upon phenanthrene exposure, altering the basement membrane surrounding the cardiomyocytes and disrupting cell signaling (23). Additionally, expression of SERCA2a Ca^{2+} pump and its direct regulator, *tbx5*, are both decreased. Calcium plays a vital role in myocyte contraction, and decreased expression of these genes results in cardiomyocyte dysfunction (46). Morphological cardiac defects such as an enlarged ventricle with thin ventricular wall, abnormal heart looping, and misplaced chambers are also characteristic of phenanthrene exposure (Table 1: Row 31).

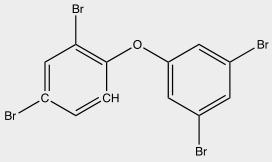
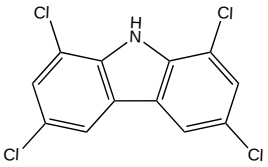
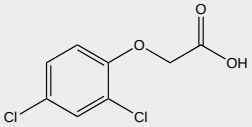
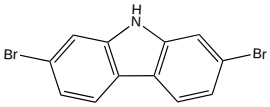
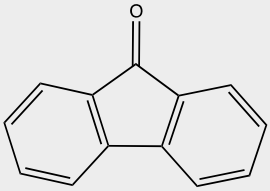
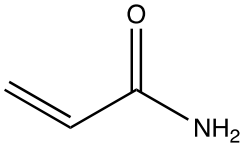
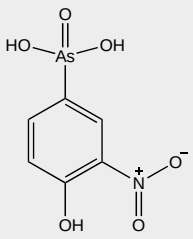
IId. Pyrene

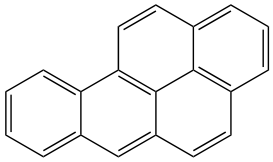
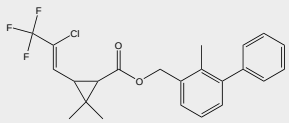
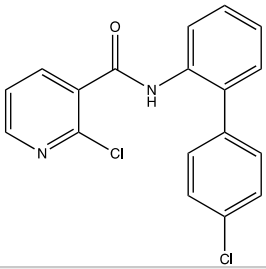
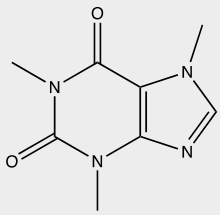
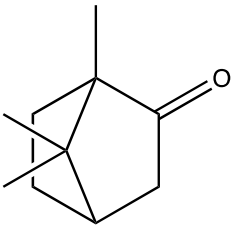
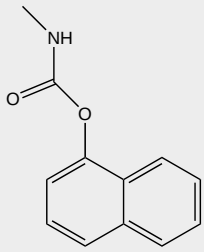
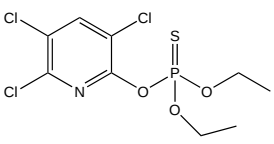
Pyrene is a polycyclic aromatic hydrocarbon (PAH) and a weak AHR agonist (Table 1: Row 34). Like several other toxins, the cardiotoxicity of pyrene acts in a dose-dependent fashion, with more severe abnormalities being directly proportional to increasing concentrations of the toxin (24). Exposure to pyrene results in the downregulation of Nkx2.5, a transcription factor that plays an essential role in the development of the cardiovascular system and in the differentiation of myocardial cells. Expression of *bmp2b*, an upstream gene of Nkx2.5, was

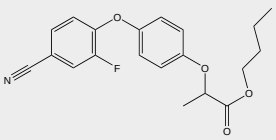
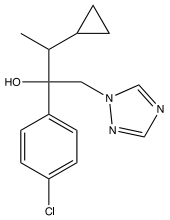
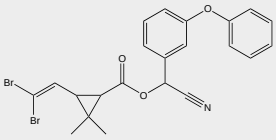
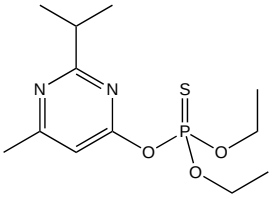
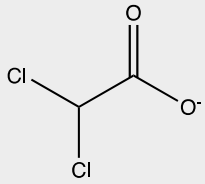
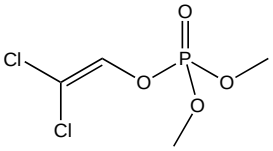
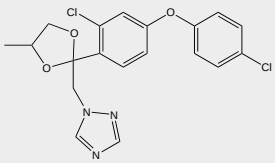
downregulated as well (24). Altered expression of these genes leads to pericardial edema and abnormal cardiac looping, along with elongated and misoriented heart chambers in zebrafish (Table 1: Row 34).

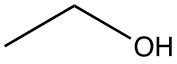
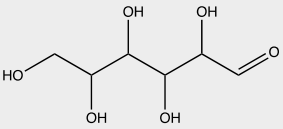
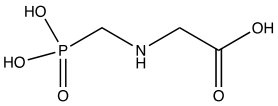
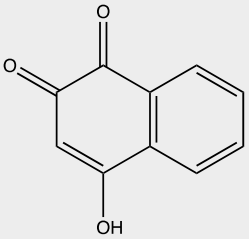
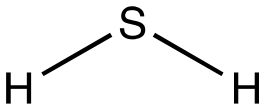
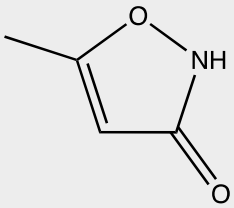
IIe. Benzo-a-pyrene

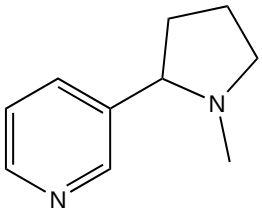
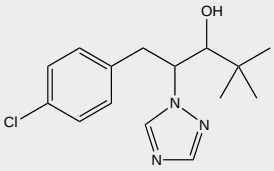
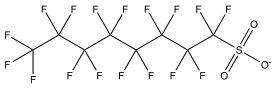
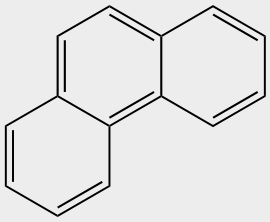
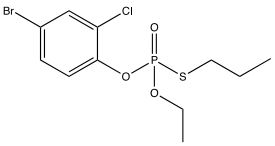
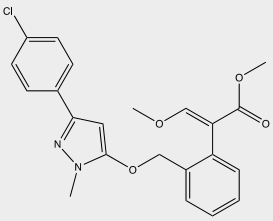
Like several other PAHs, cardiac toxicity of benzo-a-pyrene [B(a)P] is related to AHR binding and the disruption of gene expression. Heart abnormalities occur in a dose dependent manner, with increasing concentration of B(a)P leading to a higher frequency of abnormal cardiac morphologies, like pericardial edema, bradycardia, and abnormal heart looping (Table 1: Row 8) (27). A network of genes responsible for regulating cardiogenesis, listed as follows, were disrupted by exposure to B(a)P – the genes *prox1*, *tbx5* and *pak2a* were downregulated upon B(a)P exposure, while *ahr1b*, *cyp1c1*, *cyp1a1*, *mmp9*, *prox1*, *tbx5*, *pak2a*, and *mmp2* were upregulated (27). Exposure to B(a)P results in elongated hearts, a thin atrium and small ventricle, and misplaced, non-overlapping chambers (Table 1: Row 8) (27).

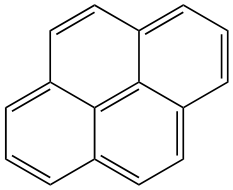
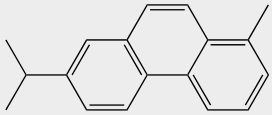
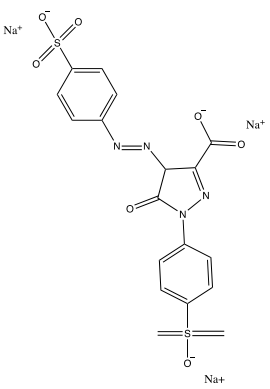
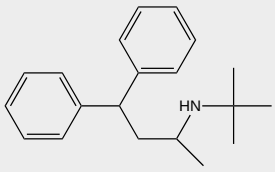
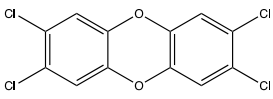
TOXICANT NAME	CHEMICAL STRUCTURE	CARDIAC PHENOTYPE	ORIGIN	REFER-ENCES
2,2,4,4-tetrabromodiphenyl ether		Tachycardia Arrhythmias	Plastics and flame retardants	(47)
2,3,6,7-tetrachlorocarbazole		Pericardial edema Elongated heart	Organic pollutant	(48)
2,4-dichlorophenoxyacetic acid		Pericardial edema Tachycardia	Pesticide; synthetic plant hormone	(49)
2,7-dibromocarbazole		Pericardial edema Elongated heart	Organic pollutant	(48)
9-fluorenone		Bradycardia	Organic pollutant	(50)
Acrylamide		Bradycardia Cardiomyocyte proliferation reduced Abnormal atria and ventricles Failed differentiation of the atrioventricular canal	Hydrogenation of acrylonitrile	(51)
Arsenic		Tachycardia Abnormal cardiac morphogenesis	Water contaminant	(52), (53)

Benzo-a-pyrene		Bradycardia Pericardial edema Abnormal heart looping Irregular heartbeat Smaller ventricle and thinner atria	Forest fires; volcanic eruptions; oil spills; automobile emissions	(27), (54)
Bifenthrin		Pericardial edema	Insecticide	(20)
Boscalid		Pericardial edema Bradycardia	Pesticide	(21)
Caffeine		Tachycardia	Found naturally in some plants Can be synthetic	(55)
Camphor		Pericardial edema	Found in camphor trees; insecticide	(56)
Carbaryl		Bradycardia Heart malformation	Insecticide	(57)
Chlorpyrifos		Bradycardia	Pesticide; acetylcholinesterase inhibitor	(58)

Cyhalofop-butyl		Pericardial edema Bradycardia	Herbicide	(59)
Cyproconazole		Pericardial edema	Fungicide	(60)
Deltamethrin		Pericardial edema Tachycardia Decreased blood flow	Insecticide	(20), (61)
Diazinon		Cardiac edema	Pesticide; acetylcholinesterase inhibitor	(58)
Dichloroacetate		Tachycardia (<144 hpf) Bradycardia (>144 hpf)	Chlorination of drinking water	(62)
Dichlorvos		Bradycardia	Pesticide; acetylcholinesterase inhibitor	(58)
Difenoconazole		Bradycardia	Fungicide	(63)

Ethanol		Pericardial edema Reduced heart size Reduced ventricular thickness Abnormal heart looping Cardiac bifida Cardiac fusion	Fermentation of yeast	(64), (45)
Glucose		Severe arrhythmia Myocardial hypertrophy Myofibril loss	Plants and fruits	(65)
Glyphosate (Roundup Ultramax)		Bradycardia	Herbicide	(66)
Henna		Pericardial sac edema Slow heartbeat	Leaves of *Lawsonia inermis*; ink for hair dye and tattoos	(67)
Hydrogen Sulfide		Tachycardia (low sulfide concentrations) Bradycardia (high sulfide concentrations) Pericardial edema Absence of heartbeat and blood circulation	Petroleum refineries	(68)
Hymexazol		Bradycardia Cardiac edema	Fungicide	(69)

Nicotine		hERG blocker Tachycardia High blood pressure	Tobacco plant	(70), (71)
Paclobutrazol		Pericardial edema Abnormal heart looping Bradycardia	Fungicide	(72)
Perfluorooctanesulfonate (PFOS)		Pericardial edema Tachycardia (larvae) Bradycardia (embryos) Heart malformations	Breakdown of perfluorinated chemicals (PFCs).	(73)
Phenanthrene		Pericardial edema Abnormal heart looping Enlarged ventricle Thin ventricular wall Tachycardia	Burning of fossil fuels, oil, wood, and organic matter; polycyclic aromatic hydrocarbon	(23)
Profenofos		Pericardial edema Abnormal heart looping Bradycardia Thin atria wall	Pesticide	(74)
Pyraoxystrobin		Pericardial edema Bradycardia Arrhythmia	Fungicide	(75)

Pyrene		Pericardial edema Abnormal heart looping Tachycardia Decreased stroke volume	Incomplete combustion of crude oil and coal	(24)
Retene		Pericardial edema Reduced blood flow Enlarged atria with thinner walls Reduced ventricle size Underdevelopment of bulbus arteriosus	Crude oil; paper mills	(22)
Tartrazine		Pericardial edema Bradycardia	Artificial colorant	(76)
Terodiline		2:1 AV block	Organic compound	(77)
Tetrachlorodibenzo-*para*-dioxin (TCDD)		Bradycardia Decrease in overall size of heart Defects in AV valves Pericardial edema	Byproduct of incomplete combustion	(78), (79)

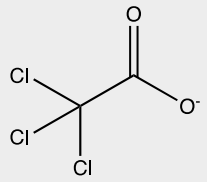
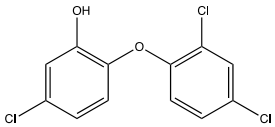
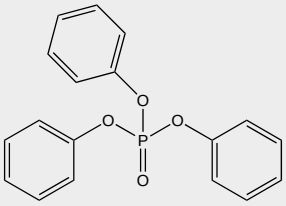
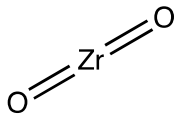
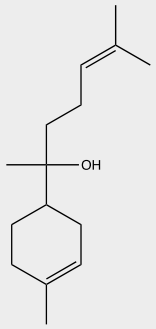
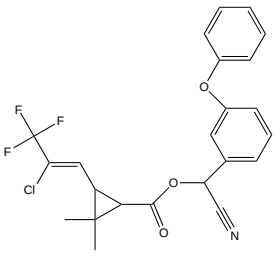
Trichloroacetate		Pericardial edema	Chlorination of drinking water	(80)
Triclosan		Pericardial edema	Personal care products; antibacterial and antifungal agent	(81)
Triphenyl Phosphate (TPP)		Blockage of cardiac looping Pericardial edema Bradycardia	Flame retardants; aryl phosphate ester	(82), (83)
Zirconia Oxide nanoparticles		Pericardial edema	Bioceramic metal compound	(84)
α-Bisabolol		Pericardial edema Bradycardia Thrombosis Hemorrhage Increased SV-BA distance Cardiac arrhythmia	Personal care products and pharmaceuticals	(85)
λ-cyhalothrin		Pericardial edema	Insecticide	(20)

Table 1. Environmental toxins related to congenital heart disease highlighted in this study. Toxins are listed alphabetically. Tachycardia and bradycardia are abnormally rapid and slow heart rates, respectively. Pericardial edema is the accumulation of excess fluid in the pericardial sac surrounding the heart, putting pressure on the heart and affecting its ability to contract properly. Arrhythmias are abnormal or irregular heartbeats, occurring when the electrical circuit of the heart is malfunctioning. Stroke volume (SV) is the amount of blood pumped by the ventricle in one contraction. The bulbus arteriosus (BA) is the outflow tract component of the zebrafish heart that maintains constant blood flow to the gills, and the sinus venosus (SV) is the inflow tract of the heart, delivering blood into the atrium. A 2:1 atrioventricular (AV) block is a conduction block in the heart that prevents cardiac impulse transmission between the atria and ventricle. A thrombosis is a blood clot in the circulatory system. A hERG (human ether-á-go-go gene) is a potassium channel gene that, if disrupted, can cause lethal arrhythmias.

DISCUSSION

I. *ccdc141* gene expression

Through the use of in situ hybridization, we observed *ccdc141* expression in the yolk syncytial layer (YSL) at 50% epiboly stage; mesoderm at bud stage; polster and paraxial mesoderm at 3s stage; anterior lateral plate mesoderm (ALPM) and somites at 10s stage; heart and somites at 26 hpf stages; and heart, somites, and head in 36 hpf stage. These findings indicate that variants/mutations of *ccdc141* could be involved in the development of CHDs and are important for understanding the role of *ccdc141* in heart development because the location(s) and time(s) at which a gene is expressed provide insight into its function. For example, if a gene is expressed only during development and not in adult organisms, the gene likely only functions in development; or if a gene is not expressed in a tissue, it is unlikely to serve a function in that tissue.

Due to time constraints, we were unable to observe the 16s, 22s, 48 hpf and 72 hpf stages as originally planned. Studies of the 16s and 22s stages would provide more information on *ccdc141* expression throughout the segmentation period where growth is rapidly occurring, as the presented results provide data on only the first half of the period. These stages correspond to a specific time period in cardiac development: 16s when cardiomyocytes are undergoing angular movement to perform cardiac fusion and 22s when cardiac fusion has completed. Analysis of the 48 hpf stage would provide information on the hatching period, and the 72 hpf stage would offer data on expression at the last stage of development before the start of the larval period, when cardiac trabeculation is starting.

These results lay a foundation upon which further study of the role of *ccdc141* in development can be performed. We found that *ccdc141* is expressed in both cardiac

primordium/ALPM mesoderm and in neural tissue. Expression in the neural tissue is consistent with previous studies implicating *ccdc141* in neuronal migration; thus, *ccdc141* might serve a similar function in the ALPM, which is undergoing collective migration towards the midline of the zebrafish to fuse and create a single heart. We also found that *ccdc141* is expressed in the heart and somite tissue, which will become trunk muscle, indicating that *ccdc141* may be important for musculoskeletal development as well. In light of this information, future ISH experiments could be conducted to determine *ccdc141* expression in the larval, juvenile, and adult periods of development. This would allow for a more thorough and detailed view of expression throughout the entire lifespan and, together with the presented data, would help determine if *ccdc141* is expressed and functioning only in development, or if it plays a role in adulthood as well. Results on function can then be extrapolated to humans, as *ccdc141* in zebrafish is orthologous to that in humans and thus may retain the same function. This use of zebrafish as model organisms is a key component in research because it bypasses many of the ethical concerns that come with research on humans, which allows us to study diseases and test possible treatments in a practical way.

II. Toxicology

Through extensive research, a table of 44 known cardiotoxins was compiled to be used in the publication of a review chapter, written by the Bloomekatz lab, regarding the genetic and environmental components of CHDs (86). The mechanism by which the environmental toxins presented induce cardiotoxicity is unknown. In line with the research on the role of toxins in developmental abnormalities more broadly, we see that the severity of abnormal cardiac

phenotypes is dependent on both the duration and concentration of exposure, as well as the developmental stage at which exposure occurs.

Distinct types and classes of chemicals produce similar phenotypes. For example, polycyclic aromatic hydrocarbons (PAHs), like phenanthrene, pyrene, and benzo-a-pyrene, disrupt the aryl hydrocarbon receptor (AHR) pathway. AHR is important transcription factor in the regulation of toxicity in cells. PAHs act as ligands for AHR, and upon binding, can be bioactivated to produce toxic metabolites (87). Phenotypes typical of PAHs include elongated hearts and misplaced heart chambers. Pesticides, insecticides, and fungicides also produced similar phenotypes; nearly all of these compounds result in pericardial edema, bradycardia, or tachycardia. In all of the toxins presented, tachycardia, bradycardia, and edema were the most common phenotypes.

It is my hope that this table can be used as an at-a-glance reference of environmental toxicants that contribute to the development of CHD, as well as a basis for further studies. In combining the two parts of my thesis, an interesting line of experimentation could involve analyzing the connection between *ccdc141* and environmental toxins in CHDs. For example, determining if *ccdc141* expression through ISH during development is disrupted in zebrafish embryos exposed to the cardiotoxins presented in this work. Lastly, future experiments could be performed to determine what correlation, if any, exists between toxicant exposure and normal expression of *ccdc141*.

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