Development of a zebrafish model for inherited cardiac electrical disorders

by

Kyle Simpson

B.Sc. (Biological Sciences), Simon Fraser University, 2016

Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
Doctor of Philosophy

in the
Department of Biomedical Physiology and Kinesiology
Faculty of Science

SIMON FRASER UNIVERSITY
Spring 2024

Declaration of Committee

Name:	**Kyle Simpson**
Degree:	**Doctor of Philosophy (Biomedical Physiology & Kinesiology)**
Title:	**Development of a zebrafish model for inherited cardiac electrical disorders**
Committee:	**Chair:** **Dawn Mackey** Associate Professor, Biomedical Physiology and Kinesiology
	Thomas Claydon Supervisor Professor, Biomedical Physiology and Kinesiology
	Esther Verheyen Committee Member Professor, Molecular Biology and Biochemistry
	Glen Tibbits Committee Member Professor, Biomedical Physiology and Kinesiology
	Megan Barker Committee Member Senior Lecturer, Biological Sciences
	Peter Ruben Examiner Professor, Biomedical Physiology and Kinesiology
	Eric Accili External Examiner Associate Professor, Cellular and Physiological Sciences University of British Columbia

Ethics Statement

The author, whose name appears on the title page of this work, has obtained, for the research described in this work, either:

a. human research ethics approval from the Simon Fraser University Office of Research Ethics

or

b. advance approval of the animal care protocol from the University Animal Care Committee of Simon Fraser University

or has conducted the research

c. as a co-investigator, collaborator, or research assistant in a research project approved in advance.

A copy of the approval letter has been filed with the Theses Office of the University Library at the time of submission of this thesis or project.

The original application for approval and letter of approval are filed with the relevant offices. Inquiries may be directed to those authorities.

Simon Fraser University Library
Burnaby, British Columbia, Canada

Update Spring 2016

Abstract

Long-QT Syndrome (LQTS) is a cardiac electrical disorder distinguished by irregular heart rates and sudden cardiac death. LQTS Type II (LQTS2), which accounts for approximately 40% of cases, is caused by loss-of-function mutations in the Kv11.1 (hERG) channel. LQTS-associated hERG channel variants are typically studied in heterologous expression systems, or induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs); however these single cells/tissue layers do not recapitulate the complexities of a whole organ or organism. Zebrafish have a similar cardiac electrophysiological profile to humans and a well-defined genome, and have thus emerged as a powerful tool to study LQTS-associated variants in an organismal context. In this thesis I explain why zebrafish are such a powerful model for studying both inherited and acquired LQTS. I focus on inherited LQTS2, and the genetic approaches to model this in zebrafish. I describe the development of a CRISPR system that can be used to efficiently edit and detect clinically relevant LQTS2 point mutations, using a novel exon replacement strategy with fluorescent reporter gene incorporation. This approach is paired with an early phenotyping pipeline for efficient characterization of the impact of LQTS variants on cardiac structure and electrical activity. Since some variants may produce embryonic lethal phenotypes, and because compensatory mechanisms may confound measurement of the effect of variants introduced in zebrafish embryos, I then developed an inducible CRISPR system that could be triggered in adult fish. To achieve this, I designed and constructed a photoinducible CRISPR system that could be used to introduce the Cas9 gene into the zebrafish genome so that editing can be triggered in adult stages with endogenously produced inducible Cas nucleases. The final part of my thesis includes a pedagogical research study on teaching the same genetic engineering tools that I use in my thesis, with the goal of facilitating undergraduate student involvement in research. Overall, my thesis work extends the utility of zebrafish as a disease modeling platform, providing knowledge consolidation, novel gene-editing approaches that can account for developmental compensatory influences and can be applied to multiple genes of interest, and opportunities for enhanced undergraduate education in the fundamentals of CRISPR technologies.

Keywords: zebrafish; CRISPR; LQTS; hERG; pedagogy; genetic engineering

Dedication

This thesis is dedicated to my grandmother, Kathleen Dunphy, who in her 90s still asks me every time we speak “explain your research and thesis to me again?”

Acknowledgements

Since I was young, I was always interested in the life sciences, spending lots of time on the island catching crabs or looking through tidal pools. My parents were a huge part of that, wanting to stoke curiosity in me early. It worked, and they have stuck with me the entire *way too long* I've been in school. They are the first people I want to thank, I would not still be doing my studies without their support. Alongside my parents, my brother has always kept me on my toes but I couldn't ask for a better person to listen.

Grad school itself has been a complicated journey, having started in a different lab and department, before having to leave and find a new home. Dr. Thomas Claydon was the only person to give me a chance, and for that I am eternally thankful. He has been an amazing mentor, and provided me opportunity I could only ever dream of. Dr. Glen Tibbits and Dr. Esther Verheyen have both been amazingly supportive, of both my research and teaching. As committee members they have challenged me, and as people I've worked with they have always been in my corner. Dr. Megan Barker, though I met her later in my program, has been a huge inspiration for the teaching career path I am now embarking on. In terms of teaching opportunity, nobody has been a bigger help and mentor than Megan. Outside my committee, Dr. Kathleen Fitzpatrick has been another monumental mentor when it comes to teaching. I took three courses with her in undergrad, and from that point she already was stoking my interest in education.

A key part of grad school is friendship, and the Claydon lab has given me a lot to work with. I'd first thank Dr. Ravichandra Venkateshappa and Dr. Padmapriya Muralidharan, both were some of my best friends in the lab and I spent many hours with them either discussing the exciting work being done or arguing politics, and I wouldn't change it for anything. Jake Kemp, Dr. Christina Hull, Dr. Patrick Shi, and Dr. May Cheng from when I first joined the lab helped me fit in when I was new to the department, seamlessly integrating me into the BPK culture. Naseem Givzad, Dr. Diana Hunter, Harshini Seetharam, Shefali Vishwakarma, and Debipriya Mitra, all cornerstones of the Claydon lab. Of course the legions of undergrads I've had the privilege of working with: Raj, Shoaib, Ningning, Zhao Kai, Reo, Mandy, Galvin, and Simran. My thesis wouldn't exist without all of them. Likewise, Dr. Vera-Ellen Lucci, Erin Williams, and others from the other Dr. Claydon lab helped me branch out and meet many new people. Another particular thank you to Lisa Lin, who was a great friend and shoulder when I needed it.

Life as a grad student often feels like you don’t actually have a life, so support from outside is incredibly important. My best friend for 20 years now, Alex Strohschein, has been instrumental in keeping me plugged in to life on the outside when I needed it. And finally, my more recent found family, folks from the TSSU who have been some of the most amazing people I’ve ever met and really helped me find the community I’ve been searching for so long now. There are way too many people to name, and I’m not going to get in trouble by picking and choosing, so the one I will specify is Jess Hercus who within this new group of comrades has been one of my best friends. And of course a shoutout to the staff at Storm Brewing, for consistently making that my third place.

Table of Contents

List of Tables

List of Figures

List of Acronyms

A-V node	Atrioventricular node
AgRP	Agouti-Related Protein
AhR	Aryl Hydrocarbon Receptors
ANOVA	Analysis of Variance
AP	Action Potential
APD	Action Potential Duration
ASD	Autism Spectrum Disorders
AVB	Atrio-Ventricular Block
BBB	Blood Brain Barrier
BE	Base Editing
BPM	Beats Per Minute
Cas	CRISPR-Associated Protein
CCD	Charge-Coupled Device
CHO	Chinese Hamster Ovary
CiPA	Comprehensive *in vitro* Proarrhythmia Assay
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	CRISPR Ribonucleic Acid
DI	Diastolic Interval
DIC	Doxorubicin-Induced Cardiomyopathy
DLCs	Dioxin-Like Compounds
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
dpf	Days post-fertilization
DPU	Diphenylurea
DSB	Double-stranded Break
DTA	Diphtheria Toxin A
EADs	Early-After Depolarizations
EBD	Evan’s Blue Dye
ECG	Electrocardiogram

EEG	Electroecephalogram
ENU	Ethylnitrosourea
ER	Endoplasmic Reticulum
ERC	Electrical Restitution Curve
EROD	Ethoxyresorufin-O-deethylase
gDNA	Genomic Deoxyribonucleic Acid
GFP	Green Fluorescent Protein
GMO	Genetically Modified Organism
GOF	Gain-of-function
GWAS	Genome-Wide Association Study
HDR	Homology-Directed Repair
HEK	Human Embryonic Kidney
hpf	Hours post-fertilization
HR	Heart Rate
ID	Intellectual Disability
I_{K1}	Inward Rectifier Potassium Current
I_{Kr}	Rapid Delayed Rectifier Potassium Current
I_{Ks}	Slow Delayed Rectifier Potassium Current
IND	Investigational New Drug
iPSC-CM	Induced Pluripotent Stem Cell Derived Cardiomyocyte
JoVE	Journal of Visualized Experiments
KIs	Knockins
KOs	Knockouts
LED	Light-Emitting Diode
LGMD	Limb Girdle Muscular Dystrophy
LOEC	Lowest observed effective concentration
LOF	Loss-of-function
LPS	Lipopolysaccharide
LQTS	Long-QT Syndrome
LQTS1	Long-QT Syndrome Type I
LQTS2	Long-QT Syndrome Type II

hERG	Human ether-à-go-go Related Gene
LQTS3	Long-QT Syndrome Type III
miRNA	Micro Ribonucleic Acid
MO	Morpholino
mRNA	Messenger Ribonucleic Acid
MS-222	Tricaine Methane Sulfonate
NHEJ	Non-Homologous End-joining
PAM	Protospacer Adjacent Motif
PAS	Per-Arnt-Sim
PCR	Polymerase Chain Reaction
PE	Prime Editing
PFPiAs	Perfluoroalkyl Phosphonic Acids
PRRP	Post-Refractory Repolarization Period
qPCR	Quantitative Polymerase Chain Reaction
QTc	Heart Rate-Corrected QT Interval
rbERG	Rabbit ether-à-go-go Related Gene
RFFL	Rififilyn
RFP	Red Fluorescent Protein
RNA	Ribonucleic Acid
ROI	Region of Interest
ROS	Reactive Oxygen Species
SA node	Sinoatrial node
SA	Sinus Arrest
SEMA3	Semaphoring 3
sgRNA	Single Guide Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
SQTS	Short-QT Syndrome
SSB	Single-stranded Break
ssODN	Single-Stranded Oligodeoxynucleotide
SSS	Sick Sinus Syndrome
STEM	Science, Technology, Engineering, Mathematics

TALENs	Transcription Activator-like Effector Nucleases
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TdP	Torsades de Pointes
TE Buffer	Tris-EDTA Buffer
tracrRNA	Trans-Activating CRISPR Ribonucleic Acid
UV	Ultraviolet
VF	Ventricular Fibrillation
VIS	Visnagin
VT	Ventricular Tachycardia
VUS	Variants of Unknown Significance
WT	Wildtype
YFP	Yellow Fluorescent Protein
zAgRP	Zebrafish Agouti-Related Protein
zERG	Zebrafish ether-à-go-go Related Gene
ZFIN	Zebrafish Information Network
ZFNs	Zinc-Finger Nucleases
ZIRC	Zebrafish International Resource Center

Chapter 1. Introduction

1.1. Zebrafish as a model organism

1.1.1. The zebrafish, *Danio rerio*

Zebrafish, *Danio rerio*, are a small freshwater teleost fish, approximately 2.5-4 cm long, found natively in south and south-eastern Asia, primarily around and just east of India. Zebrafish are commonly found in shallow slow-moving streams and associated still pools. They can withstand a wide range of conditions, having been found in temperatures between 12-39 °C, pH levels from 5.9-9.8, and salinities ranging from 0.01-0.8 ppt. In the wild they commonly eat both larval and adult insects, zooplankton, small crustaceans, as well as algae and plant material (Parichy, 2015; Del Vecchio et al., 2022). In laboratory settings, artemia (brine shrimp) nauplii and processed feeds formulated to maximise lipid and dietary protein are commonly used. Such a mix of processed and live feed combines an optimized nutritional profile of processed feed with the enrichment of live feed, which is particularly beneficial in earlier larval stages (Watts et al., 2016; Del Vecchio et al., 2022). The life cycle of the zebrafish can be difficult to categorize, as they develop in a continuous stream of changes rather than having distinct divisions, as seen in Figure 1. Embryos are typically defined as ending at hatching, which is around 3 days-post-fertilization (dpf). At this point the fish is considered a larvae, and this stage lasts approximately 6 weeks. During transition to juvenile they will lose their caudal fin fold, developing an actual caudal fin, and develop scales. Juveniles morphologically resemble adults but are not sexually mature; sexual maturity occurs around the 3 month old mark (Singleman and Holtzman, 2014). In the wild, zebrafish typically survive one year, however in research settings the average lifespan is approximately two years (though with proper husbandry techniques they can be kept up to five years). An important determining factor in how long they are kept in captivity is breeding age, since the breeding capacity of the zebrafish is diminished after approximately 1.5 years. Breeding follows a circadian cycle, with males releasing pheromones at night and spawning commencing in the morning. In natural environments, zebrafish will spawn in shallow streams, and so in lab settings it is common to simulate this environment in breeding tanks with shallow false bottoms. This is also necessary as zebrafish will eat hatchlings. Breeding is asynchronous, with females releasing eggs into the water before males fertilize them, and clutches can range from

several hundred to 1,000, depending on frequency of breeding (Parichy, 2015; Del Vecchio et al., 2022). This abundance of embryos and their rapid life cycle are two of many reasons, further expounded on below, for the popularity of zebrafish as a model organism.

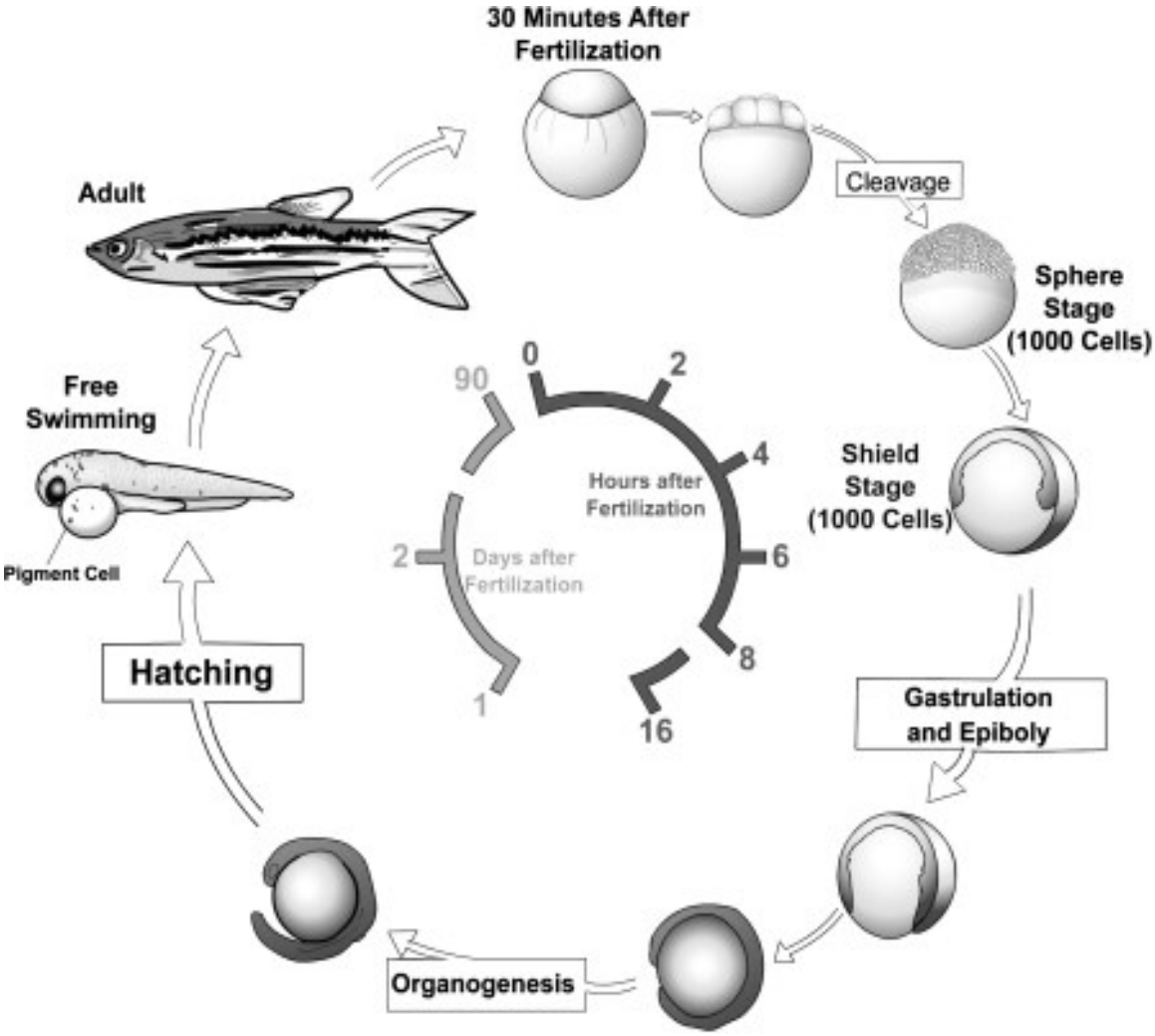


Figure 1: Life cycle of a zebrafish.
Zebrafish start as a single cell sitting on top of a yolk sac, where they obtain their nutrients until approximately 5 dpf. The single cell starts dividing after approximately 45 min., and hatches at 2-3 dpf. At 3 months zebrafish are typically sexually mature and can reproduce.

Note: Image obtained from (D'Costa and Shepherd, 2009). Permission obtained from Mary Ann Liebert Inc. license #: 5678460425653

Zebrafish are commonly used as a genetically tractable model in developmental, toxicological, physiological, and genetic studies. According to Teame *et al.*, the number of publications utilizing zebrafish as a model has increased significantly over recent years (Teame et al., 2019). At the time of writing, a search of the Pubmed database using the

terms “zebrafish” and “biomedical” as conducted by Teame *et al*., shows that the number of annual publications has continued to increase after their review article was published, peaking at 563 publications per year in 2021. This abundance of published research on zebrafish also results in a vast amount of information and resources available for researchers to use in their own work. The Zebrafish Information Network (ZFIN), a database for zebrafish genetic and disease model information formed in 1994, provides a huge resource of free data and also links with the Zebrafish International Resource Center (ZIRC), an institution that supplies zebrafish lines, probes, and other services. Currently there are 1,632 labs identified by ZFIN as doing zebrafish research (Bradford et al., 2022). This popularity as a model organism is due to multiple features and characteristics that make them a powerful model system. Zebrafish larvae are transparent, allowing for simple visualisation of development as well as phenotypes associated with genetic or pharmacological intervention. For example, early zebrafish studies by Melby et al. 1996 recognized the advantage of translucent zebrafish embryos to perform cell fate-mapping experiments. In these studies, embryos labelled approximately 5.5-6 hours-post-fertilization (hpf) with 1-2% rhodamine dextran, showed precursor cells for various embryonic structure formations such as the notochord were identified and cell fate maps are now available (Melby et al., 1996). The zebrafish genome has been well annotated; according to the most recent version of the annotated zebrafish genome (Ensembl GRCz11), they possess 1,373,471,384 base pairs with 25,545 protein-coding genes. According to Howe *et al.*, 71.4% of human genes have at least one zebrafish ortholog, and as of 2013 82% of known morbid human genes are represented in these zebrafish orthologues. Beyond these known morbid genes, of the 4,023 genes implicated in genome-wide association studies (GWAS) of human diseases, 76% have at least one zebrafish ortholog (Howe et al., 2013). Based on these similarities, many tools have been developed to further zebrafish research and these will be discussed further in Chapter 1.2. The standard strong model characteristics are also present: small size, relatively simple husbandry protocols, high offspring numbers, and a rapid development time. Here, I provide an overview of the use of zebrafish in various areas of research and provide justifications for their use in each case, with a summary in Figure 2.

1.1.2. Zebrafish as a model of neurological function

Development and function of various physiological systems show high conservation between mammals and zebrafish, including the brain and mechanisms of neural development, and zebrafish have been used extensively to model neurological development and function. For example, zebrafish have been used to study autism spectrum disorders (ASD). Kozol et al 2015 studied two highly suspected ASD-risk genes (syngap1b and shank3a) in zebrafish and used targeted morpholino-knockdown to understand their importance. Knockdown of gene function resulted in fewer GABAergic neurons in the midbrain and hindbrain of the zebrafish. This is significant because a reduction in number of inhibitory GABAergic neurons relative to that of excitatory glutamatergic neurons is associated with increased excitability and ASD symptoms (Hussman, 2001; Powell et al., 2003; Kozol et al., 2015). Zebrafish have also been used to investigate epilepsy. Zebrafish larvae exhibit robust seizure phenotypes in response to both drug-induced and inherited epilepsy-associated variants. Seizure phenotypes include well-characterized convulsive and circling movements that allow for easy phenotyping of epilepsy-relevant behaviours (Baraban et al., 2005, 2013). Electroencephalogram (EEG) recordings from zebrafish have also been used to quantify the effects of risk-associated variants in epileptic seizure activity. For example, EEGs recorded in larval zebrafish carrying a variant in the homolog of SCN1A, *scn1Lab*, that is associated with Dravet syndrome showed the power of zebrafish to both model dysfunction, and to identify a potential compound to inhibit seizures (Baraban et al., 2013). In this study, a homozygous variant identified from a previous chemical mutagenesis screen, *didy*^{s552}, showed ictal-like events (the period of a seizure wherein there is the most electrical activity in the brain, which is associated with seizure activity) compared to age-matched zebrafish controls, which showed no such heightened electrical activity. Variant zebrafish also expressed phenotypes that are consistent with epilepsy, such as whole-body convulsions and rapid undirected movements, not observed in age-matched control zebrafish. Following demonstration of *scn1Lab* variant phenotype, the authors tested the ability of 320 compounds to inhibit seizure activity. From this screen, clemizole was found to reduce swim velocity and seizure activity, as well as EEG seizure events, such as burst duration, frequency, time spent seizing). This study allowed for the discovery of clemizole as a pharmaceutical approach to suppress seizures, and highlights the use of the zebrafish model. Subsequent studies using zebrafish to model Dravet Syndrome have identified

additional compounds with similar mechanisms of action to clemizole, such as fenfluramine and trazodone that may have therapeutic potential (Zhang et al., 2015; Sourbron et al., 2016; Griffin et al., 2017). Another area of research involving zebrafish is intellectual disability (ID), where overexpression gene variant studies have been performed in zebrafish. For example, two missense variants in the human *RHEB* gene have been associated with both ID and macrocephaly, and overexpression of mRNA harbouring this variant in zebrafish caused macrocephaly, while a CRISPR knockout of the gene caused microcephaly. This informed the authors that the observed variant is likely gain-of-function (GOF) enabling the development of future therapeutic opportunities (Reijnders et al., 2017).

1.1.3. Zebrafish vascular models

Another physiological system well defined in zebrafish is the vascular system. As larval zebrafish are translucent, and develop major vessels within 48 hours post-fertilization (hpf), they provide a potent model for studying vascular development and disease. As an example, in 2015, Clay and Coughlin made a vascular injury and repair model using larval zebrafish. They induced mechanical vessel injury in the caudal vein using a hooked needle, and then measured time to coagulation. Using markers for different components of innate immunity, as well as time-lapse photography, their studies provided an unparalleled view into the potential of zebrafish as a vascular repair model (Clay and Coughlin, 2015). This technique has since been used to model effects of SARS-Cov-2 spike protein on blood coagulation, injecting the protein into the common cardinal vein and causing a mechanical injury to the posterior cardinal vein. The bleeding time after injection of the spike protein was then compared to the control. This technique aided the authors in furthering understanding of role of covid-19 in coagulation and inflammation in patients (Zheng et al., 2021).

Another example shows the insight provided into blood brain barrier (BBB) permeability. The BBB is an integral regulator of homeostasis in the brain, and one of the most tightly-regulated sites of vascularisation proving impermeable to toxic substances. However this impermeability also presents a complication for drug development since many compounds are not able to cross. An in-depth understanding of the BBB system is therefore important to drug development, and zebrafish have emerged as a strong potential model. The impermeability of the BBB is primarily due to tight junctions, which

have been detected at the zebrafish BBB (Jeong et al., 2008). There are multiple proteins commonly associated with BBB tight junctions, such as claudins, occludins, and ZO-1. Claudins form the structure of tight junctions, regulating the size-based permeability of tight junctions. Occludins are involved in regulating permeability of the tight junction, however don't have the same structural effects. ZO-1 proteins are responsible for anchoring tight junction proteins to the cytoskeleton, and potentially facilitating polymerization of claudins at tight junctions. The ZO-1 protein was found via western blot analysis using anti-ZO-1 antibodies, localised to cell-cell borders in all cerebral blood vessels. Claudin-5, one of the most commonly found tight junction proteins, was found via immunohistochemistry techniques to be localised in cell-cell border regions as well. Following this, in 2011 Eliceiri et al. described a number of simple techniques for tracking permeability of the BBB using various staining and microscopy techniques, demonstrating the applicability of zebrafish in studying this important system. This discovery of a functionally similar BBB in zebrafish, as well as the visualisation techniques, have allowed for toxicology studies examining environmental contaminants with potential for toxic effects (Eliceiri et al., 2011). In 2023, Zhang et al. studied the effects of perfluoroalkyl phosphonic acids (PFPiAs), a commonly used chemical in pesticides, food packaging, detergents, and fire-fighting foam. These compounds have been previously detected in the household dust from 102 Vancouver homes, as well as in more than 50% of 50 human sera samples in the United States (Lee and Mabury, 2011; De Silva et al., 2012). Zhang found that the presence of PFPiAs induced increased lipopolysaccharide (LPS) expression, which in turn via Evan's Blue Dye (EBD) tracing was found to have increased BBB permeability. Western blot analyses showed that expression of ZO-1, occluding, and claudin-5 were all significantly lower than control zebrafish (Zhang et al., 2023). The visualisation techniques and understanding of the BBB from Eliceiri et al. resulted in a stronger understanding of the toxic effects of commercially used chemicals found in our everyday lives.

Outside of vascular repair and regulation, zebrafish are a powerful model to examine vascular development. In 2001 Isogai et al. mapped angiogenesis in the developing zebrafish from 1-7 dpf. Using a combination of confocal microangiography, which uses fluorescent linked latex beads injected into the sinus venosus of the embryonic heart that then rapidly pass through the developing vasculature to allow for fluorescent imaging, to produce 2-D and 3-D images, and Berlin-Blue dye injections to create wiring

diagrams the authors tracked new vessel growth. The authors found that the developing vasculature of the zebrafish followed a similar plan to other vertebrates, demonstrating a conserved pathway of development (Isogai et al., 2001).

1.1.4. Using zebrafish to study toxicology

Zebrafish have been commonly used as a toxicological research model, both due to the reasons explain in Chapter 1.1.1 as well as the fact that embryos and larvae can absorb aqueous compounds through the chorion and skin. Studies done on molecule uptake found that there is some size discrimination in molecules passing the chorion, and by 60 hpf the larval fish is taking in compounds primarily via ingestion alongside epidermal transfer (Pelka et al., 2017; Bauer et al., 2021). Given this simple method of toxin delivery, this is a robust model for studying environmental toxins. An example of a potent and ubiquitous category of environmental toxins are dioxin and dioxin-like compounds (DLCs) (Rappe et al., 1987; Black et al., 2012; Xu et al., 2015; Fang et al., 2016). One of the more potent DLCs, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), is produced in both commercial processes, and natural occurrences, such as paper bleaching, incineration of chlorine-containing substances, manufacturing of certain organochlorine chemicals, volcanoes, and forest fires. These chemicals, while now banned in production, are still found in the environment due to strong bioaccumulation (Black et al., 2012; Xu et al., 2015). A high throughput model for detecting toxins like TCDD is useful, and Xu et al. in 2015 did this using its effect on aryl hydrocarbon receptors (AhR). These receptors, when bound by a ligand, congregate in the nucleus and activate transcription of multiple genes, including cyp1a. The authors used a GFP under a cyp1a promoter to detect the presence of TCDD and to measure how much this increased cyp1a expression. The author's found that TCDD, along with other DLCs, induced heavy GFP expression in the kidney, liver, and gut compared with DMSO controls. The ethoxyresorufin-O-deethylase (EROD) test is the standard for testing AhR agonists in water samples, but it is time-consuming and laborious requiring isolation of microsomes, incubation with the appropriate cofactors, and fluorometric analysis of the resulting resofurin compound. In contrast, the transgenic zebrafish simply requires exposure to the water sample and fluorescent screening, also providing the option for integration into a high-throughput system. When examining effectiveness via lowest observed effective concentration (LOEC), the two screens have comparable efficiency in larval fish (approximately 10 pM in both, though the transgenic

larvae showed some GFP expression barely above WT at 1 pM) while the transgenic zebrafish screen was an order of magnitude more effective than using the EROD assay with adult livers (the usual method of testing) (Xu et al., 2015).

Aside from environmental toxins, zebrafish are a model for pharmaceutical toxicology, used as a high-throughput platform for both drug discovery and drug toxicology. Doxorubicin is an effective chemotherapy drug, however in a dose-dependant manner is also associated with cardiotoxicity. There are many proposed mechanisms for this, including an increase in production of reactive oxygen species (ROS), reduction of DNA methylation, stimulation of apoptosis, and upregulation of autophagy (Liu et al., 2014; Ma et al., 2018; Rawat et al., 2021). In 2015, Liu et al. developed an embryonic zebrafish doxorubicin-induced cardiomyopathy (DIC) model and then used this model as a drug-screening platform in search of an adjuvant to attenuate the negative effects of doxorubicin without inhibiting its anti-tumour properties. The authors treated embryos at 1 dpf with 100 μM doxorubicin, and by 3 dpf these fish exhibited symptoms comparable to DIC in humans including increased apoptosis of cardiomyocytes, reduced numbers of cardiomyocytes overall, reduced heart rate, and a compromised contractility. Upon recapitulation of the DIC phenotypes, they then screened 3000 compounds and identified two that were protective at low concentrations (less than 1 μM): visnagin (VIS) and diphenylurea (DPU). These compounds are structurally distinct, with VIS being in the furanochromone family, and DPU a derivative of urea. Regardless, both compounds reduced cardiomyocyte death almost completely *in vivo* after doxorubicin treatment. Both compounds were further tested in mammalian models to determine translatability of these results and found they were effective at both recovering DIC phenotypes and not impeding the anti-tumour effects of doxorubicin (Liu et al., 2014).

1.1.5. Zebrafish as a model of metabolic disease

Demonstrating the versatility of zebrafish in disease modeling, they are commonly used as a model for obesity (Song and Cone, 2007; Klaauw et al., 2019; Leibold et al., 2022). As discussed previously there is great genetic homology between humans and zebrafish, however they also recapitulate many anatomical and physiological phenotypes.

Genetic bases for obesity have been modeled in zebrafish examining both neural (Song and Cone, 2007) and non-neural (Klaauw et al., 2019) irregularities in adiposity.

Leptin is a key hormone, increasing in concentration in response to available energy levels. Detecting leptin levels is a key method of energy homeostasis, which Song and Cone sought to study in zebrafish in 2007. Given the complexity of the system, having a robust vertebrate model to potentially discover new interacting genes involved in regulating obesity is valuable. Zebrafish possess melanocortin receptors, involved in an integral neural signaling pathway required for energy homeostasis, as well as agouti-related protein (AgRP), an antagonist for melanocortin receptors. The authors injected zebrafish embryos with zebrafish AgRP (zAgRP) and then crossed them down to the F3 generation. Fish in the F1 generation were weighed throughout the first year of their life, showing that transgenic fish were significantly heavier than WT: depending on the founder, up to 100% larger. F2 and F3 fish were homogenized and chloroform extracted, with triglyceride measurements taken from the lipid extracts. Transgenic fish were found to have 141% more triglycerides than WT, demonstrating obesity phenotypes. There were also increases in adipocyte size, number, and linear growth of the transgenic fish, consistent with melanocortin receptor disfunction in mammalian systems (Song and Cone, 2007). This demonstration of zebrafish utility as an obesity model paved the way for further research, such as van der Klaauw et al. in 2019. The authors studied the formation of the melanocortin circuits, developed in the hypothalamus. Rare semaphoring 3 (SEMA3) variants, a protein involved in axonal growth and guidance, have been linked with obesity in humans, The authors used CRISPR to mutate multiple points along the SEMA3 pathway, and 7 genes were identified to increase somatic growth, body weight, and percent body fat. Alongside associations with obesity in humans, and similar studies done in mice, the authors concluded that SEMA3 is involved in development of melanocortin circuits in the hypothalamus, potentially explaining the relevance of these rare variants in human obesity (Klaauw et al., 2019).

1.1.6. Zebrafish as a model of tissue and organ regeneration

Zebrafish possess remarkable regenerative capabilities, with the first studies demonstrating their use as a regenerative model in 1995 when Johnson and Weston performed the first genetic screens to identify genes associated with fin regeneration. In this seminal study, they identified 7 temperature-dependent mutations that expressed phenotypically as terminating fin regeneration, slowing regeneration, or development of tumours during the regenerative process (Johnson and Weston, 1995). Since these early

studies, zebrafish have been used extensively to study tissue regeneration (Poss et al., 2002; Wang et al., 2011; Gemberling et al., 2013; Aguirre et al., 2014; Ghosh and Hui, 2016; Beffagna, 2019; Massoz et al., 2021). The study of cardiac regeneration is a key component of these zebrafish regenerative studies, because human cardiac tissue does not significantly regenerate after events such as scarring, which is a major cause of cardiac-related mortality. In 2002, Poss *et al.* discovered that after surgical removal of 20% of an adult zebrafish's heart, they were able to recover to the point of normal activity and behaviour after a week; after 60 days the surface area (which had been reduced by 19% from the surgery) had completely recovered to its previous state. By staining both DNA and histone-3 in cardiomyocytes near the injury site, it was shown that these cells showed an increase in both DNA replication and mitosis (Poss et al., 2002). This demonstrated the value of zebrafish in studying cardiac regeneration. Even at larger scale damage, using a genetic cell ablation model that destroys more than 60% of ventricular cardiomyocytes, Wang *et al.* demonstrated near complete regeneration in a matter of weeks. One line of zebrafish were injected with a chemical inducible Cre-recombinase expressed in cardiomyocytes, while another expressed a loxP that targeted diphtheria toxin A chain (DTA) to the Cre-expressing cells. When these lines were intercrossed, and exposed to 4-HT (to induce Cre-recombinase expression) via injection or bath, approximately 60% of cardiomyocytes were terminated. These fish demonstrated sensitivity to extreme environmental stressors such as heat shock, as well as exercise-induced stress. However after 2 weeks, most showed complete recovery phenotypically. Using a nuclear DNA stain, the researchers observed that ventricular cardiomyocytes proliferated throughout the chamber, suggesting a proliferative regeneration process rather than cell hypertrophy. Interestingly, this regenerative capability seemed more robust than with mechanical injury, occurring on the scale of weeks rather than months (Wang et al., 2011). These examples of the zebrafish's regenerative capacity in the cardiac system, something not possible in mammalian systems, are of great interest to studies of human cardiac injury. Using this understanding of zebrafish cardiac regeneration, in 2014 Aguirre *et al.* identified microRNAs with increased expression during zebrafish cardiomyocyte proliferative events, and determined which were highly conserved across vertebrates. The authors identified two families, miR-99/100 and let-7a/c, associated with three proteins, SMARCA5, FNTβ, and GATA4, that were all associated with cardiomyocyte dedifferentiation. Silencing these miRNAs in primary mammalian cardiomyocytes, increased formation of beating colonies, suggesting increased proliferation as expected

from zebrafish regeneration mechanisms. Injection of anti-miR into murine models with myocardial infarction resulted in significant reduction in scarring, increase in ventricular wall thickness, improved ejection fraction, and reduced infarct size. Upon histological analysis, the authors found that an increased number of cardiomyocytes had reduced miRNA expression, demonstrating the increased proliferation hypothesized from the zebrafish model. This proof of concept demonstrated that these conserved regenerative mechanisms can be activated in mammalian systems where they have been dormant to facilitate recovery from otherwise permanent cardiac damage (Aguirre et al., 2014).

1.1.7. Zebrafish as a model of cardiac function

In earlier sections I have described how zebrafish have been used as a valuable model to study the cardiac biology in the areas of cardiac regeneration and pharmacological toxicity. Zebrafish have also been used specifically as a model to study embryonic development of the cardiac system. Much of cardiac development is conserved among vertebrates, in all the heart is the first organ to form and function (Bakkers, 2011). Atrial and ventricular myocardial precursors are separated along the lateral marginal zone very early, up to 5 hpf. These authors used caged fluorophore techniques, a fluorophore that is not activated until exposed to UV light, to label individual blastomeres at 40% epiboly (5 hpf) before allowing them to further develop and track cell proliferation and movement. Epiboly refers to a developmental stage and movement in zebrafish (as well as some other organisms such as sea urchins and amphibians) wherein the ectoderm spreads around the yolk while both endoderm and mesoderm layers internalise. They found that at 5 hpf, myocardial precursors were strictly ordered along the lateral marginal zone, while endocardial precursors were evenly distributed. This suggests myocardial progenitors are spatially organized prior to gastrulation, but not when chamber fates are determined. The authors suspected Nodal signalling is involved in this process, so they injected embryos with *lefty1* mRNA, a known Nodal signalling antagonist. Focussing on a region wherein all myocardial progenitors formed ventricular myocytes, they noted that with Nodal signalling block a mix of atrial and ventricular fates developed. This informed that not only were myocardial progenitors organized by 40% epiboly, but Nodal signalling was involved in chamber fate mapping (Keegan et al., 2004).

The earliest functional form of the heart is a linear tube, with an outer myocardium surrounding the inner endocardium layer. In 2007, Bussmann et al. investigated the

formation of the endocardium, which was poorly understood at the time. VEGF is a signalling molecule involved in vasculogenesis and angiogenesis, and was one of the receptors found in zebrafish to only be expressed in endothelial precursors. The authors created a transgenic line of this particular receptor, fusing it with a reporter gene, and used time lapse photography to track proliferation and movement of endothelial precursor cells. Fluorescence was first detected in the 10-12 somite stage (14-15 hpf), and at the 14 somite stage endocardial precursors grouped in two bilateral populations in the posterior region of the anterior lateral plate mesoderm. By the 18 somite stage, these populations migrated posterior and started fusing from the anterior end. Finally the cells migrated leftward where the heart tube forms in an asymmetric fashion around the 22 somite stage. These discoveries that endocardium precursors are specified and then migrate into the region of heart tube formation drove our understanding of endocardium formation (Bussmann et al., 2007). By approximately 24 hpf, the heart starts to beat, and blood circulates through the major vessels by 36 hpf.

Zebrafish have also been used to study cardiac conduction and electrical excitation, and are shown to present many similarities to human function that elevate their use above for example small mammal models. Zebrafish have also been used as models of inherited cardiomyopathies and ion channelopathies, which perturb electrical and structural properties of cardiac function, and are associated with sudden death in humans. These features are considered in detail in Chapter 3, where I discuss a semi-systematic approach to investigating zebrafish models of electrical disturbances.

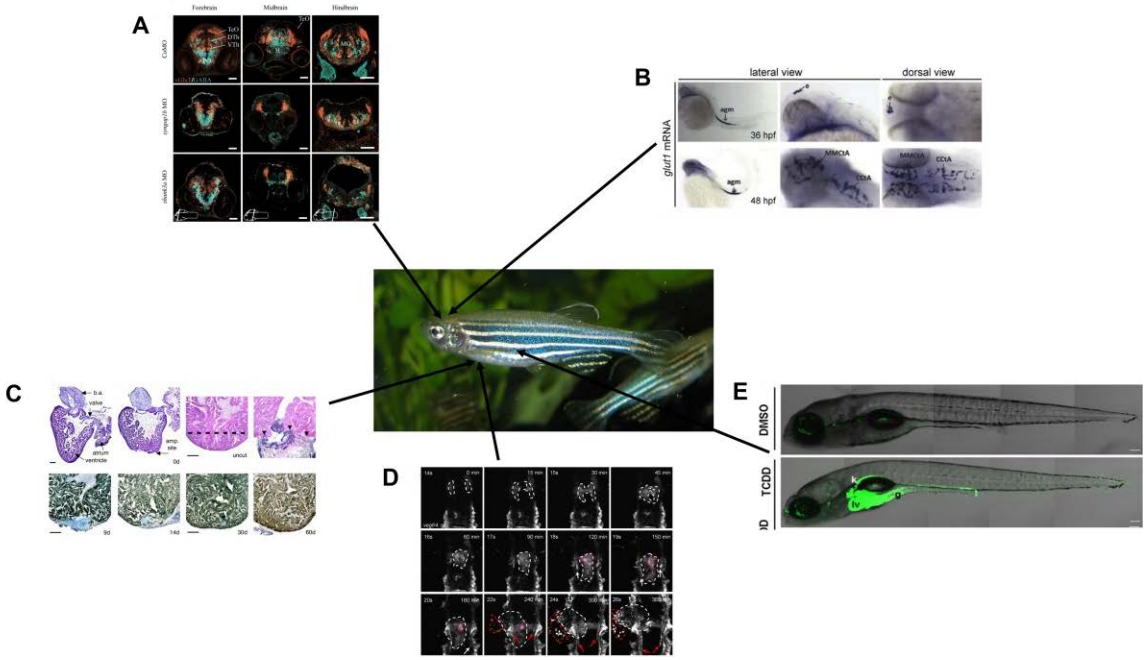


Figure 2: Examples of the utility of zebrafish in different fields of research.
A. Expression of excitatory and inhibitory neurons in mutants associated with autism spectrum disorder, using immunohistochemical staining methods (Kozol 2015). B. mRNA expression of glut1, a glucose transporter found uniquely in the blood-brain barrier. By 48 hpf, expression is seen in the capillaries of the BBB. (Quinonez-Silvero 2020). C. Stain of the zebrafish heart showing regeneration after 20% resection. The bottom row shows fibrin sealing the resected region, before being replaced by cardiac muscle tissue (myosin heavy chain stain). (Poss 2002). D. Formation of the zebrafish heart, from initial endocardium migration, to formation of the heart tube, and finally leftward migration of the heart. E. Transgenic zebrafish capable of detecting environmental TCDD, an organic pollutant, via GFP expression. The top photo shows the zebrafish exposed to a DMSO control, and the bottom exposed to TCDD. (Xu 2015).

Note: 2A image obtained from (Kozol et al., 2015), permissions obtained from Oxford University Press license #: 5684430673241; 2B image obtained from (Quiñonez-Silvero et al., 2020), permissions obtained from Elsevier license #: 5684440475071; 2C image obtained from (Poss et al., 2002), permissions obtained from The American Association for the Advancement of Science license #: 5684451359792; 2D image obtained from (Bussmann et al., 2007), cited work published under the terms of a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/) and is unmodified from the original; 2E image obtained from (Xu et al., 2015), permissions obtained from Springer Nature license #: 5684440781242.

1.2. Genetic tools in zebrafish

The zebrafish genome shares 70% of its gene identity with the human genome, alongside structural chromosomal similarities (Barbazuk et al., 2000; Howe et al., 2013). Synteny, the chromosomal position relations between genes, has also been shown as highly conserved between zebrafish and humans. Barbazuk et al. mapped 523 zebrafish genes with predicted human homologs and demonstrated that 80% belong to conserved synteny groups, meaning they are linked in similar ways to their human homologs (Barbazuk et al., 2000). The extensive mapping and annotating of the zebrafish genome,

and measured similarities to the human genome, have resulted in large databanks and consortiums such as The Zebrafish Information Network (ZFIN), which provide a detailed database including information such as gene annotations, maps, mutations, antibodies, gene expression data, and transgenic lines. A centralised databank such as this facilitate further genetic studies in zebrafish, including the ability to screen for use in developmental and disease modelling. Genetic screens can be conducted in a variety of ways depending on the research goal in question. Forward genetics involve determination of genes responsible for an observed phenotype, while reverse genetics involve targeted mutation of specific genes and determination of the phenotypic effect. In the following sections, I describe some examples of how these approaches have been used in zebrafish to understand the genetic basis of a variety of biological functions.

1.2.1. Identifying genes via forward genetics

Forward genetics screens are conducted via insertional mutagenesis, using genetic elements such as transposons to create random mutations, or via chemical means using compounds such as ethylnitrosourea (ENU), an alkylating agent that modifies single bases via transfer of an ethyl group. Inheritance of these mutation phenotypes is then linked to known genes to study their location in the genome, a process called linkage analysis (Granato et al., 1996; Haffter et al., 1996). These screens can also be performed with naturally occurring mutations. An early example of zebrafish genetic screens comes from 1996, when Haffter et al. used ENU to mutagenize adult male fish and then propagate their lines to study F2 phenotypes. F2 embryos were scored for structural and behavioural abnormalities, and this resulted in 372 unique genes with mutations identified. The number of unique genes was confirmed via complementation testing, a method of genetic crosses to determine whether similar phenotypes result from the same gene mutation or different ones. If two unique genes are causing the phenotype, once crossed, the offspring should exhibit a wildtype phenotype with the variants “complementing” each other (Haffter et al., 1996). Based on this screen, Granato et al. identified 48 genes affecting locomotion behaviour in larval zebrafish. Larvae from the screens performed by Haffter et al. were subjected to motility tests between 48-60 hpf, being stimulated by a needle at the tail and observing motility responses. Those who demonstrated abnormal motility behaviour were anaesthetised and had their trunk muscle examined: 63 mutants out of 166 identified had reduced muscular development and showed mutations in 18 different genes (Granato et

al., 1996). One of the mutations discovered led to the first muscular dystrophy models in zebrafish, a human disease model still studied today (Findlay et al., 2023).

Forward genetics screens, such as those using ENU-mutagenesis techniques, are not as prevalent; however they are still used. Currently, techniques such as gene-breakage and trapping are used to both knock-out function and report presence of proteins (Clark et al., 2011; Ichino et al., 2020; Ding et al., 2022; Findlay et al., 2023). A transposon possessing a red fluorescent protein (RFP) gene missing a start codon, and followed by a poly-A signal, truncates the native protein while producing a fluorescent fusion to detect location of expression. Using this transposon technique, Ichino et al. in 2020 generated 1200 mutant zebrafish lines, which were further enriched by Ding et al. in 2022 for those with cardiac expression. The authors were looking for models of Sick Sinus Syndrome (SSS), and by detection of cardiac fluorescence identified 35 mutant lines for further ECG phenotyping. 3 lines showed sinus arrest (SA) events, and 1 showed atrio-ventricular block (AVB). Further interrogation of one particular mutant, dnajb6b, revealed susceptibility to external stimuli: atropine, carbachol, and verapamil. These resulted in reduced heart rate and increased SA incidence, supporting dnajb6b mutants being arrhythmogenic (Ichino et al., 2020; Ding et al., 2022). Later studies on the dnajb6b gene showed that it is also responsible for limb girdle muscular dystrophy (LGMD). The work showing arrhythmogenicity was mentioned as an important consideration when examining gene knockouts for the dominant dnajb6b mutation as haploinsufficiency may result in an arrhythmogenic state for patients (Findlay et al., 2023).

1.2.2. Examining phenotypes via reverse genetics

Reverse genetics, a more commonly used screening technique, involves targeted mutation of particular genes and observation of the phenotypic effect. This utilizes techniques to either increase or decrease the function of a specific gene of interest, via targeted molecular manipulation. Initial studies took advantage of morpholino (MO) knockdowns, a technique that uses an analog of nucleic acids to block mRNA from undergoing translation. They bind the mRNA molecule via complementary base-pairing, preventing the initiation complex in translation as well as modifying RNA splicing (Moulton, 2006). Morpholino knockdown was first explored in zebrafish in 2000, by Nasevicius and Ekker, who demonstrated its capabilities to selectively knockdown a number of developmental genes, as well as human disease-associated genes. Developmental genes

such as *chordin (chd)*, *one-eyed-pinhead (oep)*, and *no tail (ntl)* were targeted via MOs and upon knockdown developmental defects were observed consistent with the role for these genes in early developmental signalling pathways. *Chordin* is involved in dorsal-ventral patterning, with loss of function resulting in somite deformities. *One-eyed-pinhead* is both a maternal and zygotic effect gene, meaning the gene product comes from both the mother as well as within the zygote. At low MO concentrations only the zygote-produced *oep* was lost, while at higher concentrations both zygotic and maternal were affected. One difficulty with the *oep* experiment, which highlights a disadvantage of the MO knockdown approach, is that the 5' UTR of the mRNA is important for MO binding, and if there are polymorphisms in this region, knockdown can be inhibited as occurred in some strains of zebrafish with this gene. Finally, *ntl* interacts with *oep* for formation of somitic mesoderm, and reduction in function for both of these genes results in loss of somitic mesodermal markers. The *uroporphyrinogen decarboxylase* gene was also targeted and its knockdown recapitulated the hepatoerythropoietic porphyria condition, a defect in heme production that manifests with fluorescent and photosensitive red blood cells (Nasevicius and Ekker, 2000).

These studies are an example of the strength of the MO approach to understand gene function. However, there are limitations to their use. Due to their transient nature, Mos are not able to completely block expression, rather incompletely reducing expression. Another limitation is that because MOs target and block mRNA, rather than editing DNA, specific edits or generation of clinically relevant variants are not possible using this approach.

The development of specific gene-editing tools such as transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeats (CRISPR) has enabled investigation of target gene function and this has been readily adopted in the zebrafish field (Doyon et al., 2008; Huang et al., 2011; Zu et al., 2013; Hwang et al., 2014; Shah et al., 2015). These direct gene-editing technologies cause a breakage in DNA at a specific location and utilize the cellular repair mechanisms to fill the gap. This can be in the form of non-homologous end-joining (NHEJ) wherein the broken ends of the nucleic acid are ligated together resulting in indel (insertion and deletion) formations, or homology-directed repair (HDR) which uses a template (normally the homologous chromosome, but also introduced synthetic templates) to guide repair of the damaged sequence. Taking advantage of these more specific

targeting techniques and repair mechanisms allowed for much more robust disease modelling, and for recapitulation of human disease-associated variants. The mechanisms and uses of these different approaches to reverse genetic screening will be outlined in turn.

Zinc-finger nucleases (ZFNs) were the first nuclease complexes recognised as gene-editing tools. Pavletich and Pabo in 1991 resolved the crystal structure of the zinc-finger-DNA complex to provide the inspiration for zinc-finger-based DNA-binding proteins (Pavletich and Pabo, 1991). After Li, Wu, and Chandrasegaran observed that the FokI restriction enzyme contains multiple domains, separating cleavage activity from DNA site-recognition, the Chanrasegaran group went on to fuse the cleavage domain of FokI with multiple zinc-finger proteins, demonstrating the versatility and binding-specificity of the zinc-finger proteins (Li et al., 1992; Kim et al., 1996). This led to current ZFN construct designs, pairing two zinc-finger proteins to the FokI nuclease cleavage domain. In 2008, ZFNs were first used in zebrafish to target the *golden* (*gol*) and *no tail* (*ntl*) genes, causing loss-of-function (LOF) phenotypes. The *gol* gene encodes SLC24a5, a transmembrane cation exchanger. In zebrafish, loss of function is known to reduce the number of melanosomes causing reduction in skin pigmentation, a simple trait for phenotyping. Two days after injection of ZFN constructs, 32% of embryos showed pigmentation mosaicisms in the eyes compared to wildtype controls, presenting as patches of pigmentation in the eyes. After sequencing, evidence of NHEJ repair events including both insertions and deletions in the ZFN target range were identified, suggesting the ZFNs successfully targeted and caused DNA breakage at the target location. The *ntl* gene encodes a transcription factor vital for early development of mesoderm. These studies showed that *ntl* null mutants do not develop the notochord or tail. This target was chosen due to the importance of the gene in early embryonic development, and the use of zebrafish as an early developmental model. There was approximately a 20% transmission rate to the F1 generation, demonstrating for the first time the ability to generate precise mutations that are germline heritable (Doyon et al., 2008).

Transcription activator-like effector nucleases (TALENs) were developed in 2010, combining TALE transcription factors that specifically bind recognised DNA sequences with FokI domain nucleases (Christian et al., 2010). Miller *et al.* tested these fusions in endogenous expression systems such as K562 cells, demonstrating both NHEJ- and HDR-mediated repair in the *CCR5* gene. A double-stranded plasmid was co-nucleofected

with the TALEN constructs to induce HDR repair with insertion of a BglI restriction site (Miller et al., 2011). The first use in zebrafish was in 2011, by Huang *et al.*, testing mutations at two genes, *tnikb* and *dip2a*. The *tnikb* gene encodes the *TRAF2 and NCK interacting kinase b* protein, a kinase predicted to function in the zebrafish central nervous system, and is orthologous to human genes wherein loss of function is linked to intellectual developmental disorders. Mutations in this gene were verified via both restriction fragment length analysis (the TALEN target was a BamHI site, absent once a double-stranded break was induced) and sequencing. Nine of these *tnikb* variant zebrafish were also screened for transmission of the variant to F_1 embryos, with three positively identified. Additionally, the authors developed software to detect potential off-targets for the TALEN target sites, finding nine for the *tnikb* sequence; however after sequencing there was no significant increase in altered sequence compared to wildtype. As additional proof of concept, the *dip2a* gene was also targeted and verified in a similar fashion. This gene is predicted to be involved in axon formation but not much is known about it. This study demonstrated heritable NHEJ events, with both mutations appearing in F1 generations through germline transfer (Huang et al., 2011).

1.2.3. CRISPR: function, mechanism, and adoption in zebrafish studies

While ZFNs and TALENs both successfully generated zebrafish variants, these approaches are limited by their complexity and time-consuming nature. ZFNs are difficult to make, requiring assembling three zinc-finger arrays to bind 9 bp of sequence. In one study, 168 arrays were assembled for targeting 104 DNA sites, but a bacterial two-hybrid assay showed that 79 of these sites did not have a functional zinc finger assembly (Ramirez et al., 2008). TALENs are simpler to design, however construction requires an approximately 3 kb cDNA for each new target site. This presents complications for generation of multiple variants. CRISPR is an alternate technique that is based on bacterial adaptive immunity and was developed to allow simpler precise generation of mutations.

CRISPR functions by the endonuclease, Cas (CRISPR-associated protein), being led to a particular sequence of interest by an RNA complex. In bacteria this consists of a crRNA (CRISPR RNA) associated with a tracrRNA (trans-activating CRISPR RNA), both forming a complex with the Cas enzyme and directing to a target sequence. The crRNA

is derived from viral DNA that has been integrated by the bacteria immune system during a previous infection. For research purposes, an sgRNA takes the place of the crRNA-tracrRNA complex, and can be synthesized *in vitro*. The sgRNA contains a backbone that is analogous to the tracrRNA, and this complexes with the Cas endonuclease, and a spacer sequence analogous to the crRNA, which recognizes the target sequence. In order to successfully cleave the DNA, each Cas enzyme has a particular PAM (protospacer adjacent motif) sequence, a short 3-8 bp sequence that must be immediately downstream of the target sequence, otherwise the Cas endonuclease may not properly bind and cleave the DNA. A standard PAM sequence for the commonly used spCas9 is -NGG. It is thought that this PAM site provides self- and non-self discrimination in the bacteria, as the particular PAM sequence is typically found in the viral genome but not adjacent to the immunized viral DNA in the bacterial genome.

After the Cas enzyme creates a double-stranded break (DSB) in the target sequence, the cell uses one of multiple innate repair mechanisms, the two most frequently used being NHEJ and HDR. NHEJ utilizes a type IV ligase to attach the ends of the break, in a process that typically results in random errors through insertions and deletions. This mode of repair is appropriate for knockout studies, but due to its imprecise nature less so for precise alterations. HDR allows for use of a designed template to undergo homologous recombination with the cut site, acting as a template to fill in the DSB. Using this pairing of precise cuts and cellular repair mechanism, any modification can be made to any point along the genome of any organism.

CRISPR has challenges, two significant ones being the low efficiency of targeted repair and the potential for off-target Cas-mediated cleavage activity. Efficiency, while not actually an issue with the CRISPR DSB process, is relevant when making precise edits through HDR. This repair process necessitates DNA being accessible for homologous recombination, and a homologous template being present, restricting utilization of HDR to S and G_2 phases of the cell cycle. Meanwhile, NHEJ repair can happen at any time, greatly reducing the relative efficiency of HDR. Off-target cleavage activity may occur when the sgRNA anneals at another genomic location with similar enough sequence identity. As long as a PAM site is present, and the first ~8-12 bases adjacent to the PAM (called the seed sequence) are identical, the Cas can bind and cleave these locations. Due to this possibility, considered guide design is essential and online software used to design sgRNA will provide possible off-target locations and rank potential guides according to this

off-target potential. Figure 3 shows the association between RNA guide and Cas9, along with the repair mechanisms.

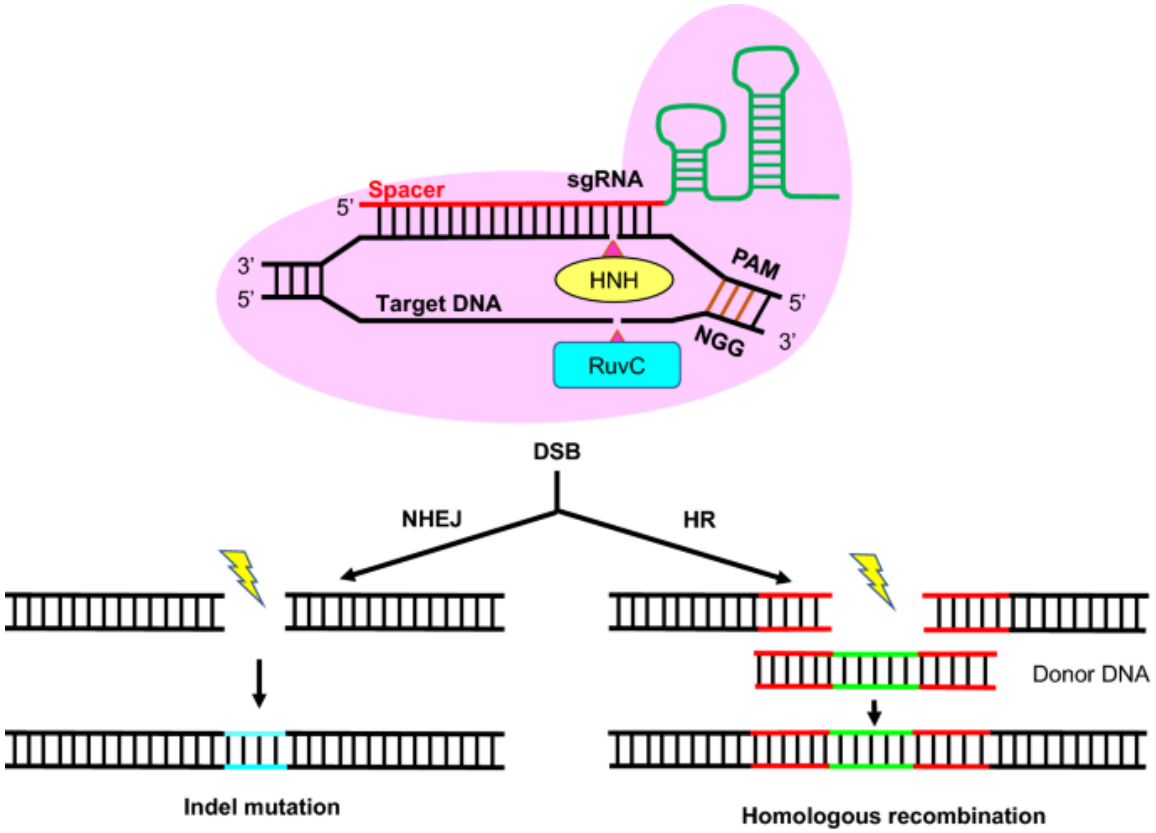


Figure 3: A schematic of the CRISPR mechanism.
The sgRNA forms a complex with the Cas enzyme via the scaffold sequence (green). The Cas-sgRNA complex then detects PAM sequences, and the spacer sequence (red) will bind homologous sequence. If there is homologous sequence and binding is successful, there is a conformational change in the HNH domain, allowing for cleavage of the target strand. This conformational change allows for the RuvC domain to cut the non-target strand, resulting in a double-stranded break. This break is then repaired via either homology-directed repair or non-homologous end joining.

Note: Image obtained from (Cai et al., 2019). Cited work published under the terms of a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/) and is unmodified from the original.

1.2.4. A history of CRISPR

The bacterial origins of the CRISPR editing system were first discovered in 1987 though not modified into a gene-editing tool until 2012. In 1987, Ishino *et al.* sequenced the *iap* gene in *E. coli*, and discussed at the end of the publication an unusual set of repeat sequences. At the time the author hypothesized that these may play a role in stabilizing mRNA, but left this as an unknown (Ishino et al., 1987). In 2000, in a correspondence to the Molecular Microbiology journal, Mojica *et al.* described a series of repeats found in a

wide variety of prokaryotes, however without any suggestions as to function (Mojica et al., 2000). In 2004 however, Mojica *et al.* suggested that the intervening sequences between repeats were related to extrachromosomal elements such as bacteriophage genetic elements, which failed to infect bacterial strains containing these intervening sequences. It was found that 65% of spacer sequences shared identity with phage or other external genetic elements associated with the given bacterial strain, typically sequences known to be involved in transposition or other homology-driven mobilization. While this study did not directly address functional assessment, the authors observed that bacterial strains with a given spacer resisted infection from related external genetic elements and this implied an immunity function (Mojica et al., 2005). Further computational studies have linked likely nuclease protein families (Cas proteins) with these repeat regions, further supporting the link between this system and immunity (Makarova et al., 2006). Actual functional experiments started in 2007, with Barrangou *et al.* determining the mechanism and function of spacer sequences between the CRISPR repeats. Infecting bacteria with particular bacteriophage, they determined using comparative genome analysis that additional spacers were added that matched the particular strains of bacteriophage used experimentally. Additionally, they removed specific spacers with homology to each particular bacteriophage and transferred them to WT bacterial strains, demonstrating that the viral resistance was transferred with these spacers (Barrangou et al., 2007). In 2012, Jinek *et al.* described the potential use of CRISPR systems as a gene-editing tool, adapting the CRISPR mechanism by providing synthetic single-stranded RNA to guide the nuclease to a particular sequence of interest (Jinek et al., 2012). This paved the way for the use of CRISPR as the premier gene-editing tool, being more simple to design and implement than either ZFNs or TALENs (Nemudryi et al., 2014).

The first use of CRISPR in zebrafish was in 2013 by Hwang *et al.* who demonstrated that CRISPR had comparable efficiencies to both ZFNs and TALENs in zebrafish. Using NHEJ as a repair mechanism, the authors used CRISPR to successfully edit 9 out of 11 targeted sites, which included two sites that TALENs were unable to target. Combined with the improved ease of construction, the CRISPR approach was considered to provide a robust gene-editing system (Hwang et al., 2013). In the same year, Chang *et al.* were the first to use CRISPR to generate a precise repair using an HDR template in zebrafish, inserting an mloxP site into the etsrp gene that is involved in vascular development. Using a single-stranded DNA template, 1 out of 12 randomly selected

injected embryos possessed the expected mloxP insertion without other damage to the surrounding sequence (Chang et al., 2013). Together, these studies confirmed that CRISPR can be used for efficient knockouts as well as more precise mutations and the generation of clinically relevant variants in zebrafish and paved the way for extensive future investigations.

1.2.5. The future of CRISPR development

The CRISPR mechanism is constantly being modified and updated, from different guide structures that allow for more specific targeting and lower off-target activity (Kocak et al., 2019)(Kocak 2019) to different Cas enzymes that can target a variety of PAM sites, extending the ability to edit the genome (Bandyopadhyay et al., 2020). An example of a Cas modification that was developed to mitigate off-target potential is the Cas9 nickase, a Cas9 with only a single cleavage domain instead of the usual two. This results in single-stranded breaks (SSB) rather than DSBs. Two of these SSBs in close proximity can act as a DSB, while reducing the likelihood that they will be found in proximity in an off-target location (Cong et al., 2013). Particular advancements of note are base editing (BE) and prime editing (PE), modifications to the CRISPR mechanism that do not require DSBs or an HDR template. Instead, these fuse a Cas9 nickase and either deaminase or reverse transcriptase, respectively. The BE system uses a deaminase to chemically modify a nucleotide, converting it into a different base, and then the cell uses the SSB from the nickases to replace the base-paired nucleotide, thereby creating a point mutation (Komor et al., 2016). Prime editing uses reverse transcriptase and a guide RNA that also acts as a template, bypassing the need for either a double-stranded break or a template while inserting more than a single point mutation (Anzalone et al., 2019)

In this thesis, I describe novel approaches that I have developed using the CRISPR-Cas9 system to create a platform for studying the inherited cardiac electrical disorder, Long-QT Syndrome, in zebrafish. I then explore modifications to this system, to more precisely influence spatial and temporal activation of gene editing in the zebrafish system. The following section provides a brief introduction to the Long-QT Syndrome electrical disorder, which is considered in more detail in subsequent chapters.

1.3. The cardiac electrical disorder, Long-QT Syndrome

1.3.1. An overview of the heart

The heart is one of the earliest developing organs, responsible for driving circulation via electrical control in the heart (Kemmler et al., 2021). This is true in both zebrafish and human embryos, with a very similar developmental pathway expounded on more in Chapter 3. At the gross structural level, zebrafish hearts differ from human hearts, with zebrafish possessing only 2 cardiac chambers and a much thinner ventricular wall (see Figure 4). Despite this, the electrical pathway through the heart is fairly similar in zebrafish and humans. In humans, action potentials initiated in the sinoatrial (SA) node, cause the atria to contract, and are then propagated through the atrioventricular (AV) node. This delays conduction of the action potential, allowing for coordinated contraction of the ventricles. In the zebrafish, equivalent structures for both the SA and AV nodes exist, fulfilling similar roles as in the human heart. While there is only a single atrium and ventricle, propagation of the action potential through these tissues follows a generally similar path. There are differences in conduction in the ventricle, and these are discussed further in Chapter 3.

The currents responsible for shaping the ventricular action potential are similar between human and zebrafish. As shown in Figure 5, the action potential can be divided into 5 phases (named 0-4). Each of these phases is dictated by the presence of particular ion channels that selectively pass currents through the cardiomyocyte membrane. In both human and zebrafish, the I_{Na} current drives the initial depolarization of phase 0, though in zebrafish there is also contribution from a T-type calcium channel passing the $I_{ca,T}$ current. Phase 1 repolarization, caused by the transient outward current, is observed in some regions (e.g. epicardial) of the human ventricle, but is not observed in zebrafish cardiac tissue. The L-type calcium channel in human hearts is responsible for the Phase 2 plateau that prolongs the action potential and this current is present in zebrafish. Phase 3 repolarization is driven primarily by the I_{Kr} current, passed by the hERG potassium channel. Described further in chapter 1.3.4, these channels have unique gating properties that enable them to drive repolarization in phase 3 of the action potential. The I_{Ks} current is another potassium current that contributes to Phase 3 repolarization in humans. In zebrafish, only low levels of I_{Ks} current have been detected, and the role of this current is likely of lesser importance. The Phase 4 resting membrane potential in both humans and

zebrafish is maintained by the inward rectifier I_{K1} current, which also sets the initial membrane potential for the next depolarization (Nemtsas et al., 2010; Echeazarra et al., 2021).

The electrical activity in each chamber of the heart translates to the defined waveforms of the surface ECG. The P wave represents depolarization in the atria, the QRS complex representing depolarization in the ventricles, and the T wave representing repolarization of the ventricles. Thus, an action potential in the ventricle is represented by the QT interval, the time between the Q wave and T wave. Figure 6 shows a comparison of a human and zebrafish ECG, highlighting the striking resemblance of one another.

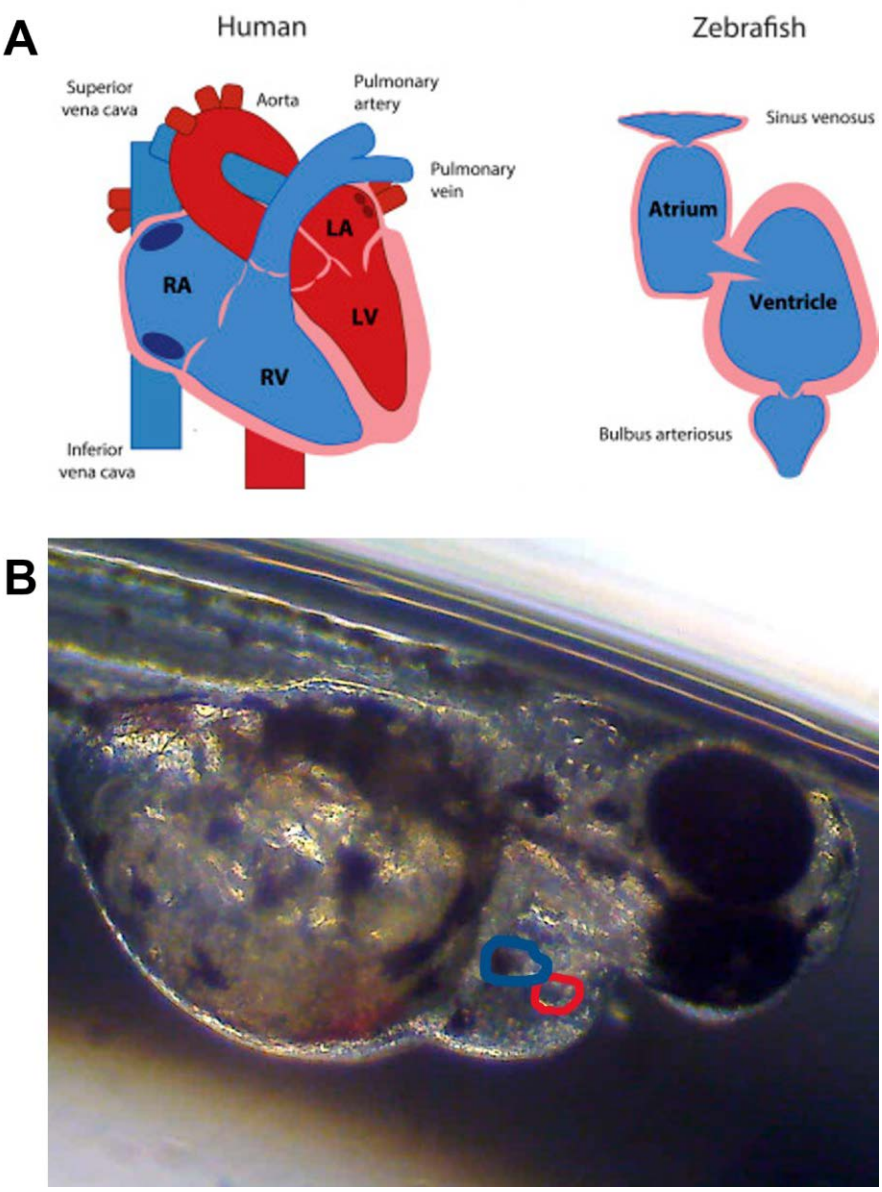


Figure 4: Structures of human and zebrafish hearts.
A. The human heart consists of four chambers, two atria and two ventricles. The zebrafish heart consists of two chambers, having a single atrium and ventricle. B. The zebrafish heart outlined in the pericardial sac. The atrium is outlined in red, and ventricle in blue.

Note: 4a image obtained and modified from (van Opbergen et al., 2018b), permissions obtained from Elsevier license #: 5777921492612

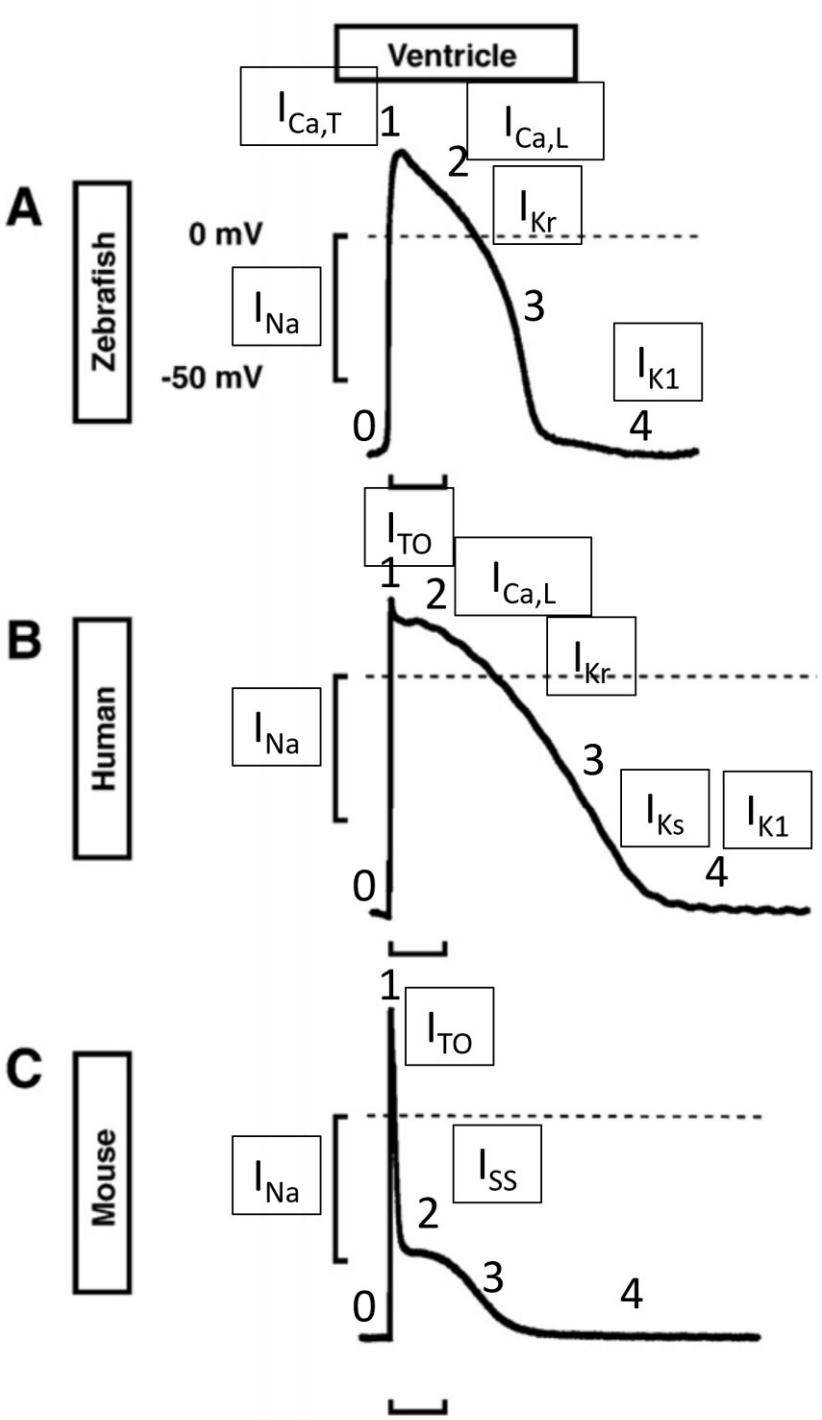


Figure 5: A comparison of ventricular action potentials between zebrafish, human, and mouse. Each action potential shows the phases as well as currents responsible for each phase in the action potential. The zebrafish heart was spontaneously beating at 149 ± 8 bpm at 28 °C while the human and mouse myocardial tissues were stimulated at 60 bpm (1 Hz) at 37 °C.

Note: Image obtained and modified from (Nemtsas et al., 2010), permissions obtained from Elsevier license #: 5777941126849

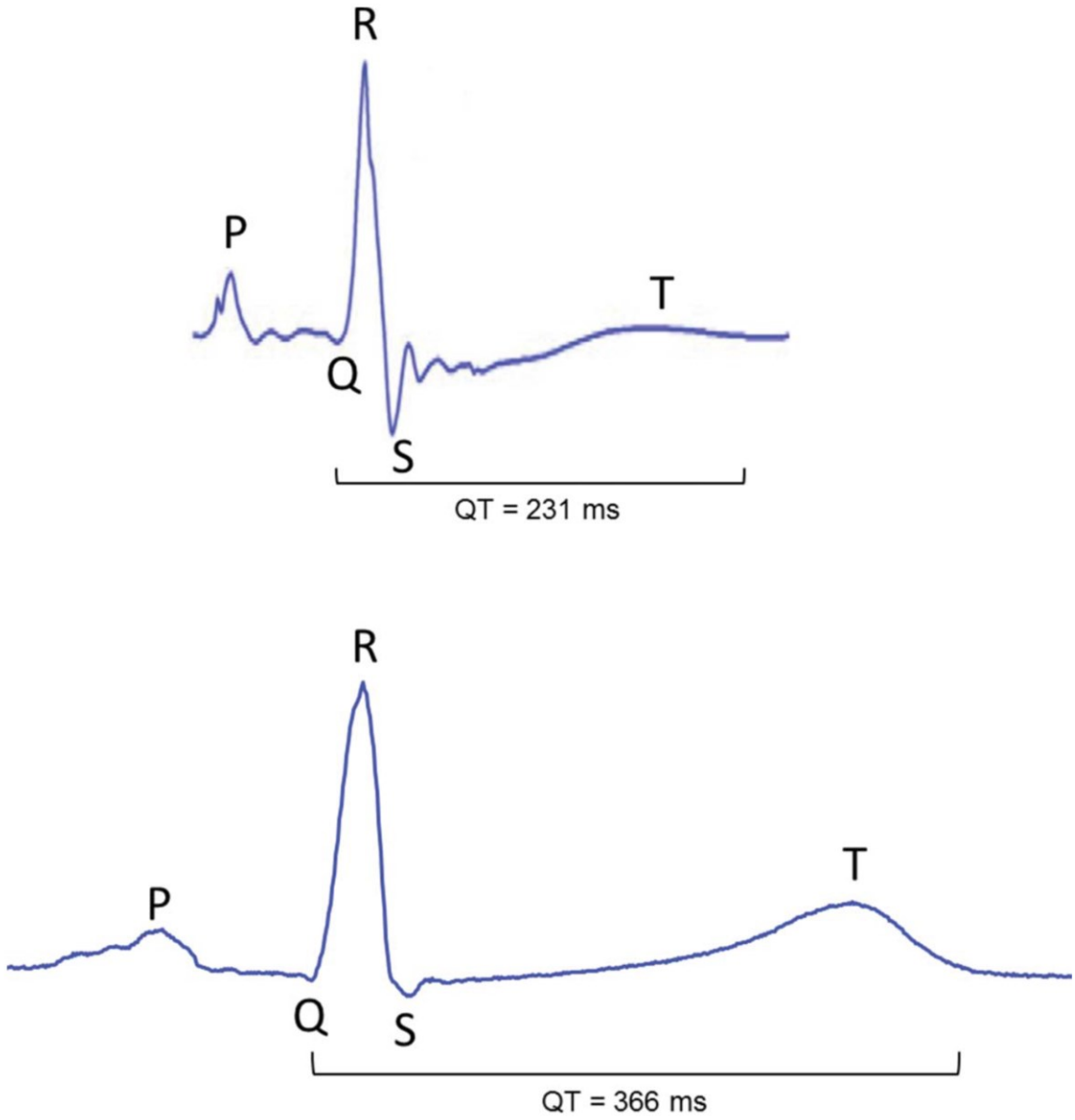


Figure 6: Comparison of zebrafish and human ECG.
Electrocardiograms for zebrafish (top) and human (bottom). P wave, QRS complex, and T wave are labelled. These recordings are on the same timescale, with QT intervals labelled.

Note: Image obtained from (Vornanen and Hassinen, 2016). Permissions granted by Taylor and Francis publishing for theses and dissertations.

1.3.2. Varieties of LQTS

Long-QT Syndrome (LQTS) is a disorder of the cardiac electrical system wherein the heart rate-corrected QT interval (QTc) in the surface ECG is prolonged and there are irregularities in the T wave (see Figure 7) (Alders et al., 1993; Schwartz et al., 2012a; Giudicessi and Ackerman, 2013). This provides a substrate for cardiac arrhythmias, i.e.

irregularities in the beating of the heart. LQTS can be both heritable and acquired, though this thesis focusses on the inherited variety. Inherited LQTS has approximately 17 varieties, with variants LQTS1, LQTS2, and LQTS3 accounting for 75% of cases. LQTS1 derives from LOF mutations in the Kv7.1 channel, encoded by *KCNQ1*. This channel passes the I_{Ks} current, important as an adaptive measure when heart rate increases. This is due to I_{Ks} acting as a potential reserve, providing increased repolarizing current when heart rate increases (Jost et al., 2005; Printemps et al., 2019). LQTS3 is caused by mutations in the *SCN5A* gene, encoding the NaV1.5 channel, which is primarily responsible for depolarisation in the ventricular action potential. Gain of function variants increase the depolarising current, which extends the action potential duration (APD) by providing a more persistent inward sodium current, offsetting repolarizing currents, providing a substrate for early-after depolarizations (EADs). EADs are depolarizations that occur during late repolarization, when the membrane potential is still depolarized and stimuli can potentially trigger premature action potentials (Schwartz et al., 2012a; Pérez-Riera et al., 2017). LQTS2, is caused by LOF mutations in the *KCNH2* gene, encoding the Kv11.1 channel. This channel is responsible for the I_{Kr} current, the major repolarising current in the ventricular action potential. With a reduced I_{Kr} current, repolarisation (and the QT interval) can be prolonged, allowing for EADs to form and arrhythmogenic events . Each of these LQTS varieties, though caused by variants in different channels, typically manifest with a prolonged repolarization and platform for EADs to trigger premature action potentials. These action potentials can cause irregular beating and arrhythmias such as torsade-de-pointes (TdP), leading to potential sudden cardiac death (Alders et al., 1993; Schwartz et al., 2012a).

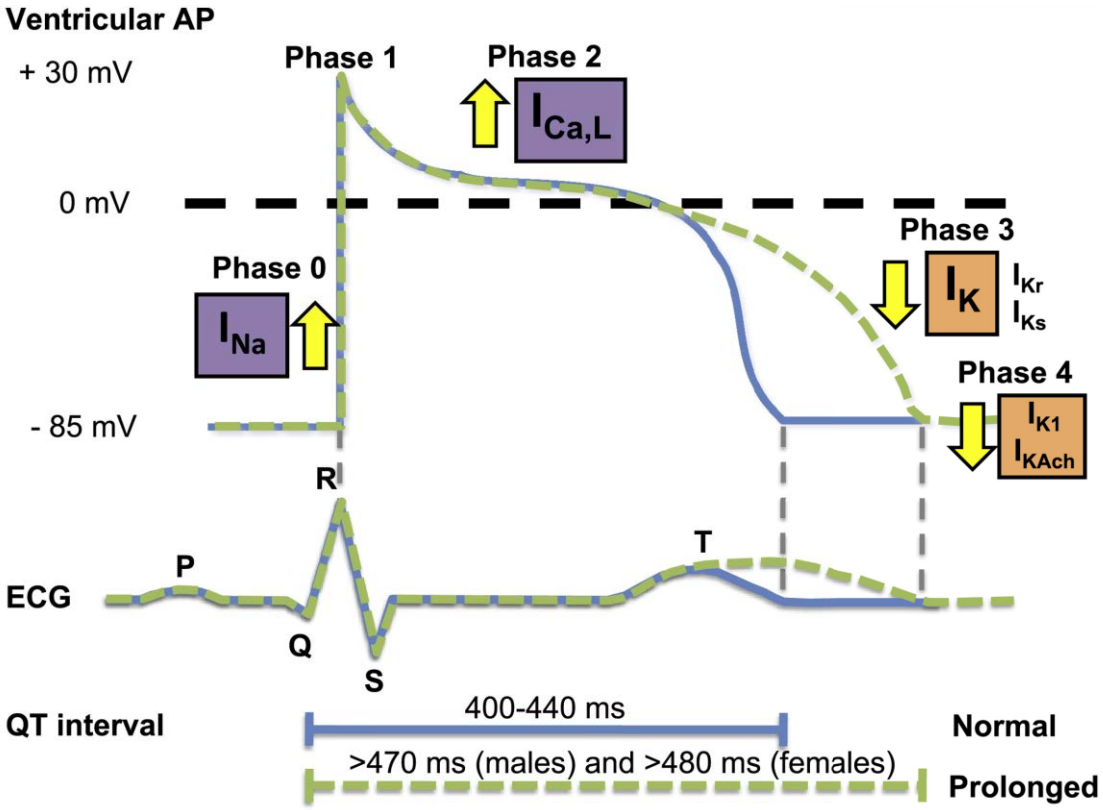


Figure 7: A comparison of WT and LQTS cardiac electrical measures.
A) ventricular action potentials, B) ECG, and C) QT intervals. Blue represents a healthy heart, green represents an individual with LQTS.

Note: Image obtained from (Giudicessi and Ackerman, 2013). Permission obtained from Elsevier license #: 5679080822669

1.3.3. Complications and risk stratification

Cardiac events due to LQTS typically occur in patients up to 40 years of age, mostly manifesting from preteen years through the 20s (Guettler et al., 2019). LQTS is found in approximately 1:2,000, with some estimating closer to 1:1,000 (Schwartz et al., 2009a). This disparity is partially due to how LQTS manifests, in that patients do not always show an abnormal QTc interval, which causes difficulty in risk stratification of this syndrome. Currently a points system is used to diagnose LQTS, accounting for ECG morphology, clinical history, and family history (Alders et al., 1993). However this is reliant on a typical pathology for LQTS, which is not always the case. 25% of individuals with LQTS have a normal QTc range, and in Chapter 1.3.3 I describe one variant recently reported by our lab group to affect the protective current potential of the hERG channel while manifesting a normal APD and QTc interval (Priori et al., 2003; Goldenberg et al.,

2006). An important factor is the location of the variant in the *KCNH2* gene, with those near the pore and transmembrane regions being more pathogenic (Tseng, 2001; Huo et al., 2008; Al-Moubarak et al., 2020). This, alongside functional assays of any variants found, are important when assessing pathogenicity and risk stratification. Aside from genes directly associated with LQTS-related phenotypes, there are "modifier" genes which have an association with the phenotype or interact with channel subunits in a fashion that promotes LQTS. Together this makes studying variants a particular challenge.

So far I have discussed inherited LQTS, but this is one of two categories of the disorder. Acquired LQTS, caused by interaction between pharmaceuticals and the hERG channel specifically, or conditions such as hypokalemia and hypomagnesemia, are not the focus of this thesis but are a relevant factor in development of robust LQTS models. This form of LQTS and the role zebrafish can play is explored more in Chapter 3.

1.3.4. hERG channels and LQTS2

Both the inherited LQTS2 and acquired LQTS forms are caused by dysfunction of the Kv11.1 channel, either by loss-of-function mutation or pharmacological blockage, respectively (Alders et al., 1993; Schwartz et al., 2009a, 2012b; Shah et al., 2019). LQTS2-associated loss-of-function Kv11.1 channel mutations are typically inherited heterozygous gene variants with homozygous mutations being embryonic lethal. Kv11.1, which is also often known as the human Ether-à-go-go-Related Gene, or hERG, is a delayed rectifier potassium channel, primarily responsible for phase 3 repolarisation in the ventricular action potential. The hERG channel normally conducts the I_{kr} current, which repolarizes the membrane during phase 3 of the action potential. The channel slowly activates during depolarization but rapidly inactivates, via C-type inactivation. As the membrane starts to repolarize, hERG channels rapidly recover from inactivation and pass an outward, slowly deactivating current (Shah and Carter, 2008; Vandenberg et al., 2012). This serves multiple potential roles: it allows for suppression of repolarisation during the plateau phase of the action potential (due to rapid channel inactivation), resurgent repolarisation during phase 3 (due to recovery from inactivation into the open state), and protective repolarising drive in response to premature depolarisations during the refractory period (due to slow channel closing). These features, provide temporally regulated repolarising current that counteracts potential EADs through phase 3 and 4 of the ventricular action potential. See Figure 5 for a comparison with the zebrafish and mouse action potential.

The channel itself comprises 4 subunits, each consisting of 6 transmembrane segments as well as an N-terminal Per-Arnt-Sim (PAS) domain and C-terminal cyclic nucleotide binding domain (CNBD) (see Figure 8). The S1-S4 segments form the voltage sensing domain, with the S4 instrumental in triggering opening and closing of the pore as a result of a high density of charged amino acid side chains that sense changes in the transmembrane voltage producing conformational shifts. In the classical electromechanical coupling model, the pore domain is connected to the S4 via the S4-S5 linker, and movements in the S4 segment translates to movement in this linker and subsequently to the pore domain S5 and S6 segments, opening and closing the channel.

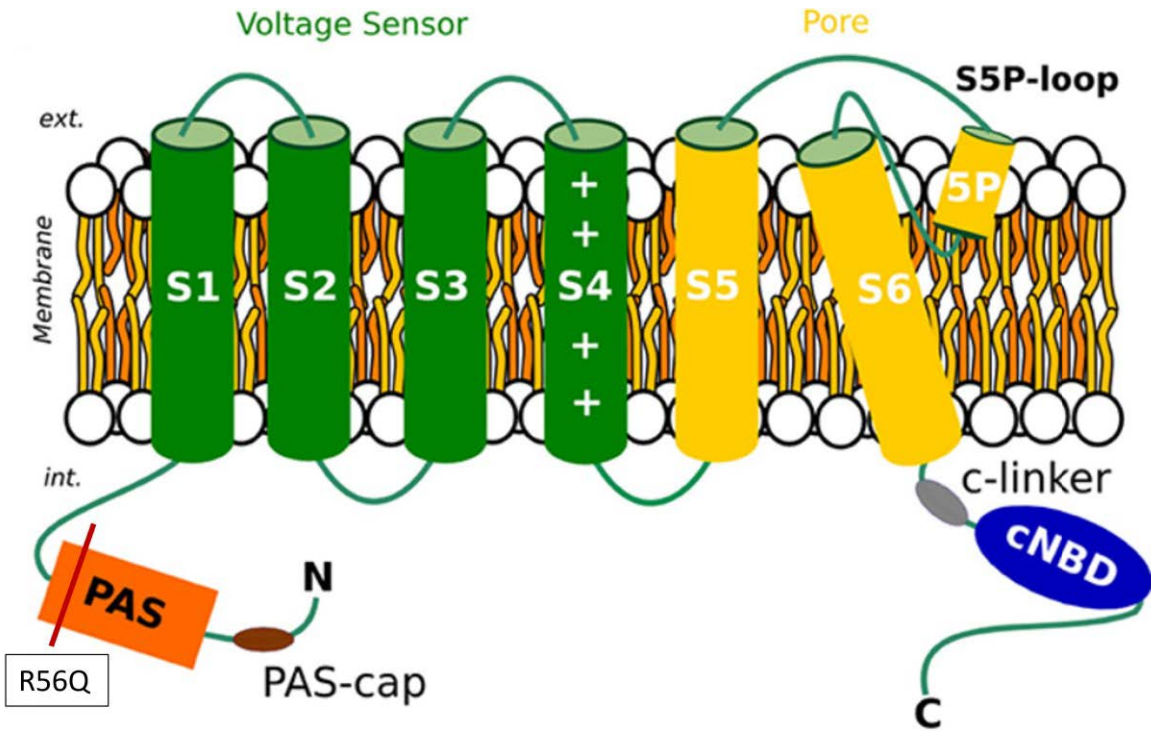


Figure 8: A schematic of the membrane topology of the hERG channel.
The hERG channel contains 6 transmembrane domains, organized into a voltage-sensing domain and pore domain. The cytosolic side of the protein contains an N-terminal PAS domain and C-terminal domain.

Note: Image obtained from (Perissinotti et al., 2018). Cited work published under the terms of a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/) and is unmodified from the original.

As mentioned in Chapter 1.3.1, variants in hERG are responsible for inherited LQTS2. However, many variants that are discovered in patients are considered to be variants of unknown significance (VUS), where the identified variants has no functional studies conducted to determine their effect. As of 2021, approximately 81% of known hERG variants were considered VUS (Oliveira-Mendes et al., 2021). This suggests a need for a high throughput platform to characterise variants, something addressed in part in this

thesis. Additionally, there are variants that do not present with typical LQTS phenotypes in that they are associated with sudden cardiac death, but not prolongation of the APD or QTc. For example, it has been hypothesized that R56Q, a variant in the Per-Arnt-Sim (PAS) domain of the channel, reduces hERG channel protective currents that resist EADs without prolonging the APD (Kemp et al., 2021). This produces a challenge for risk stratification, as there is not a noticeable clinical phenotype as we would typically expect for LQTS.

When examining characterized hERG variants, the majority are trafficking-deficient, wherein they are prevented from, or degraded before, reaching the cell membrane. As of 2020, 84% of hERG variants whose cell surface expression has been examined were found to be trafficking-deficient. An example is G604S, a mutation in the pore region of the hERG channel. Expressed on its own, this variant was found to not traffic to the cell membrane. Using Western blots, WT hERG would normally show a 155 kDa band representing glycosylated protein at the cell membrane, and a 135 kDa band representing immature protein still in the endoplasmic reticulum (ER). When expressing just the variant form in HEK293 cells, only the 135 kDa band was seen, suggesting the channel is not reaching the cell membrane as a mature protein. Additionally, when co-expressing G604S with WT hERG, the 155 kDa band was weaker than just WT, suggesting that the variant was reducing expression at the cell membrane, measured at 49.5% less (Huo et al., 2008). Finally, there can be modifier genes, separate genes that interact with hERG in some fashion to cause a phenotype as if the hERG gene itself was mutated. This can be a confounding factor. An example of a modifier is a SNP in the gene encoding rififilyn (RFFL), a ubiquitin ligase that is linked to variation in the QTc interval. In 2019, Roder *et al.* explored the role this SNP plays in hERG function by studying the homolog (rbERG) in rabbit cardiomyocytes as well as a heterologous expression system (239A cells). Expression of this RFFL variant reduced rbERG expression and activity. In 239A cells, the researchers found via western blots that RFFL interacted with the core hERG subunit, and in fact that it causes increased ubiquitination and degradation of hERG (Roder et al., 2019).

Together, this demonstrates the difficulty in studying hERG variants and channel defects. While there is relatively strong understanding, much of the available information comes from data in heterologous expression systems that have less biological relevance to humans. While effects of variants on measures such as APD can be inferred, a whole

organ model is required to observe actual arrhythmogenesis. Given this uncertainty in expressed phenotype, when studying VUS, observing a more holistic cardiac model is a necessity in order to make predictions of pathogenicity or functional mechanisms of action. When translating to cardiac systems, a cardiac system must be studied, and the zebrafish is a powerful tool for this. In this thesis, I will demonstrate why this is the case and how a pipeline for these studies can be constructed.

1.3.5. Zebrafish hERG orthologs and paralogs

As discussed in Chapter 1.1.1, approximately 71.4% of human genes have at least one zebrafish ortholog. Alongside these orthologs, due to genome duplication events, zebrafish often have multiple paralogs of these genes. Genome duplication events can occur via allopolyploidy or autopolyploidy, which describe combinations of the genome of different species or doubling of a genome within a species, respectively. Duplicated genes have been shown to diverge through multiple paths: either changing in function, location of expression, or remaining as a duplicate. It is thought that 25% of zebrafish genes have a duplicate that has not diverged.

The zebrafish ortholog to the human KCNH2 gene is *zkcnh2a*, but after further study the cardiac equivalent was discovered to be *zkcnh6a* (Vornanen and Hassinen, 2016). In total there are 5 related *zkcnh* genes (*zkcnh2a*, *zkcnh2b*, *zkcnh6a*, *zkcnh6b*, *zkcnh7*), with *zkcnh2a* and *zkcnh6b* paralogs resulting from the teleost genome duplication event (Genge et al., 2024). An analysis of amino acid homology with human KCNH2a shows that although *zkcnh2a* has a greater homology than *zkcnh6a*, examining the expression patterns via qPCR shows that *zkcnh6a* is expressed more in the ventricle than *zkcnh2a* (Figure 9) (Genge et al., 2024). Examining gene and amino acid comparisons, showed that both *zkcnh2b* and *zkcnh6b* are truncated, missing the C-terminal and N-terminal domains, respectively (Figure 10) (Genge et al., 2024). *zkcnh6a* encodes the same protein domains as human KCNH2, as well as an amino acid homology of 64% in the PAS domain, 82% in the voltage sensing domain, and 91% in the pore domain.

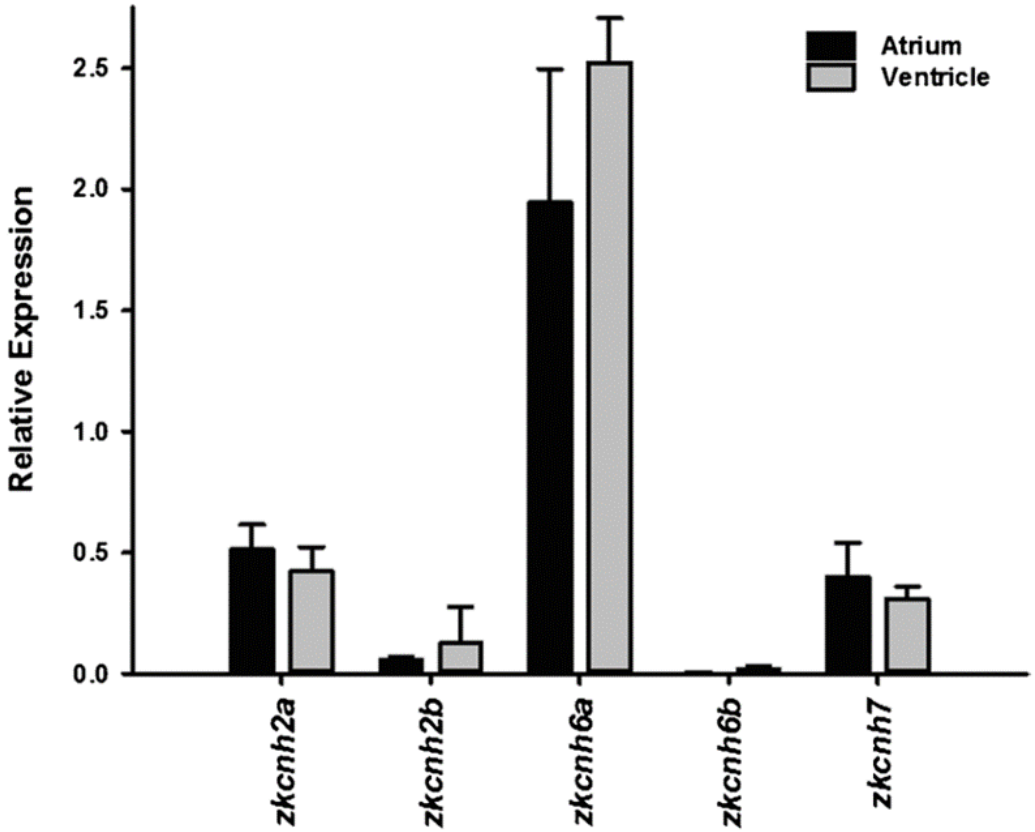
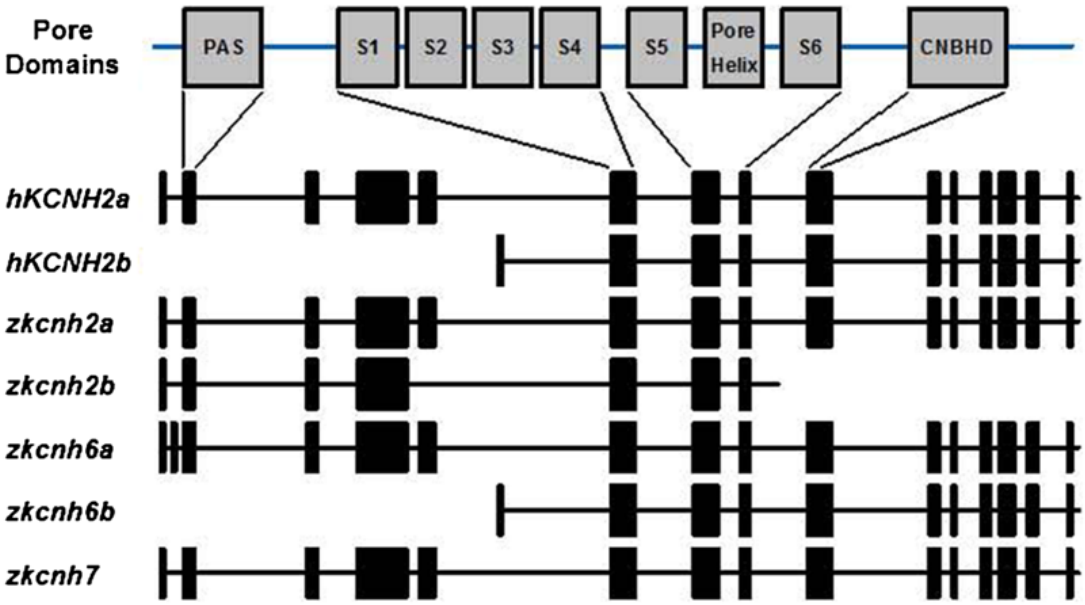


Figure 9: Comparison of *zkcnh* transcripts in the heart.
zkcnh6a is the predominant transcript expressed in both chambers of the zebrafish heart, implying it is the functional equivalent of human KCNH2 rather than *zkcnh2*.

Note: Image obtained from (Genge et al., 2024). Permissions obtained from Springer Nature, license #: 5777951052548



% Amino Acid Homology	PAS	S1-S4	S5, Pore Helix, S6
hKCNH2a / zkcnh6a	64%	82%	91%
hKCNH2a / zkcnh6b	–	84%	88%
hKCNH2a / zkcnh2a	78%	83%	94%

Figure 10: A comparison of *zkcnh* exons mapped to protein domains and amino acid similarity. *zkcnh6a* possesses the same protein domains as hKCNH2a, as well as sharing up to 91% amino acid homology in the pore domain. While it possesses less homology than *zkcnh2a*, the zebrafish ortholog, expression data suggests that *zkcnh6a* is the functional equivalent.

Note: Image obtained from (Genge et al., 2024). Permissions obtained from Springer Nature, license #: 5776221036143

Chapter 2. General Methods

This methods section contains general approaches primarily relating to zebrafish husbandry, breeding, injections, and general molecular techniques. Design strategies and methodology for the genetic approaches that I have developed during my thesis research will be described in detail in Chapters 4 and 5.

2.1. Zebrafish Housing

Zebrafish are housed in a Techniplast ZebTEC rack under the guidance of Simon Fraser University Office of Animal Care Services. This self-sustaining system holds up to 500 adult (>3 months old) zebrafish and provides automated pH, salinity, and temperature control. Temperature is maintained at 28 ± 1 °C, salinity at 500 ± 25 µS, and pH at 7.5 ± 0.2. Each tank can hold up to 10 fish, with an even mix of males and females. For enrichment there are plastic plants within the tanks and laminated printouts of rocks underneath to simulate their natural habitat. Water is at a constant flow, with 10 L of the entire system's water exchanged each day. The sump of the rack system contains a series of filters: a mesh net for physical filtration, a bio-filter seeded with bacteria for breaking down nitrates, a carbon filter to remove impurities, and a UV filter to neutralize any potential pathogens or microbial life (Figure 11).

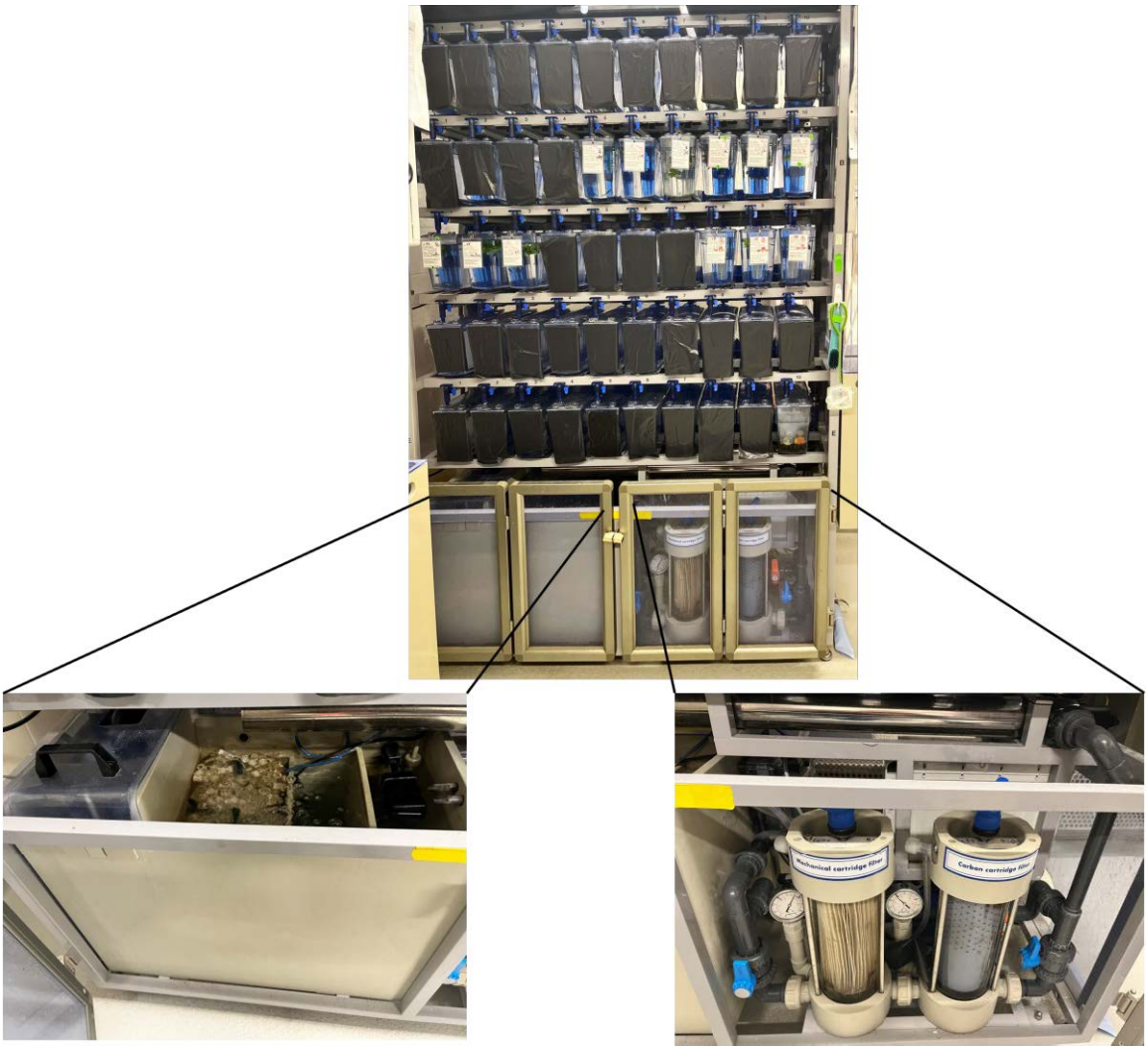


Figure 11: A photograph of the ZebTec zebrafish rack system, with magnified portions showing the filtration systems.
The bottom sump contains a series of 4 filtration systems. Lower-left: Bio-filter rings containing bacterial colonies for denitrification of water. Lower-right: Two cartridge filters for both mechanical and charcoal filtering. Above these is a UV filter as the last stage before water is recirculated.

2.2. Zebrafish Feeding

From fertilization until 6 days post-fertilization (dpf) larval zebrafish are maintained in 100 mm petri dishes with E3 media (see Table 3) supplemented with approximately 0.1 mL of methylene blue (an anti-fungal agent). Larval zebrafish subsist off their yolk sac until 6 dpf, at which point larvae are transitioned into a custom-built aquarium tank with rotifer polyculture. Rotifers (*Brachionus plicatilis*) are a saltwater planktonic species that are maintained for polyculture with the larval fish. Rotifers are maintained in a 14 L culture at a salinity of 10 ppt, and drip fed an algae mixture (Reed Mariculture). Three times per week I disposed of and replaced 4 L of the culture to remove older rotifers, and once a month the entire 14 L was moved into a clean culture system (Figure 12A and 12B). For the polyculture, an aquarium tank is maintained at a salinity of 5 ppt which allows for survival of both the zebrafish and rotifers. Larval fish are kept in 5 net inserts that house 50 larvae each, while the rest of the tank houses a rotifer culture. This allows the fish to exist in a smaller volume, concentrating rotifers, without forcing them to swim large distances to feed. There are enough rotifers and algae added to keep the population alive and self-sustaining while the larval fish feed on them.

At 10 dpf, the larvae are transferred into the adult ZebTEC system in specialized inserts that provide shallower housing. Up to 50 larval fish are housed per tank, whose shallow water ensures that the juvenile zebrafish are able to swim the height of the water column and obtain food. At this point, the larvae are fed the Gemma 150 particle size processed feed (Skretting) during a morning feed, in addition to rotifers in the afternoon. The rotifers are from the main culture and must be filtered to remove excess salt.

At 15 dpf, *Artemia* (brine shrimp) live feed is phased in. These are saltwater plankton commonly used as a live feed option for zebrafish. We decapsulate the brine shrimp cysts to remove the outer layer (which can otherwise be hazardous if consumed) by hydrating them in 1 L of dechlorinated water and adding approximately 10% of the volume in bleach for 5 minutes, and then rinsing and storing in a saturated salt solution (300 g/L) to preserve for a week at a time. Multiple times per week these are hatched, and then fed to the zebrafish for a period of 2 days before a new batch of cysts are hatched (Figure 12C). This timing ensures that we do not have to feed and maintain the shrimp,

and they are still nutritious for the fish. *Artemia* are fed to the zebrafish starting at 15 dpf, and throughout the rest of their lives.

At 30 dpf, the zebrafish are moved out of the specialized inserts and into regular system tanks, and the delivery of processed feed is switched to the Gemma 300 size processed feed. This is the feed that they will be maintained on long term in the mornings, alongside *Artemia* in late afternoons. A standardised nutrient profile for zebrafish, as exists for other models such as mice, has not been developed at this point. Evidence suggests that while zebrafish can be maintained on processed feed exclusively, there is benefit to a mixed diet of processed and live feed (Farias & Certal 2015).

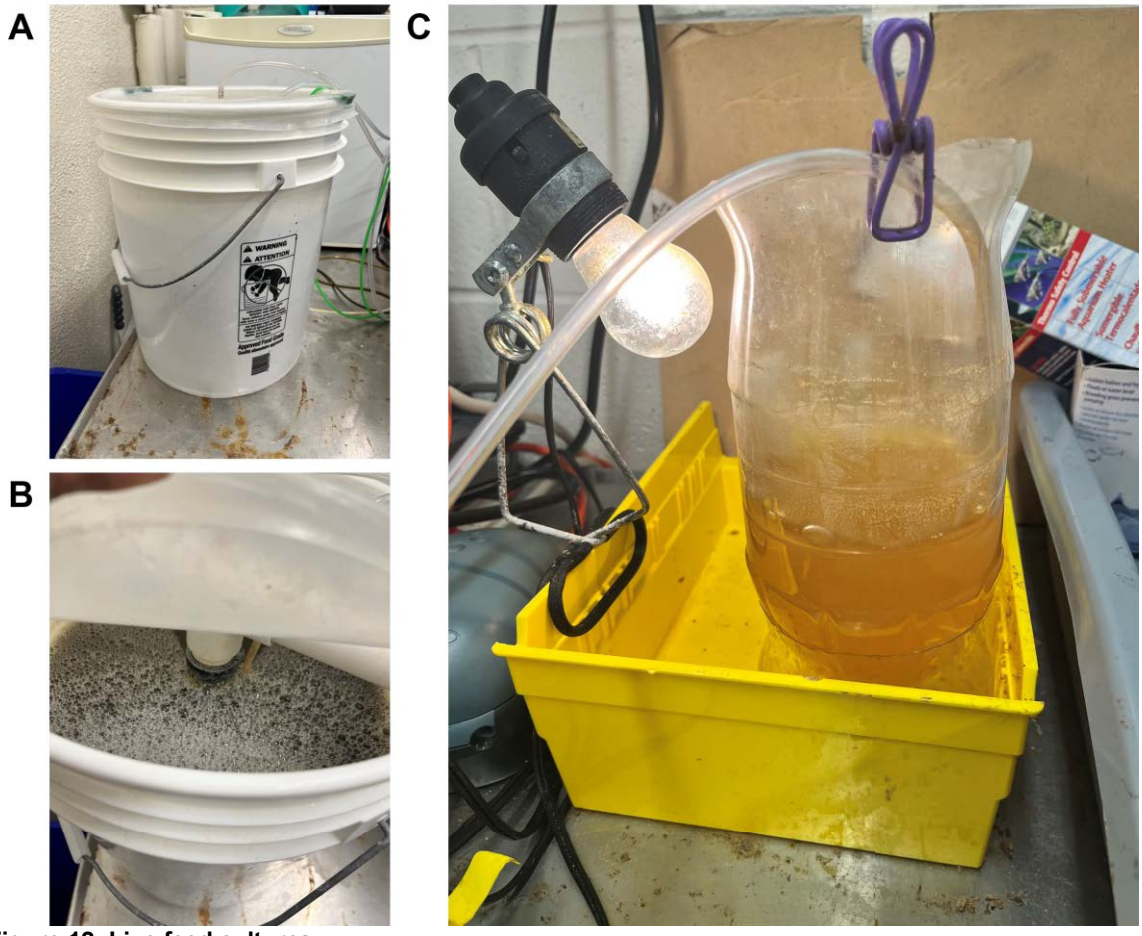


Figure 12: Live feed cultures.
A) The exterior of the rotifer culture bucket. The transparent tubing supplies aeration, while the green tubing doses algae at regular intervals to sustain the rotifers. B) Interior of the rotifer culture bucket. Bubbling denotes a healthy rotifer population ready for feeding. C) The artemia culture bottle. Decapsulated artemia cysts are grown in a plastic bottle, with constant aeration and light. The water contains 24 g/L of marine salt. Artemia are grown for up to 3 days, afterwards the leftover culture is disposed of and a new culture is grown.

2.3. Zebrafish Breeding

Zebrafish breed based on light cycles, initiating breeding as they are exposed to light in the morning. In the tank system, the lights are programmed to turn on at 8 am and off at 8 pm, giving a 12-hour circadian rhythm. With regards to breeding, this means at 8 pm the selected adults are separated in specialized breeding tanks, a smaller tank with a perforated false bottom to allow eggs to fall through and a divider to separate male and female fish, see Figure 13. Adults are chosen in either an even ratio or a 2:3 ratio of males:females. Adults are chosen from within the same tanks; this allows for both easy tracking of which fish are breeding more efficiently as well as maintaining internal hierarchies within tanks. While the fish theoretically breed normally in mixed tanks, females can become egg-bound if they are not purposely bred often enough. This is when oocytes clog the oviduct resulting in buildup of eggs, and difficulty in releasing these eggs into the water for fertilization. As these tanks are separate from the main rack system, they are kept in a water bath maintained at 28 °C overnight while the fish are contained within. Due to the perforated false bottom, water is shared between the two halves, and overnight the fish will release pheromones to stimulate breeding behaviour in the morning. At 8 am, the divider is removed and the fish are allowed to breed for 20-60 minutes, depending on the purpose of the fish breed. If injecting, the eggs need to be fertilized and injected within approximately 40 minutes, while breeding for expanding the colony requires maximizing the number of eggs, this allows for a longer (up to 60 minutes) breed. This means for injections, fish are allowed to breed for 20 minutes to allow time for transportation of the eggs to the lab. After this period, the fish are moved back into their respective tanks in the main rack system, and the false bottom of the breeding tank is removed to allow access to any eggs. These eggs are pipetted out and placed in petri dishes (up to 50 per dish). These eggs are suspended in E3 media along with 2 drops of methylene blue to combat potential fungal contamination. While the eggs/larval fish don't need to be fed until 6 dpf, they should be checked daily to remove any debris, hatched egg casings, or other contaminants. Otherwise, microbial growth can compromise larval fish survival. An important note is that over time there will be a build up of recessive mutations, as the

colony interbreeds, therefore it is useful to have unrelated zebrafish kept for outcrossing in order to introduce genetic variability into the colony.

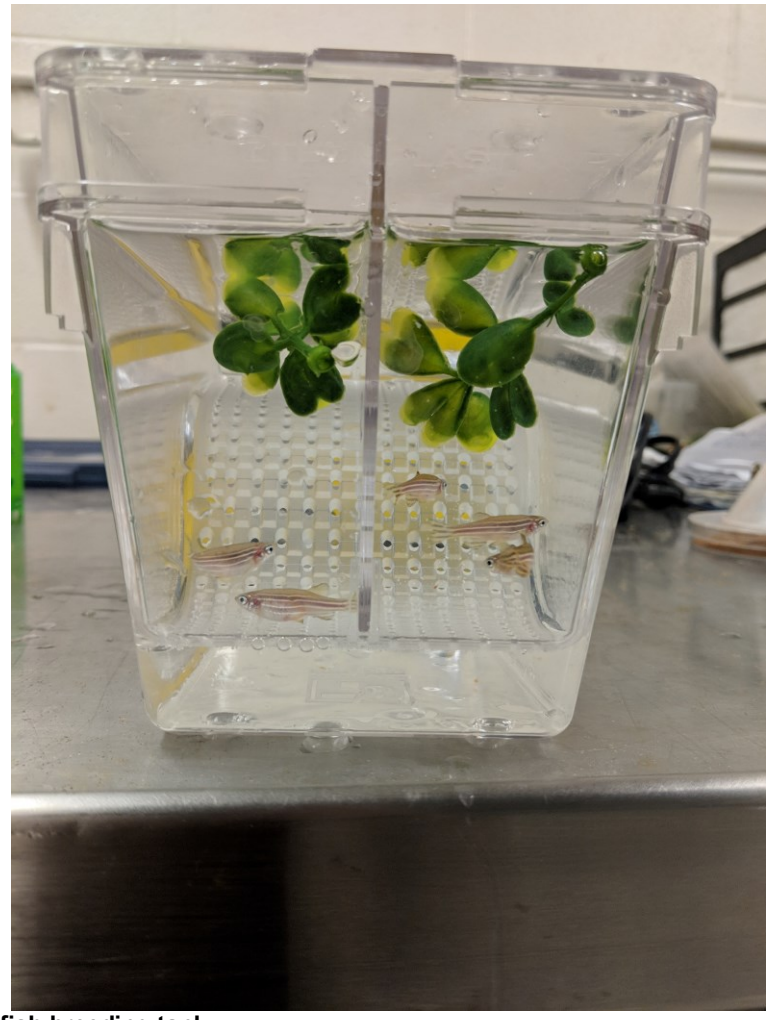


Figure 13: Zebrafish breeding tank.
Males and females are separated by the divider the previous night. Overnight the fish release pheromones which are able to pass underneath the divider and stimulate the other fish for breeding when the lights turn on. Plastic plants are provided to simulate vegetative cover in the water. The sloped false bottom provides both a shallow area for the fish to breed, preferred in their natural environments, as well as a space for the eggs to fall, ensuring the parents do not consume them.

2.4. Zebrafish Embryo Microinjections with CRISPR Components

Microinjection pipettes are fashioned on the day of injections from borosilicate glass capillaries (1 mm outer diameter from Harvard Apparatus) that are pulled in a P-1000 Flaming/Brown micropipette puller to create a fine tip that is then manually broken under a stereoscope to provide a 4 µm inner diameter. Injection pipettes are back-filled with 4 µL of injection materials (injectant) ensuring there are no air bubbles present in the tip.

When performing embryo injections to engineer CRISPR edits, it is important to limit the fish to breed no more than 20 minutes. This is because the cells typically begin division at approximately 45 minutes, and enough time is required to inject before the 2-cell stage. If injections are performed at later stages, there is no guarantee that each cell will receive the CRISPR components and chimeric mutations may result (only some cells in the fish will receive the mutation rather than all). The eggs are brought to the lab and lined up in an empty petri dish along a glass microscope slide, oriented for ease of injection. Injection settings used are an input 100 psi to the picoinjector, 1.0 psi output, and 0.3 s duration. Using a micromanipulator, the injection pipette is inserted into the yolk sac of the egg, and the injectant is dispensed in response to the air pulse controlled via a foot pedal or button control on the picoinjector. The volume injected for each egg is approximately 10% of the total egg volume, and this can be seen and confirmed visually through the stereoscope. See Figure 14 for the setup and injection process.

After 50 eggs are injected, the dish is filled halfway with E3 media and the eggs are rinsed off the microscope slide. 2 drops of methylene blue are added, and the dish is labeled dish with what has been injected, date, # of eggs, and is placed in a 28 °C incubator. These injections should be recorded in a database, keeping track of how many eggs were laid, how many fertilized, number of injections performed, and which construct(s) were injected.

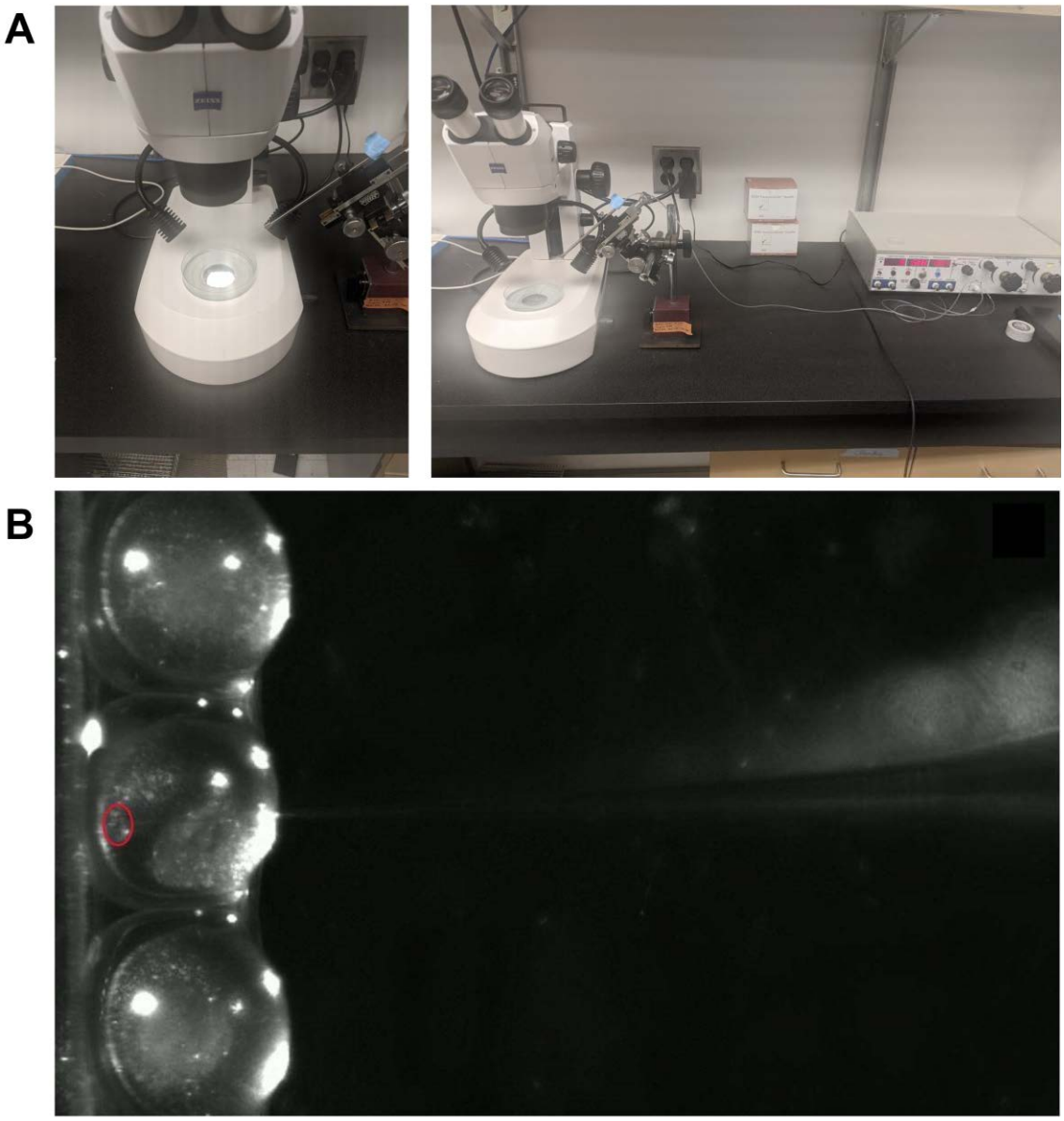


Figure 14: Zebrafish oocyte injection setup.
A) The left image shows a closeup of the injection space on the stereoscope, using a micromanipulator to perform the fine movements to inject the zebrafish eggs. The right image includes the PLI 100A Picoinjector, using air pressure to deliver a precise amount of injectant through the needle. B) A photo through our stereoscope showing the injection process. Circled red is the tip of the needle, pierced into the yolk sac of the egg. The injectant dispensed is approximately 10% of the total egg volume.

2.5. Zebrafish Genotyping and Phenotyping

At 3 dpf, the larval zebrafish are phenotyped visually, genetically, and electrically. Before phenotyping, presence of the YFP that is integrated into the HDR template (design shown in Chapter 4) is used to screen for successful HDR events. A fluorescence-capable stereoscope equipped with blue-light 500nm LEDs is used to screen the larval zebrafish for green eyes, using a 490BP20 excitation filter to isolate the desired wavelength from the range present in the LEDs, and a 535BP30 emission filter to remove environmental white light. All injected larval zebrafish are placed in 24-well plates, screened for fluorescence, and those positive for green eyes are further phenotyped as well as an equal number without green eyes as a control group.

For visual phenotyping, images of the pericardial sac are used to assess pericardial effusion, using a Blackfly U3-23S6M-C microscope camera attached to a Stemi 305 stereoscope. Images are captured using the Micromanager plugin for ImageJ, and the pericardial sac area normalized to the area of the eye is measured in each fish by manually drawing the boundaries of each and computing the area within the boundary as described in detail in Chapter 4. Simultaneous video capture of beating frequency for 10 s at 20 frames per second is used to measure heart rate. Video capture of heart rate is analysed through a custom algorithm designed by Dr. Damon Poburko. This algorithm uses changing pixel density to measure heart rate within a given region of interest (roi).

For genotyping, genomic DNA was sequenced from tail clip samples. After visual phenotyping, fish are anaesthetized in 0.2% tricaine (MS-222) until they lose their righting reflex. They are then transferred via pipette to a length of tape in a petri dish lid in a drop of water under a stereoscope. A syringe needle is used to cut the tip of the tail, and the fish is then transferred into a well of a 24-well plate with fresh E3 media for recovery. The section of tail that was clipped is placed in a PCR tube with 10 µL of 50 mM NaOH and broken down via a thermal cycler heating protocol. The reaction is neutralized via 10% Tris-HCl and centrifuged at 13,000 rpm for 5 min to isolate the DNA-containing supernatant. PCR is then performed on the isolated DNA to amplify the sequence of interest and the PCR product is sent for Sanger sequencing. Both on- and off-target sequencing is conducted at this point to clarify accuracy and precision of the CRISPR-induced edit, details on design of these protocols and identification of off-targets is explained in Chapter 4.

Cardiac electrical phenotypic characterization involves measurement of the ECG of the larval fish, as described in detail in Chapter 4. Briefly, glass microelectrodes of 2 μM diameter are pulled using a P-1000 Flaming/Brown micropipette puller. Microelectrodes are made from 1 mm borosilicate glass capillaries (Harvard Apparatus). Microelectrodes are filled with a 3 M potassium acetate solution and placed on the ventral surface of the larval zebrafish at the heart and signals are detected using an Axoclamp 900A amplifier from Axon Instruments. Solution compositions are listed in table 3. The bath solution was E3 media supplemented with 0.3 mg/mL tricaine anesthetic to limit movement artefacts in recorded ECG signals.

Chapter 3. Utility of zebrafish models of acquired and inherited Long QT Syndrome

This chapter is based on the review article cited below that was published in Frontiers in Physiology in 2021 with minor formatting edits to suit the thesis style.

Simpson, K.E., Venkateshappa, R., Pang, Z.K., Faizi, S., Tibbits, G.F., and Claydon, T.W. (2021). Utility of zebrafish models of acquired and inherited Long QT Syndrome. *Frontiers in Physiology* 11. doi: 10.3389/fphys.2020.624129.

I was the lead author on this study, with intellectual contributions from Dr. Venkateshappa, Zhao Kai Pang, Shoaib Faizi, and Dr. Claydon. Dr. Tibbits, and Dr. Claydon assisted in reviewing the manuscript. This chapter is intended to provide a background and justification for the use of zebrafish in cardiac research, specifically in studying Long-QT Syndrome.

3.1. Long-QT Syndrome and hERG channels

Long QT syndrome (LQTS) is characterized by prolongation of the heart rate-corrected QT interval (QTc) and dysmorphic T-waves on surface electrocardiogram (ECG) recordings, and increases risk of cardiac arrhythmia (Alders et al., 1993; Roden, 2008; Schwartz et al., 2012a). QTc prolongation occurs as a result of aberration in one of several cardiac ion channels resulting in anomalous depolarization or repolarization of cardiomyocytes and prolongation of the action potential duration (APD). APD prolongation that increases the QTc above the 95th percentile of the normal range (350-450 ms) is used in the risk stratification of LQTS (Postema and Wilde, 2014). LQTS can be acquired or congenital, the latter accounting for approximately 1 in 2,500 people, with the former being more prevalent and attributable to electrolyte imbalances or adverse drug effects (Schwartz et al., 2009b). Inherited LQTS has been linked to mutations in several cardiac ion channels with *KCNQ1* (LQTS1), *KCNH2* (LQTS2), and *SCN5A* (LQTS3) being the most common LQTS genes, accounting for ~90% of all genotype-positive cases (Schwartz et al., 2012a). Altered ionic currents in these cases prolongs the APD, which increases the susceptibility to early after depolarizations (EADs) and triggered activity and also creates dispersion of repolarization across the ventricular wall creating a substrate for arrhythmias (El-Sherif et al., 2019). A prolonged QT interval predisposes individuals to a form of ventricular tachycardia, torsade de pointes (TdP), which can degenerate to ventricular fibrillation and syncope, cardiac arrest, and sudden death (Alders et al., 1993; Schwartz et al., 2012a).

Variants in the *KCNH2* gene that cause channel dysfunction (loss of trafficking or gating changes that reduce the time channels spend in the open state) are linked to LQTS2, which accounts for ~40% of LQTS cases (Schwartz et al., 2012a). More than 500 *KCNH2* gene mutations associated with LQTS2 have been identified (Alders et al., 1993; Schwartz et al., 2009b; Shah et al., 2019). *KCNH2* encodes the α-subunit Kv11.1 (also known as the human Ether-à-go-go-Related Gene, hERG), which is responsible for the major repolarizing current, I_{Kr}, during phase 3 of the cardiac action potential (Shah and Carter, 2008). As such, hERG channels are important for physiological suppression of EADs and triggered activity (Smith et al., 1996). Reduced hERG currents due to genetic variants prolongs repolarization, increasing the susceptibility to triggering cardiac events such as EADs, which can lead to lethal arrhythmias and sudden death. Acquired forms of

LQTS, thought to be more prevalent than the inherited form, are mostly due to adverse drug effects or electrolyte imbalance and are almost exclusively associated with blocking of hERG channels and reduced repolarization.

Drug-induced cardiotoxicity as a result of hERG channel block is one of the major hurdles in drug discovery and development and has been a primary reason for the withdrawal of many clinically approved drugs from the market (Mandenius et al. 2011; Kannankeril et al. 2010). hERG assays using mammalian heterologous expression systems (e.g. HEK-293, CHO cells) have been the gold standard in predicting cardiotoxicity. However, translatability of this assay is limited due to the lack of complexity of ion currents that are expressed in native cardiomyocytes, which may lead to the loss of potential drug candidates in the early stage of drug discovery (Liang et al., 2013). As such, there is significant interest in the generation of screening platforms that use more complex physiologically relevant cell or animal models. Furthermore, there is a need to better understand the complexity of genotype-phenotype correlations and the underlying fundamental mechanisms of inherited LQTS to be better able to risk-stratify variants and to develop and test effective targeted therapeutics. Thus high-throughput translational models that enable study of the complex mechanisms of cardiac repolarization and its alterations in congenital and acquired LQTS are highly sought after. In recent years, the use of zebrafish to provide such a model has gained traction (van Opbergen et al., 2018a; Tanaka et al., 2019). He we briefly discuss the utility of zebrafish as a cardiac model and then review the use of zebrafish as a model of acquired and inherited LQTS2.

3.2. Zebrafish as a cardiac model

3.2.1. Morphological characteristics

The zebrafish heart is distinct from mammalian hearts, most clearly by the presence of two, rather than four, cardiac chambers. However, even with only one atrium and one ventricle, the zebrafish heart is remarkably mammalian-like in a number of ways that suit its use as a model system. The zebrafish heart develops, starting as a single conducting tube, at just 24 hours post-fertilization (hpf), and over 72 hours, develops nodal activity, separation of atrium and ventricle, and coordinated inter-chamber conduction (Chi et al., 2008). This rapid timeline of development allows for observation of a functional heart early in development, proving advantageous for studying, for example, toxicological

screens, modifying mutations, and developmental pathways (Bakkers, 2011; Sarmah and Marrs, 2016). These investigations are greatly aided by the transparency of zebrafish larvae, which enable direct visualization of cardiac function using simple motion capture, or genetically encoded indicators, to monitor outcome measures such as bradycardia, tachycardia, or 2:1 atrioventricular block (Garrity et al., 2002; Chan et al., 2009). Furthermore, the diminutive size of the zebrafish larvae permits oxygen exchange via passive diffusion, ameliorating the need for a functional cardiac pump and this allows for study of potentially severe cardiac defects that might otherwise induce mortality in other model systems (Kang et al., 2018).

3.2.2. Electrical properties

The sinoatrial node (SA node) is a heterogeneous cluster of cells that forms the pacemaker region, responsible for the initiation of cardiac depolarization, and chronotropic responses of the heart. In zebrafish the SA node has been shown to develop very early, with the more primitive “heart tube” showing a constant and linear conduction pathway at 24 hpf. Pacemaking activity was shown to be critical in zebrafish heart rate regulation by the homozygous *slow mo* zebrafish variant, which resulted in attenuated pacemaking in isolated cardiac myocytes (Baker et al., 1997; Warren et al., 2001). Using a genetically encoded GFP linked to a transcription factor (*Isl1*) previously identified in mammalian SA nodal progenitor cells, the zebrafish SA node was identified as a ring of tissue at the junction of the sinus venosus and atrium (Tessadori et al., 2012). Isolation and patch clamp electrophysiological assessment of GFP+ cells revealed that these cells produce spontaneous action potentials, demonstrating their role in pacemaker activity (Tessadori et al., 2012). Subsequent work using transgenic zebrafish lines expressing GFP in conducting tissue combined with *hcn4* and *shox2* nodal-specific markers confirmed the presence of conducting cells in the SA node region of the zebrafish heart (Poon et al., 2016). These studies also identified dense innervation around the GFP-labelled cells, consistent with autonomic nervous system chronotropic regulation of the pacemaker site (Poon et al., 2016).

At approximately 2 days post-fertilization (dpf), a canal of tissue separates the zebrafish atrium and ventricle. This tissue appears to function similarly to the mammalian atrioventricular node (A-V node), in that it delays electrical propagation between the atrium and ventricle, allowing for coordinated contraction of the two chambers (Sedmera et al.,

2003; Milan et al., 2006a; Chi et al., 2008). Using optical mapping approaches, Sedmera et al., first identified a slowing of current through the junction between atrium and ventricle (Sedmera et al., 2003). A subsequent study mapped action potential morphology in the atrioventricular canal and demonstrated the presence of an action potential configuration that was distinct from that observed in either atrium or ventricle, and that contained a slow diastolic depolarization phase consistent with mammalian atrioventricular electrical activity (Chi et al., 2008). Interestingly, Stoyek et al., found that the atrioventricular canal functioned as a secondary site of pacemaker activity; upon vagal nerve stimulation, the source of spontaneous depolarization shifted from the SA node to the A-V node as is observed in mammalian hearts upon vagal stimulation (Stoyek et al., 2016).

Zebrafish hearts function as a syncytium with cell-to-cell communication afforded by similar connexin proteins to those in mammals, with orthologs of Cx40, Cx43 and Cx45 (Cheng et al., 2004; Christie et al., 2004; Chi et al., 2010). Christie et al., identified and characterized zfCx45.6 showing that it possessed 63% sequence identity with human Cx40 (Verheule and Kaese, 2013). Functional assessment of zfCx45.6 in dual voltage clamp *Xenopus* oocytes demonstrated functional gap junction formation with similar conductance and voltage-dependence to mammalian Cx40 (Christie et al., 2004). Knockdown or mutated variants (*dco*^{s226}) of zfCx48.5 (also described as zfCx46) produced uncoordinated contractions and decreased cardiac output (Cheng et al., 2004; Chi et al., 2010) indicative of a role in gap junction formation in the zebrafish heart. zfCx43 is the zebrafish ortholog to mammalian Cx43 showing 71% sequence identity with human Cx43 and expresses in the developing heart in a similar pattern to the mouse (Chatterjee et al., 2005; Iovine et al., 2005). The Cx45 ortholog, zfCx43.4, has high identity with human Cx45 (Essner et al., 1996), but no data have localized zfCx43.4 to the heart, and its function may differ from mammalian Cx45 (Barrio et al., 1997; Desplantez et al., 2003).

In terms of whole organ electrical propagation, recordings of ECG in larval and adult zebrafish demonstrate similar temporal sequence of activation and relaxation to that observed in human hearts (Milan et al., 2006b). Recently, ECG and high resolution optical detection of temporal voltage changes within the ventricle during activation and relaxation were used to correlate ECG waveforms with voltage gradients in the adult zebrafish heart (Zhao et al., 2020). These studies suggested that zebrafish hearts rely on epicardial gradients more strongly than transmural gradients as in humans, perhaps as a result of the differences in myocardium thickness of the ventricular wall, and this may have

implications for translation of findings related to arrhythmia initiation and maintenance in zebrafish to the human (Zhao et al., 2020).

3.2.3. Cardiac electrophysiology

The zebrafish ventricular action potential is remarkably similar to that in humans, more so than other small mammalian systems, such as the murine model. Most noticeably, the significant plateau in phase 2 is pronounced in zebrafish ventricular myocytes as it is in the human, and aside from the rapid repolarization observed in epicardial regions during phase 1, all other action potential phases are shared (Vornanen and Hassinen, 2016). Direct comparison of action potentials recorded from adult zebrafish ventricular cells with those from human papillary muscle and murine ventricular strips revealed the closely associated morphology of zebrafish and human ventricular action potential (Nemtsas et al., 2010). As a result, measurements of APD in adult and larval (e.g. 3dpf) zebrafish hearts report values of ~140-230 ms (see Table 1), which are remarkably dependent on temperature (Rayani et al., 2018), but reflect the duration of the human ventricular action potential reasonably well (Alday, 2014; Vornanen and Hassinen, 2016; Hull et al., 2019; Shi et al., 2020; Zhao et al., 2020). The QT interval measured from ECG recordings in zebrafish demonstrates comparable resting heart rate and conduction intervals with humans and the QT interval has a near linear relationship with the RR interval (Milan et al., 2006b), features that are imperative for a model examining LQTS that involves delayed ventricular repolarization and prolongation of the APD and QTc interval.

Table 1: Acquired LQTS2-associated drug effects on cardiac electrical activity in zebrafish larvae and adults. NSD, not significantly different.

Larval zebrafish hearts				
Drug		**Heart Rate**	**A-V Dissociation**	**APD**
100 compounds		Bradycardia with 36 (22 known to increase QT and TdP)[5]		
11 hERG blockers		Bradycardia with all[6]	2:1 block with all (at higher concentrations)[6]	
9 QT-prolonging compounds		Bradycardia with all[10]	2:1 block with all (R^2= 0.93; IC_{50} and 2:1 block)[10]	
35 compounds			2:1 block with 14 of 17 known to prolong QT[8]	
Amiodarone 20 µg/mL		Bradycardia (HR reduced by 30%)[18]		
Astemizole	60 µg/mL	Bradycardia (HR reduced by 50%)[18]		
	1 µM		2:1 block[9]	
	5 µM		Heart failure[9]	
Cisapride	5 µM		2:1 block[9]	
	10 µM		Ventricular failure[9]	

	20 μM		Heart failure[9]	
Dofetilide	10 nM			Increased by 64 ± 45 ms (+28%)[1]
	12 nM		2:1 block[1]	
Haloperidol	5 μM		2:1 block[9]	
	10 μM		Ventricular failure[9]	
	50 μM		Heart failure[9]	
Tamoxifen 20 μM		Bradycardia (HR reduced by 75%)[18]		
Terfenadine	Unknown		2:1 block[2,8]	
	100 nM			Increased from 231 ± 5 ms to 245 ± 7 ms (6%)[3] Increased by 58 ± 15%[2]
	1 μM	Bradycardia[9]		
	5 μM		2:1 block[9]	
	10 μM		Rescue of *reggae* phenotype in 54% of larvae[7]	
	20 μM		Heart failure (20 μM)[9]	
	25 μM		2:1 block[7]	

2-MMB 50 μM			Suppressed 2:1 block in *breakdance*[4]		Decreased APD in *breakdance* from 570 ± 23 to 376 ± 66 ms (-34%)[4]
Flurandrenolide 50 μM			Suppressed 2:1 block in *breakdance*[4]		Decreased APD in *breakdance* from 482 ± 83 to 338 ± 44 ms (-30%)[4]
Adult zebrafish hearts					
Drug		**R-R interval**	**APD**	**QT**	**Other markers of arrhythmogenicity**
hERG blockers					
Astemizole 50 μM		Decreased from 308 ± 77 ms by -16 ± 6%[11]		Increased from 242 ± 54 ms by +18 ± 9%[11]	
Dofetilide	50 nM		APD_{75} increased from 159 ± 8 to 193 ± 9 ms (+21%)[14]		Increased triangulation (APD_{75} - APD_{25}) from 75 to 89 ms (+16%)[14]
	100 nM		APD_{90} increased by 75 ms[15]		
	10 μM				Increased APD_{90} and Peak-Peak variance[17]
E-4031	1 μM		APD_{90} increased from 144.1 to 179.9 ms (+20%)[13]		Increased triangulation (APD_{90} - APD_{30}) from 10 to 16 ms (+60%)[13]

	10 μM	Bradycardia[12]	Increased	QTc increased from 439 ± 39 to 529 ± 27 ms (+21%)[12]	
	20 μM + 1 μM Isoproterenol		APD_{90} increased to >3,000 ms[16]		Spontaneous EADs (1 s^{-1})[16]
Haloperidol 100 μM		Decreased from 308 ± 77 ms by -38 ± 14%[11]		Increased from 242 ± 54 ms by +16 ± 11%[11]	
Pimozide 10 μM		NSD from 308 ± 77 ms[11]		Increased from 242 ± 54 ms by +17 ± 9%[11]	
Terfenadine	200 nM		APD_{75} increased from 183 ± 7 to 216 ± 8 ms (+17%)[14]		Increased triangulation (APD_{75} - APD_{25}) from 92 to 94 ms (+2%)[14]
	100 μM	NSD from 308 ± 77 ms[11]		Increased from 242 ± 54 ms by +11 ± 6%[11]	
hERG activators					
NS1643 20 μM			APD_{75} NSD from 209 ± 13[14] APD_{25} decreased from 126 ± 6 to 101 ± 6 ms (-19%)[14]		Increased triangulation (APD_{75} - APD_{25}) from 83 to 101 ms (+18%)[14]

PD-118057 40 µM			APD_{75} decreased from 193 ± 6 to 176 ± 6 ms (-8%)[14]		Decreased triangulation (APD_{75} - APD_{25}) from 82 to 69 ms (-16%)[14]
RPR260243	10 µM				Decreased APD_{90} and Peak-Peak variance in dofetilide treated hearts[17]
	30 µM		APD_{75} decreased from 196 ± 9 to 164 ± 7 ms (-16%)[14] APD_{90} decreased from 132± 4 to 111 ± 2 ms (-16%)[17]		Decreased triangulation (APD_{75} - APD_{25}) from 95 to 71 ms (-25%)[14] Decreased triangulation (APD_{90} - APD_{25}) from 74 ± 3 to 45 ± 3 ms (-39%)[17] Increased max slope of ERC from 0.69 ± 0.08 to 1.36 ± 0.26 (+97%)[17] Increased Post-Repolarization Refractory Period (PRRP) from 58 ± 4 to 75 ± 7 ms (+23%)[17]

Note: Data from Milan et al., 2009[1]; Arnaout et al., 2007[2]; Alday, 2014[3]; Peat et al., 2011[4], Milan et al., 2003[5]; Langheinrich et al., 2003[6]; Hassel et al., 2008[7]; Milan et al., 2006a[8]; Letamendia et al., 2012[9]; Mittelstadt et al., 2008[10]; Milan et al., 2006b[11]; Tsai et al., 2011[12]; Nemtsas et al., 2010[13]; Hull et al., 2019[14]; Genge et al., 2016[15]; Sacconi et al., 2020[16]; Shi et al., 2020[17]; Burns et al., 2005[18]

Building on earlier work characterizing zebrafish ventricular myocyte ion currents (Brette et al., 2008), Nemtsas et al., provided the most comprehensive description of cardiac currents responsible for the zebrafish ventricular action potential to date (Nemtsas et al., 2010). Biophysical characterization and selective drug blockade under voltage clamp conditions, allowed these authors to examine Na^+, Ca^{2+}, and K^+ in isolated zebrafish ventricular myocytes. These studies demonstrated key roles for voltage-gated Na^+ channels in the action potential upstroke, and L-type and T-type Ca^{2+} channels in the maintenance of the plateau phase (Zhang et al., 2010; Nemstas et al., 2010); the prominent role of the latter being somewhat different from that in the adult mammalian ventricle (Haverinen et al., 2018). Other studies suggest a prominent role for Na^+-Ca^{2+} exchange current at depolarized voltages during the plateau phase (Zhang et al., 2010). Dependence of the ventricular resting membrane potential on I_{K1} expression was also demonstrated (Nemtsas et al., 2010), although the molecular correlate appears to be a different inward rectifier subfamily member in zebrafish and humans (Hassinen et al., 2015; Vornanen and Hassinen, 2016). Selective drug block initially indicated little functional presence of I_{Ks} during phase 3 repolarization in zebrafish (Alday, 2014; Nemtsas et al., 2010), however, expression of Kv7 K^+ transcripts in cardiac tissue (Wu et al., 2014), and more recent studies demonstrate the presence of I_{Ks} current (Abramochkin et al., 2018). A consistent feature of zebrafish cardiac repolarization is the prominent role of I_{Kr} in phase 3 ventricular repolarization. I_{Kr} is conveyed by zERG channels from the *zkcnh6a* gene (Langheinrich et al., 2003; Scholz et al., 2009; Leong et al., 2010b; Vornanen and Hassinen, 2016; Hull et al., 2019). The substantial genetic and pharmacological evidence for the importance of zERG channels and I_{Kr} as a critical driving force behind phase 3 repolarization suggests that zebrafish bear great potential as a model of both acquired and inherited LQTS as is discussed in more detail below.

3.3. Zebrafish as a model of acquired Long-QT Syndrome

3.3.1. HR, APD, and QT interval as markers of arrhythmia in zebrafish

Cardiotoxicity due to hERG channel blockade is a major hurdle in drug discovery and has resulted in high profile withdrawals from the market (Mandenius et al. 2011; Kannankeril et al. 2010). About 35 to 40% of drug candidates are dropped in the early development phase due to hERG toxicity issues, 19% due to cardiotoxicity, i.e., drug-

induced arrhythmias (Mandenius et al., 2011). A wide variety of drugs (developed for cardiovascular or non-cardiovascular diseases) including antiarrhythmics, antipsychotics, antibiotics, antihistamines, amongst others are known to block hERG channels. Drugs that block hERG currents delay ventricular repolarization, which may lead to TdP and sudden death (Kannankeril et al. 2010). Mandatory guidelines require the testing of all early drug candidates for their hERG liability before filing an investigational new drug (IND) application (Hammond and Pollard, 2005; Lu et al., 2008). A hERG block assay is routinely used as a surrogate marker for QT prolongation (Gintant et al., 2016) and direct measurement of hERG currents using the patch-clamp technique has been the gold standard (Gintant et al. 2016; Lawrence et al. 2005). Non-cardiac mammalian CHO or HEK-293 cells, which artificially express hERG, are easy to maintain, can be sub-cultured for numerous passages, allow the formation of high resistance low noise seals, and lack contaminating currents (Kannankeril et al. 2010; Gintant et al. 2016); however, while effective in predicting the hERG block, they pose disadvantages in that these expression systems differ in their cellular environment, likely lack many important ancillary proteins for hERG channel modulation, and do not accurately produce the channels in their native form (McNeish, 2004; Kannankeril et al., 2010; Liang et al., 2013). As such, hERG assays using non-cardiac cells can produce false positive or false negative results, which may lead to the loss of potential drug candidates in the early stage of drug development (Liang et al., 2013). The use of primary cardiomyocytes offers an environment with naturally occurring subunit composition and other intracellular pathways or factors that may modulate channel properties (Kannankeril et al. 2010), but they are not proliferative and need to be isolated freshly for every experiment and since hERG currents are low in magnitude, and can be contaminated with other currents, recording currents accurately is challenging (Liang et al. 2013; Kannankeril et al. 2010; McNeish 2004). More complex models, such wedge and isolated heart preparations, which use both electrophysiological and non-electrophysiological assays to test the proarrhythmic potential of the molecules during preclinical studies (Lawrence et al., 2005) are better at predicting APD prolongation and TdP risk, but lack throughput and are often costly alternatives. In recent years, zebrafish have emerged as a potential toxicological screening platform for compounds at risk of predisposing APD prolongation, TdP, and acquired LQTS in humans.

Zebrafish were introduced as a drug screening platform in the context of LQTS almost 20 years ago (Langheinrich et al., 2003; Milan et al., 2003) (see Table 1). These

studies recognized the advantages of using transparent 48-72 hpf larvae to monitor the effects of compounds on cardiac rhythmicity using relatively simple light microscopy imaging. Milan et al., tested the effects of 100 compounds on zebrafish heart rate (Milan et al., 2003). Of these, 36 caused bradycardia, and 21 of these are known to cause QT prolongation and TdP. Langenreich et al., screened 11 diverse hERG blocking compounds in zebrafish and all 11 induced bradycardia, and 2:1 atrioventricular block at high concentrations (Langheinrich et al., 2003), both of which are consequences of LQTS observed in humans (Motoike et al., 2000; Chang et al., 2004; Lee et al., 2006). These initial studies demonstrated that compounds which block hERG channels in heterologous cell assays and cause QT prolongation and TdP in humans have high affinity for the zERG orthologue in zebrafish. Rescue of the gain-of-function reggae zERG mutant cardiac phenotype by terfenadine block (Hassel et al., 2008) further demonstrated the targeted action of hERG-specific drugs in zebrafish. Indeed isolated zERG (*zkcnh6a*) channels have subsequently been shown to present similar pharmacological sensitivity to blocker compounds, such as terfenadine, as observed in hERG (*hKCNH2*) channels (Hull et al., 2019). Further developments to incorporate high-throughput zebrafish larvae screens revealed that bradycardia and susceptibility to 2:1 block provided accurate detection of QT prolonging compounds with high sensitivity and specificity (Burns et al., 2005; Mittelstadt et al., 2008; Peal et al., 2011; Letamendia et al., 2012) as shown in Table 1. Some studies have also used genetic models that prolong (*breakdance*, *bre*) or abbreviate (*reggae*, *reg*) the APD to demonstrate the effect of QT prolonging compounds in zebrafish larvae (Hassel et al., 2008; Milan et al., 2009; Peal et al., 2011) (see Chapter 3.4 for descriptions of *breakdance* and *reggae*).

To gain mechanistic insight beyond heart rate changes, several groups developed techniques to test the impact of compounds on APD and/ECG in zebrafish larvae (see Table 1). Measurements of membrane voltage from explanted paced embryonic hearts showed that zERG block by 100 nM terfenadine prolonged the APD by 58% and resulted in 2:1 block as a result, such that only every other stimulus elicited an action potential (Arnaout et al., 2007). Using optical mapping of a fluorescent dye, Milan et al., measured the effects of a range of drugs on APD in wild-type and zERG non-trafficking variant, *breakdance*, 2 dpf zebrafish (Milan et al., 2009). Homozygous *breakdance* zebrafish presented marked prolongation of the APD, action potential triangulation, 2:1 block, and spontaneous EAD formation, highlighting the importance of I_{Kr} repolarizing current in

zebrafish cardiac function. Heterozygous *breakdance* larvae presented more muted APD prolongation, but this was greatly exaggerated by the application of the hERG blocker, dofetilide, or ATX-II, which interferes with Na^+ channel inactivation. Dofetilide alone also produced 2:1 block at higher concentrations. Following this, Peal et al., screened 1,200 compounds in *breakdance* larvae using optical mapping to detect APD changes in search of compounds that might reverse the prolonged APD phenotype (Peal et al., 2011). They discovered two compounds that rescued wild-type-like APD in *breakdance* larvae, suggesting novel pathways for restoration of repolarization associated with hERG dysfunction. More recently, light-sheet imaging of a membrane dye in whole 14 dpf zebrafish hearts showed that E-4031 (a hERG blocker) application increases APD and induces the occurrence of frequent EAD formation (~1 every s) (Sacconi et al., 2020). These studies demonstrate the applicability of zebrafish larvae to model acquired LQTS and to conduct toxicological screening.

3.3.2. Electrical instability and beat-to-beat variability as markers of LQTS in zebrafish hearts

In adult zebrafish hearts, hERG blockers prolong the APD and increase the QT interval (Milan et al., 2009; Tsai et al., 2011; Genge et al., 2016; Hull et al., 2019) while hERG activator compounds reduce APD (Hull et al., 2019; Shi et al., 2020) (see Table 1). Studies using adult zebrafish hearts also permit more detailed study of markers of cardiac arrhythmogenicity, such as electrical instability and beat-to-beat variability, which is an important consideration given that although APD and QT prolongation provide a substrate for the initiation of TdP, there is evidence that QT prolongation alone correlates poorly with TdP (Mattioni et al., 1989; Shimizu et al., 1995; Fossa et al., 2002; Fenichel et al., 2004; Belardinelli et al., 2006). Frommeyer et al., suggested that the changes in AP morphology may better explain the antiarrhythmic potential of compounds (Frommeyer et al., 2011). Indeed, triangulation of the action potential (often quantified as APD_{90}:APD_{30}), which represents prolongation of late phase repolarization or abbreviation of earlier phases, is associated with development of TdP (Hondeghem et al., 2001) as a result of increased risk for the generation of EADs and triggered activity (Frommeyer et al., 2011) and an increased temporal dispersion of repolarization which promotes re-excitation by current flow (Hondeghem et al., 2001). In adult zebrafish hearts, hERG activator compounds reduce APD with a selective effect on late phase repolarization resulting in reduced

triangulation (Hull et al., 2019; Shi et al., 2020), indicating that zebrafish may be suited to screening for therapeutic compounds to ameliorate hERG channel loss of function.

Adult zebrafish hearts have also been used to study the effect of hERG activator compounds in more complex dynamic adaptation of APD to understand their antiarrhythmic potential (Shi et al., 2020). Dynamic instability of the membrane voltage is a precursor to fibrillation that arises from changes in pacing rate and can be used as a surrogate parameter in the assessment of TdP risk (Koller et al., 1995). The electrical restitution curve (ERC) provides a measure of APD stability by describing APD changes as a function of the diastolic interval (DI), the time from the end of the action potential to the activation of the following action potential (Bass, 1975; Franz, 2003). Decreasing the DI reduces the APD in the subsequent beat as a result of incomplete recovery of ion channels (Garfinkel et al., 2000; Franz, 2003; Ng et al., 2007). Changes in the shape of the ERC, in particular the maximum slope of the curve, suggest that this relationship is a promising surrogate parameter for evaluating TdP risk (Koller et al., 1995; Garfinkel et al., 2000; Franz, 2003; Ng et al., 2007). The maximum slope of the ERC measured using optical mapping of action potentials in isolated adult zebrafish hearts was increased in the presence of the hERG activator compound, RPR260243 (Shi et al., 2020), suggesting that the activator compound improved dynamic APD adaptation during acute changes in beating frequency (Franz, 2003). The increased slope of the zebrafish ERC with RPR260243 was associated with reduced beat-to-beat variability of heart rate and APD in an acquired LQTS model using dofetilide block (Shi et al., 2020) suggesting antiarrhythmic potential. Further studies using dynamic protocols to measure the ERC (using trains of stimulations with progressively shorter basic cycle lengths), which improve congruence between the observed development of alternans at short DIs and the slope of the ERC (Koller et al., 1998), promise to further enhance the predictive and translational power of ERC measurements in zebrafish hearts.

The RPR260243 hERG activator compound was also shown to increase the post-refractory repolarization period (PRRP) in zebrafish hearts (Shi et al., 2020), which has been hypothesized to be antiarrhythmic by reducing VT inducibility (Garfinkel et al., 2000; Franz, 2003; Franz et al., 2014). The PRRP is the delay in the onset of electrical restitution beyond the full repolarization of the previous action potential (Franz, 2003), i.e. it describes the phenomenon in which extra stimuli can only generate action potentials once the previous action potential has fully repolarized preventing encroachment. A longer PRRP

may be considered antiarrhythmic, since the action of suppressing early premature responses to extra stimuli and allowing a more rapid normalization of APD and conduction velocity effectively narrows the window of partial refractoriness, a substrate for the generation and maintenance of VF (Franz et al., 2014). Several studies in animals and humans demonstrate the antiarrhythmic benefits of a lengthened PRRP (Koller et al., 1995; Franz, 2003; Fedorov et al., 2008) suggesting that the effect of hERG activators to prolong the PRRP in zebrafish hearts protects against TdP induction and demonstrates antiarrhythmic potential for the treatment of LQTS. Future studies using measures of electrical instability and APD rate adaptation as surrogate markers of arrhythmogenicity will benefit from correlating findings with the propensity towards EAD formation, since this is a robust biomarker. It is however interesting to note the scarcity of reported EAD events in studies of adult zebrafish hearts. Further studies aimed at characterizing the conditions required for EAD induction and triggered activity in adult zebrafish hearts would advance the use of EAD propensity as a biomarker alongside measures of electrical instability described here.

3.4. Zebrafish as a model of inherited Long-QT Syndrome

Inherited LQTS2 in humans results from dysfunctional variants in the *KCNH2* gene, which encodes the hERG channel. In zebrafish, multiple variants have been identified as possible orthologs to *KCNH2*, with *zkcnh2* initially identified as the primary ortholog. However, screening for all possible zebrafish homologous sequences of *zkcnh2*, Leong et al., demonstrated the presence of a second variant, *zkcnh6a*, which paired more closely as the ortholog to *KCNH2* in humans (Leong et al., 2010b). Tissue-specific RNA extraction and qPCR analyses have confirmed that *zkcnh6a* is the ortholog of human *KCNH2* (Langheinrich et al., 2003; Scholz et al., 2009; Leong et al., 2010b; Vornanen and Hassinen, 2016; Hull et al., 2019), and the biophysical function and pharmacological properties of *zkcnh6a* channels are similar to those of human *KCNH2* (Scholz et al., 2009; Hull et al., 2019). Currents carried by *zkcnh6a* zERG channels produce the zebrafish cardiac current, I_{Kr}, which is the predominant repolarizing driving force in the zebrafish heart (Nemtsas et al., 2010).

Performing a screen for mutations affecting zebrafish developmental, Chen et al., discovered a zERG variant associated with aberrant electrical properties (Chen et al., 1996). The *breakdance* mutant resulted from an I59S mutation in the N-terminal region of

zERG channels and causes 2:1 atrioventricular block in zebrafish hearts (Chen et al., 1996; Milan et al., 2009; Peal et al., 2011). The cause of the A-V dissociation was subsequently show to arise from markedly delayed ventricular repolarization, which prolonged the ventricular APD such that only every other atrial depolarization resulted in ventricular capture (Milan et al., 2009) (see Table 2). These findings are consistent with clinical observations in pediatric cases of LQTS, where 2:1 block is sometimes observed (Motoike et al., 2000; Chang et al., 2004; Lee et al., 2006). Kopp et al., showed that 2:1 block in *breakdance* hearts was dependent on both temperature and development, demonstrating conversion to 1:1 rhythm at lower environmental temperatures, and beyond 4 dpf (Kopp et al., 2005). Zebrafish homozygous for the *breakdance* variant also presented markers of arrhythmogenicity, such as action potential triangulation and the presence of spontaneous EADs (Milan et al., 2009). This phenotype is consistent with LQTS in humans and the confirmation that the *breakdance* mutation is located within the zERG K^+ channel demonstrates the potential for zebrafish to model arrhythmogenic cardiac diseases.

Table 2: Effects of inherited mutations or targeted disruption of the zERG gene on cardiac electrical activity. *in silico action potential model predictions

Variant		Heart rate	A-V dissociation	APD	QT	Notes and other markers of arrhythmogenicity
zkcnh2 I59S (*breakdance*) (V59 in hERG)	Homozygous	85 ± 2 bpm[10]	2:1 block[3,4,7,10]	Increased from 225 ± 21 to 615 ± 66 ms[4] Increased from 290 ± 85 to 376 ± 66 ms[7]		Non-trafficking variant. Triangulation and spontaneous EADs.[4] Temperature and developmental stage dependence.[10] Switching between 2:1 and 1:1 rhythm[10]
	Heterozygous			Increased from 226 ± 21 to 258 ± 16 ms[4]		
zkcnh2 I462R (I500R in hERG)	Homozygous		Silent ventricle[1]			
	Heterozygous		2:1 block[1]			
zkcnh2 M521K (M554K in hERG)	Homozygous		Silent ventricle[1]			
	Heterozygous		2:1 block[1]	Increased from 330 ± 12 to 476 ± 35 ms[1]	Increased from 416 ± 8 to 469 ± 25 ms[1]	

Antisense MO knockdown of *zkcnh2*	Bradycardia	2:1 block[2,6]	APD_{90} increased from 275 ± 10 to 544 ± 15 ms[8]		MO at high concentrations produced fibrillation[2] Depolarized resting potential and ventricular systole also observed[6]
Injection of RNA coding 40 LQTS-associated mutations and 10 non-disease causing hERG SNPs following antisense MO knockdown of *zkcnh2*		Suppression of 2:1 block in 9/10 SNPs. 39/40 mutations sustained 2:1 block[6]			
zkcnh2 antisense MO + hERG N470D (N432D in zERG) RNA			APD_{90} increased to >500 ms[6]		Function of equivalent hERG variant: no current at 37°C; non-trrafficking[11,12]
zkcnh2 antisense MO + hERG A614V (A586V in zERG) RNA			APD_{90} increased to >500 ms[6]		Function of equivalent hERG variant: no current at 37°C; non-trafficking[13]
zkcnh2 antisense MO + hERG A1116V (equivalent residue in zERG is uncertain) RNA			APD_{90} increased to >500 ms[6]		Function of equivalent hERG variant: mild QTc prolongation, reduced hERG current density when combined with K897T[14]
zkcnh2 L499P (*reggae*) (L532P in hERG)			APD_{90} decreased by 19%[8]		Function of equivalent hERG variant: increased current due to right-shifted voltage dependence of

						inactivation (reduced inactivation)[15] Intermittent cardiac arrest; sinus exit block[8] Antisense MO knockdown of zERG suppressed *reg* phenotype in 48% of larvae[8] Cardiac function was normal in intercrossed heterozygous *reg* and *bre* larvae[8]
GFP expressed at the *zkcnh6a* locus	Heterozygous *zkcnh6a*$^{GFP/+}$					GFP expressed exclusively in the heart. Larvae are viable[9]
	Homozygous *zkcnh6a*$^{GFP/GFP}$		Silent ventricle			Cardiac edema[9]
Conditional *zkcnh6a*^{loxP}			Silent ventricle			Cardiac edema. Cre recombinase induces excision of *zkcnh6a* exon 6[9]

Note: Data from Arnaout et al., 2007[1]; Langheinrich et al., 2003[2]; Chen et al., 1996[3]; Milan et al., 2009[4]; Milan et al., 2003[5]; Jou et al., 2013[6]; Peal et al., 2011[7]; Hassel et al., 2008[8]; Hoshijima et al., 2016[9]; Kopp et al., 2005[10]; Zhou et al., 1999[11]; Gong et al., 2004[12]; Sakaguchi et al., 2008[13]; Crotti et al., 2005[14]; Zhang et al., 2011[15]

The utility of zebrafish as a model for inherited LQTS2 was more directly demonstrated by the identification of *zkcnh2* (likely actually *zkcnh6a*) (Leong et al., 2010b) mutations, I462R (I500R in hERG) and M521K (M554K in hERG), which caused dysfunction (likely causing non-trafficking since these variants are linked with *breakdance* via complementation testing, although this has not been tested directly) of zERG channels resulting in a corresponding loss of I_{Kr} and 2:1 block when inherited heterozygously, and a silent ventricle phenotype when inherited homozygously (Arnaout et al., 2007). Recordings of action potentials from M521K (M554K in hERG) mutant zebrafish hearts revealed that the phenotype was caused by a significantly prolonged ventricular APD (Arnaout et al., 2007) (see Table 2). These findings demonstrated a clear link between zERG dysfunction and cardiac phenotypes that could be readily observed and quantified in 48-72 hpf zebrafish enabling rapid detection. Other studies using antisense MO knockdown of zERG function showed that targeted disruption of zERG resulted in bradycardia, 2:1 block, and a prolonged APD in zebrafish embryos (Langheinrich et al., 2003; Milan et al., 2003; Jou et al., 2013). Using this as a platform, Jou et al., developed an *in vivo* cardiac assay in zebrafish embryos to screen for benign or disease-causing variants (Jou et al., 2013). Following MO knockdown of zERG and generation of 2:1 block or silent ventricle phenotype, injection of WT hERG RNA restored a WT-like phenotype in >50% of embryos or reduced the severity of the phenotype (i.e. 2:1 rather than silent ventricle). Similar recovery was observed with 9 of 10 non-disease causing SNPs, but not with 39 of 40 LQTS2-associated mutations, from which APD was measured in some and shown to be significantly prolonged (Jou et al., 2013) (see Table 2). Furthermore, the study demonstrated a clinical phenomenon, wherein a dysfunctional variant co-expressed with a SNP was capable of reducing the phenotypic severity, showing that this assay has the potential to provide useful information for practical clinical applications. Beyond testing known LQTS-causing mutations, or screening effects of mutations found in humans, zebrafish have also been used to discover and elucidate effects of novel mutations that affect cardiac repolarization. Milan et al., used dofetilide block of zERG channels to induce 2:1 block in zebrafish embryos and rescue from or exaggeration of this phenotype was used to screen for novel gene mutations affecting repolarization (Milan et al., 2009). Using this approach, the authors identified 15 novel mutations involved in cardiac repolarization.

On the other end of QT-related arrhythmia spectrum, the *reggae* mutation identified in the zebrafish zERG gene causes a Short QT Syndrome (SQTS). Hassel et

al., identified a missense mutation, L499P (L532P in hERG), which abbreviated the APD and resulted in intermittent cardiac arrest and sinus exit block (Hassel et al., 2008). Injection of antisense MO restored the WT phenotype in around half of the *reggae* embryos consistent with the idea that the cardiac phenotype resulted from zERG gain-of-function. Furthermore, crossing of *reggae* individuals with *breakdance* individuals (in which repolarization is delayed) produced offspring with no cardiac abnormalities suggesting that the two mutations complemented one another. These studies were pioneering in their demonstration of zebrafish to model inherited LQTS. More recent developments in genetic engineering approaches promise to further unleash the potential of this model species. Below, we discuss future opportunities and challenges using precise gene-editing to create zebrafish models of inherited LQTS.

3.5. Precise gene-editing approaches in zebrafish to model LQTS

Precise gene editing technologies are providing one of the most rapidly evolving tools in the repertoire of genetic manipulation, and they promise to greatly influence the utility of zebrafish to model LQTS and other inherited cardiac electrical diseases. In 2016 Hoshijima et al., demonstrated two novel approaches for genetic targeting and manipulation of the *zkcnh6a* gene (Hoshijima et al., 2016), which provide advanced tools to model and create LQTS in zebrafish. Using TALENs to target double stranded breaks in the genomic DNA immediately after the *zkcnh6a* start codon, and providing a modified synthetic DNA template for repair, which contained the coding sequence for GFP, these authors first created zebrafish lines expressing the GFP reporter gene in lieu of *zkcnh6a*. Both homozygous (*zkcnh6a*$^{GFP/GFP}$) and heterozygous (*zkcnh6a*$^{GFP/+}$) were generated from this approach. *zkcnh6a*$^{GFP/GFP}$ embryos, homozygous for the GFP knock-in in place of *zkcnh6a*, i.e. *zkcnh6a* null, presented contractile defects and pericardial edema consistent with knock-out of zERG function (Arnaout et al., 2007; Hoshijima et al., 2016). This *zkcnh6a* knockout provides further demonstration of the role of zERG channels in zebrafish cardiac electrophysiology, and furthermore, the *zkcnh6a*$^{GFP/+}$ embryos provide an additional opportunity in that heterozygous embryos, being viable (although phenotypic analyses were not performed to confirm normal cardiac function), express GFP exclusively in the heart providing a locus-specific cardiac reporter. Previous fluorescent dye membrane voltage reporters (Milan et al., 2009; Peal et al., 2011; Tsai et al., 2011) and

genetically encoded fluorescent reporters (Huang et al., 2003; Burns et al., 2005) had been used to visualize or measure cardiac activity, but the approach used by Hoshijima et al., can be used to detect cardiac activity and developmental or environmental influences on *zkcnh6a* gene function specifically.

In a second advancement, Hoshijima et al., developed a silent conditional editing approach to knock-in a GFP reporter gene within the *zkcnh6a* locus (Hoshijima et al., 2016). Again, using TALENs, targeted double stranded breaks were introduced in the intronic sequences on either side of the *zkcnh6a* exon 6. Repair was then directed to replace exon 6 using a novel DNA template that included the WT *zkcnh6a* exon 6 alongside GFP controlled by the α-crystallin eye lens promoter, all of which was flanked by loxP sites. Analyses revealed the introduction of the floxed allele and green eyes in 70% of zebrafish embryos, which were viable and morphologically normal. Injection of *cre* recombinase mRNA into embryos expressing the floxed allele resulted in efficient excision of sequence between the loxP sites flanking the α-crystallin:GFP and *zkcn6a* exon 6 leaving embryos without the GFP eye lens reporter, and with a silent ventricle phenotype and pericardial edema that is characteristic of knockout of *zkcnh6a* (Hoshijima et al., 2016).

Developments in genetic technology, such as in the use of CRISPR, promise to simplify and improve efficiency of precise gene editing approaches in model systems, including zebrafish (Chang et al., 2013; Hwang et al., 2013; Varshney et al., 2015; Liu et al., 2017b; Cornet et al., 2018). The CRISPR system is an anti-viral defense mechanism found in a variety of bacteria and archaea that has been co-opted to modify target genes within organisms (Jinek et al., 2012). CRISPR editing involves guide RNA (sgRNA) targeting of Cas-endonuclease (CRISPR associated protein) activity to a precise site within the gene of interest. Intrinsic genomic repair can occur via non-homologous end joining (NHEJ) resulting in random edits and indels, or by homology-directed repair (HDR), which can be co-opted by the provision of exogenous repair template incorporating the edit of interest. The ease of use and cost effectiveness of CRISPR has facilitated a variety of applications such as reverse genetics, improving biopharmaceutical efficiency, and allowing for the knock-out of target genes, or knock-in of point mutations within a gene of interest to generate disease models (Hruscha et al., 2013; Gupta and Musunuru, 2014; Armstrong et al., 2016). These developments hold great promise for the generation of inherited models of LQTS in zebrafish. Previous studies have shown that low success

rates of precise edits via HDR (~2-4%) (Armstrong et al., 2016; Albadri et al., 2017) using CRISPR in zebrafish can be improved by adopting a number of approaches: 1) considered design of the guide sgRNA (Doench et al., 2014; Moreno-Mateos et al., 2015; Cui et al., 2018; Michlits et al., 2020; Zhang et al., 2020a); 2) delivery of sgRNA as RNA rather than DNA (Liu et al., 2017a); 3) use of Cas9 protein instead of DNA or mRNA (Albadri et al., 2017; Zhang et al., 2018); 4) complexing Cas9 protein and sgRNA prior to injection (Burger et al., 2016); and 5) use of a plasmid-borne double-stranded repair template rather than ssODN (Irion et al., 2014). Leveraging these developments of CRISPR utility in zebrafish promises to open new possibilities for LQTS modelling in this model species. Additionally, further CRISPR-based developments, such as base-editing (Komor et al., 2016) and prime-editing (Anzalone et al., 2019) along with the development of new Cas-nucleases that utilize different recognition PAM sites (Kleinstiver et al., 2015; Hu et al., 2018) may alleviate previous limitations, such as off-target Cas9 activity and target-site restrictions. These approaches broaden the horizons and targeting range for the use of CRISPR in disease modelling in zebrafish opening the door to allow examination of the extensive array of genetic variants implicated in LQTS2 and potential underlying genetic susceptibility to acquired LQTS2, as well as other cardiac diseases.

3.6. Challenges and opportunities of zebrafish as a model of LQTS

The precision and ease with which the zebrafish genome may be modified, as described above, presents opportunities to examine clinically relevant hERG variants alongside cellular models, such as iPSC-derived cardiomyocytes (iPSC-CMs) to elucidate the phenotype of variants of unknown significance, and to inform clinical risk stratification of patients with hERG variants. Although rapidly evolving, current iPSC-CM approaches are challenged by cellular heterogeneity and immaturity, and they do not yet provide a whole organ or animal model, whereas zebrafish hearts allow for visualization of arrhythmogenicity at the tissue and whole organ level that permits examination of system-wide effects and morphological changes. There is also potential for zebrafish to be used as a whole organ companion model for the development of targeted, patient specific pharmacological approaches. The Comprehensive *in vitro* Proarrhythmia Assay (CiPA) paradigm advises inclusion of more complex approaches to arrhythmia assessment of compounds beyond heterologous expression of hERG in non-cardiac cells to include

computational modeling and iPSC-derived cardiomyocyte platforms to better predict cardiotoxicity. It may be that zebrafish offer a supporting model that could be a cost-effective whole organ early toxicological screening complex model system. Indeed, recent evidence suggests using computational approaches suggest that compound effects in zebrafish readily predict drug efficacy in human cardiomyocytes (Tveito et al., 2020).

Despite these articulated strengths that outline zebrafish as a useful model of LQTS, there are some current limitations/challenges that should be considered and that may influence interpretation or translation of findings to the human. For example, due to ancestral gene duplication events, there are alternate potential gene transcripts responsible for the zERG channel protein, and this requires knowledge and understanding of their interplay in order to carefully design targeted genetic strategies for the generation of specific LQTS-associated mutations. In addition, aside from morphological differences associated with the zebrafish two chambered heart, which might influence interpretations related to regulation of cardiac function, there are some electrophysiological differences in the zebrafish that should be considered. Although similar to hERG, zERG channel biophysical properties show some differences (Scholz et al., 2009; Hull et al., 2019) and this likely contributes to subtle differences in I_{Kr} current during the zebrafish action potential. Perhaps as a result of these biophysical differences, the effect of hERG activator drugs is somewhat greater on zERG than hERG channels, and combined with a prominent role for I_{Kr} in zebrafish hearts, this could lead to over-estimation of the effects of hERG activator compounds in zebrafish hearts and this needs to be taken into consideration. The zebrafish heart is smaller compared with the human and has reduced ventricular wall thickness, which likely contributes to altered tissue propagation and dispersion of repolarization as has been suggested (Zhao et al., 2020). With this altered electrical and physical tissue substrate, the initiation and maintenance of arrhythmias may not precisely mirror that in human hearts, and this should be taken into consideration when translating markers of arrhythmogenicity measured in zebrafish hearts. Lastly, most zebrafish experiments are conducted at 28℃, and the possible differential effects of temperature on ion channel trafficking, gating steps, and drug interactions should be considered when translating findings in zebrafish hearts to human hearts at 37℃. Notwithstanding these caveats, there remains enthusiasm for the role of the zebrafish as a translational model of both acquired and inherited LQTS that promises to continue to inform improved patient management and drug development.

Chapter 4. CRISPR-Cas9-Mediated Precise Knock-in Edits in Zebrafish Hearts

This chapter is based on the following article published in 2022 in the *Journal of Visualized Experiments (JoVE)*.

Simpson, K.E., Faizi, S., Venkateshappa, R., Yip, M., Johal, R., Poburko, D., Cheng, Y.M., Hunter, D., Lin, E., Tibbits, G.F., and Claydon, T.W. (2022). CRISPR-Cas9-Mediated Precise Knock-in Edits in Zebrafish Hearts. *Journal of Visualized Experiments*. doi: 10.3389/fphys.2020.624129

This article describes a novel method for introducing and easy detection of precise CRISPR edits in the cardiac *zkcnh6a* gene in zebrafish. This chapter is formatted as is standard for JoVE articles, outlining stepwise instructions in the active voice to explicitly lay out the exact protocols used. The formatting has been changed to follow library thesis guidelines in terms of the protocol numbering. This publication and chapter demonstrate the core CRISPR design of my thesis as well as the phenotyping pipeline I developed, which is adaptable to multiple different types of studies. This article was paired with a recorded video description of the study, which is available at the citation above.

4.1. Introduction

CRISPR-based gene editing strategies in animal models enable the study of genetically heritable disease, development, and toxicology at the whole-organism level (Li et al., 2019; Zarei et al., 2019; Lee et al., 2020). Zebrafish provide a powerful model that is closer in numerous physiological aspects to humans than murine or human-derived cell models (Gut et al., 2017). An extensive array of genetic tools and strategies have been used in zebrafish for both forward (Kegel et al., 2019) and reverse genetic screening (Shah et al., 2015). Comprehensive genetic mapping and annotation in zebrafish has facilitated gene-editing approaches as a primary technique to engineer targeted gene knockouts (KOs) and precise knock-ins (KIs) (González-Rosa, 2022).

Despite this, generating precise KI edits in zebrafish is limited by low efficiencies and difficulty of accurate detection. Although TALENs have been successfully used and optimized for Kis (Hoshijima et al., 2016), CRISPR provides an improved gene-editing strategy with simpler sgRNA targeting. Numerous studies have used CRISPR to generate precise KIs in zebrafish (Irion et al., 2014; Kimura et al., 2014; Armstrong et al., 2016; Albadri et al., 2017; Boel et al., 2018; Prykhozhij et al., 2018; Tessadori et al., 2018; Bai et al., 2020; Eschstruth et al., 2020; Wierson et al., 2020; de Vrieze et al., 2021; Levic et al., 2021), although these edits generated through CRISPR-mediated HDR tend to be inefficient with low intrinsic success rates that require genotyping as a primary screen (Irion et al., 2014; Armstrong et al., 2016; Albadri et al., 2017; Zhang et al., 2018). This demonstrates the need for an efficient KI CRISPR system in zebrafish, as well as a reliable high-throughput system for detecting precise edits.

The goal of this study was to describe a platform for generating a precise cardiac gene KI in zebrafish hearts with simple and high-throughput detection of successful edits. A CRISPR-Cas9-based two-sgRNA exon replacement approach is described, which is based on a TALEN approach(Hoshijima et al., 2016). This approach involves excision of the target sequence using two-sgRNA guides and replacement with an exogenous template sequence that contains the KI of interest as well as a genetically encoded intronic reporter gene (Figure 15). The integration of a genetically encoded fluorescent reporter within the target gene intronic sequence enables efficient detection of positive edits. A phenotyping platform is then described for assessing cardiac electrical function in zebrafish larvae for non-invasive characterization of the gene variants associated with

inherited LQTS, a cardiac electrical disorder that predisposes individuals to sudden cardiac death.

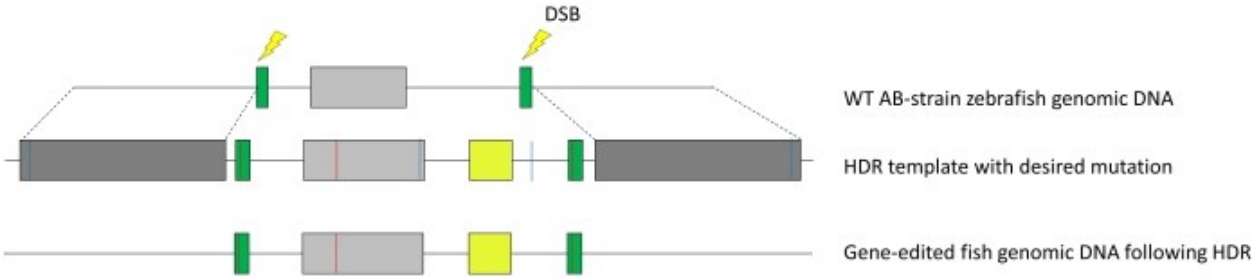


Figure 15: Integration of HDR template into the zebrafish genome.
Dark grey, homology arms; green, sgRNA guide targets with silent mutation to prevent Cas9 recutting; light grey, target exon of interest; red line, point mutation; yellow, mVenus YFP reporter gene under an α-crystallin promoter; dashed lines indicate homology. Here, the targeted precise edit was R56Q in exon 2 of the zkcnh6a gene. Abbreviations: HDR = homology-directed repair; sgRNA = single-guide RNA; YFP = yellow fluorescent protein; DSB = double-stranded break; WT = wild type.

These approaches will enhance access to, and use of, zebrafish KI gene edits to model inherited diseases and address biological and physiological questions, such as mapping gene expression patterns, and developmental regulation. Since zebrafish hearts better parallel human cardiac electrophysiological characteristics than murine models, they may be particularly attractive as a genetically tractable system for cardiac disease modeling (Nemtsas et al., 2010; Vornanen and Hassinen, 2016; González-Rosa, 2022).

4.2. Protocol

Studies using zebrafish were conducted in agreement with the policies and procedures of the Simon Fraser University Animal Care Committee and the Canadian Council of Animal Care.

4.2.1. Design of CRISPR components for precise edits

1. To design two-sgRNA guides that will be used to excise the sequence containing the KI target site, first identify the zebrafish ortholog for the gene of interest.

NOTE: Figure 16 provides a summary overview of the steps to engineer precise edits using the two-sgRNA CRISPR-Cas9 approach.

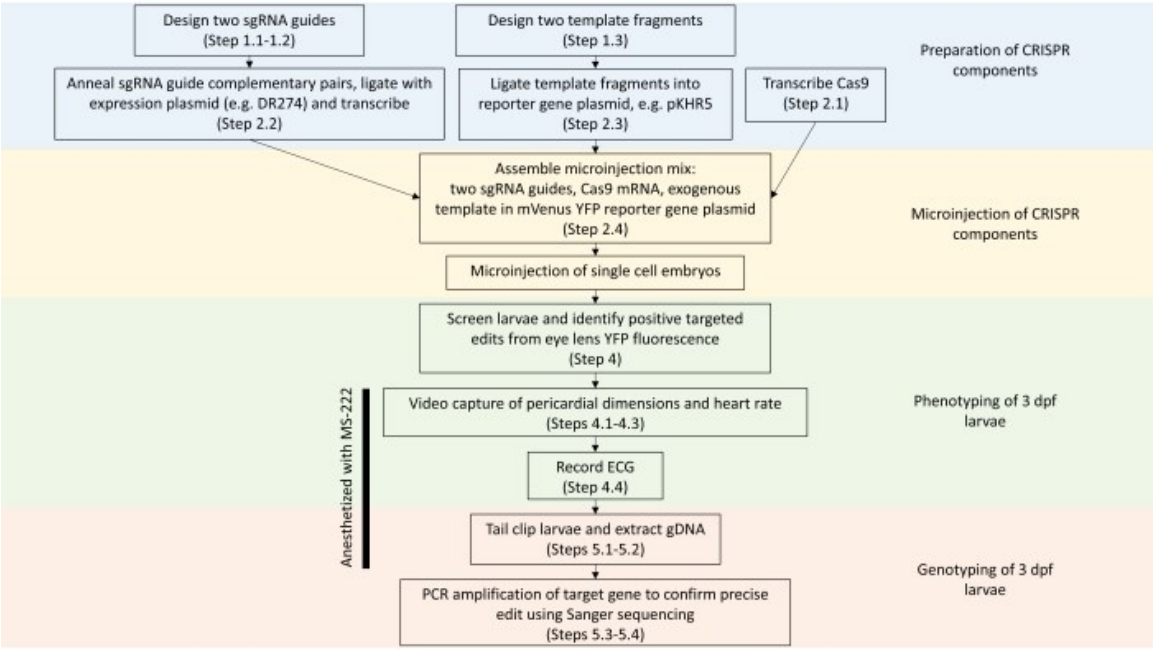


Figure 16: Summary of steps to engineer precise edits in zebrafish genes using the two-sgRNA CRISPR-Cas9 approach (related protocol step numbers are indicated in parentheses).
Abbreviations: sgRNA = single-guide RNA; YFP = yellow fluorescent protein; gDNA = genomic DNA; ECG = electrocardiogram; dpf = days post fertilization; MS-222 = tricaine methane sulfonate.

2. Next, use a design software tool, such as CRISPOR (Haeussler et al., 2016), which includes selection for *Danio rerio* as a species, and of the Cas enzyme to be used.

NOTE: The gene of interest for this study was *zkcnh6a* (Ensembl Transcript ID: ENSDART00000090809.6; UniProt Protein ID: B3DJX4), and the target mutation was amino acid, R56Q.

2.1 For the two-sgRNA approach, choose one sgRNA location that precedes the target exon and a second sgRNA that is located within the immediate downstream intron.

2.2. Ensure that the selected sgRNAs have high specificity and low predicted off-target binding. Use CRISPOR rankings to identify guides with minimal off-target binding. Do not consider guides with no mismatches in the seed sequence of potential off-targets.

2.3. Identify the most likely potential off-target sites (based on CRISPOR scores, select the top three exon potential off-target sites) for PCR-based Sanger sequencing genotyping in step 1.3 in section 4.2.6.

2.4. Once the two-sgRNAs have been selected, obtain the reverse complement for each, such that there are four oligonucleotides to be used: two complementary oligos that precede the mutation and two complementary oligos that are downstream.

2.5. On each of the four oligos, add compatible restriction sites for incorporation into a guide plasmid of choice: for integration into the DR274 plasmid, use a 5' BsaI restriction site to create an overhang. Ensure that the Bsa1 recognition site is engineered at the 5' end of the guide selected from CRISPOR, and the Bsa1 recognition site is engineered at the 5' end of the complementary strands to ensure correct orientation of the guides in the DR274 plasmid (see Figure 17).

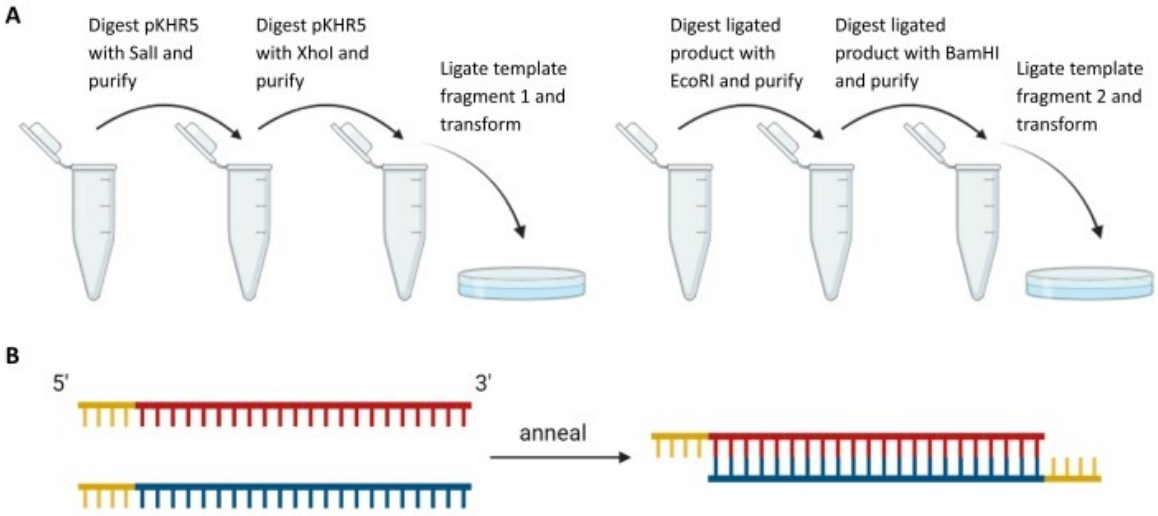


Figure 17: Preparation of exogenous template fragments and sgRNA guides.
(A) Sequential digestion and ligation of template fragments upstream and downstream of the mVenus YFP reporter gene sequence in pKHR5. (B) Annealing of complementary sgRNA pairs with restriction overhang for ligation into DR274. Abbreviations: sgRNA = single-guide RNA; YFP = yellow fluorescent protein.

3. To design the exogenous template used for HDR in zebrafish (Figure 15), choose two sequence fragments that will flank the mVenus YFP reporter gene housed in the pKHR5 plasmid.

NOTE: The intended modification/edit can be included in either the upstream or downstream fragment.

3.1. Using Ensembl, locate the target site within the gene sequence of interest, including approximately the 2 kb flanking sequence (homology arm), which will be used to make the template.

NOTE: Homology arms may be symmetrical or asymmetrical (Richardson et al., 2016; Liang et al., 2017) and approximately 1 kb each, upstream and downstream of the target site.

3.2. Divide the template into two segments that will be inserted either side of the mVenus YFP reporter gene (see Figure 18). Ensure that the split site is in an intron so that the coding sequence is not interrupted.

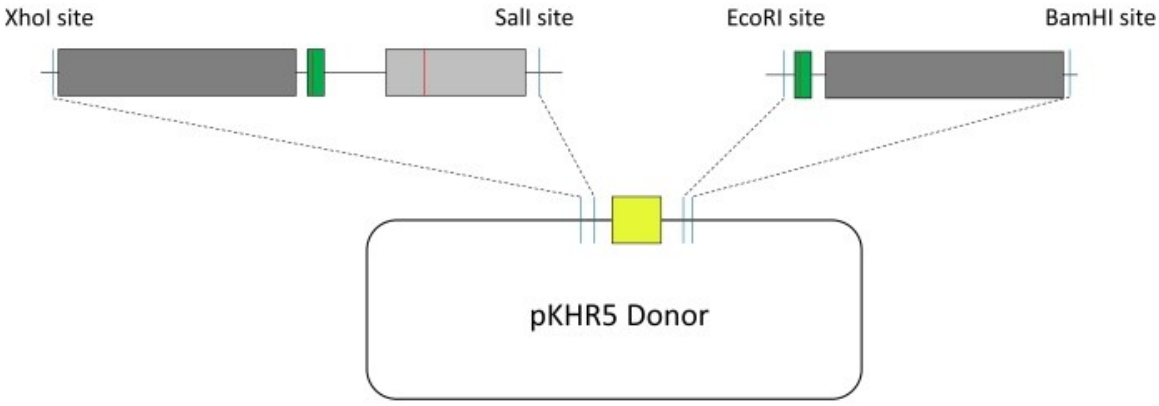


Figure 18: Construction of HDR template.
Dark grey, homology arms; green, sgRNA guide targets with silent mutation to prevent Cas9 recutting; light grey, target exon of interest; red line, point mutation; yellow, mVenus YFP reporter gene under an α-crystallin promoter; dark blue line, added restriction sites. The two template fragments are integrated into the pKHR5 plasmid donor. Abbreviations: HDR = homology-directed repair; sgRNA = single-guide RNA.

NOTE: If the gene is well characterized, check for functional roles in the intron, such as splicing sites or regulatory regions. Regions close to the 5' or 3' ends are more often involved in mRNA splicing.

3.3. Incorporate modifications into the template sequence that include: i) silent mutations in the guide Protospacer Adjacent Motif (PAM) or seed sequence (be aware of alternate PAM sites that the Cas enzyme might target) to prevent Cas enzyme recutting; ii) the modification of interest; iii) creation of restriction endonuclease sites to facilitate cloning into the pKHR5 plasmid, which contains the mVenus YFP reporter gene (see Figure 18).

NOTE: In this study, the first template segment contains XhoI upstream of the R56Q mutation site and SalI downstream, while the second template segment had EcoRI upstream of the guide target sequence and BamHI downstream. If any of the selected restriction sites are present within the template sequence, mutations to silence these will be required or alternate approaches such as Gibson Assembly can be used.

4.2.2. Preparation of CRISPR components for embryo microinjection

1. One week prior to microinjections, prepare the Cas9 for microinjection. Use Cas9 protein or prepare *Cas9* mRNA via *in vitro* transcription.

NOTE: In this study, Cas9 mRNA was used, since efficiencies tended to be higher.

1.1. Amplify bacterial cultures of commercially available XL1 Blue bacterial agar stab (containing Cas9 plasmid) using the appropriate antibiotic such as ampicillin. Use 675 µL of liquid culture (with 325 µL of glycerol) to create a backup glycerol stock for long-term storage at -80 °C.

1.2. Use the remainder of the liquid culture for a miniprep purification, according to the protocol provided with the miniprep kit. Resuspend the final purified DNA in 50 µL of the provided elution buffer. Quantify the product via a spectrophotometer to examine yield and purity.

1.3. Linearize 2 µg of the purified DNA via restriction digest using an appropriate restriction enzyme, using the appropriate buffer and incubation time as listed for the enzyme of interest.

1.4. Purify the linearized plasmid using a PCR Purification Kit, resuspending in 30 µL of the provided elution buffer.

1.5. After quantifying the product, use this as a template for *in vitro* transcription using the appropriate transcription kit for the promoter of interest. Follow the provided protocol and purify via lithium chloride precipitation (Sambrook et al., 1989b). Resuspend the purified RNA in 10 µL of nuclease-free H_2O and quantify, before storing at -20 °C for use in the microinjection mix.

2. Prepare the two sgRNA guides.

2.1. Prepare the sgRNA plasmid with a scaffold by amplifying bacterial cultures from the commercially available XL1 Blue bacterial agar stab (see Table 3 for details) in the same way as MLM3613 above (step 1.1 of section 4.2.2.), except use kanamycin instead of ampicillin.

2.2. Anneal the two pairs of complementary single-stranded oligonucleotides (ssODNs) for the sgRNA guides designed above by first resuspending the ssODNs in 1x annealing buffer to a concentration of 100 µM.

2.3. In separate reactions for each of the two sgRNAs, anneal the pair of complementary ssODNs using a thermal cycler. Mix 2 µg of each complementary ssODN pair with 50 µL of annealing buffer and incubate at 95 °C for 2 min, then cool to 25 °C over 45 min.

2.4. Digest a commercially available plasmid that contains a gRNA scaffold. Digest 2 µg of the DR274 plasmid using 1 µL of BsaI, 2 µL of appropriate buffer, and ddH_2O to 20 µL at 37 °C for 1 h. Confirm linearization (optional: purify using a PCR Purification Kit) using gel electrophoresis (Sambrook et al., 1989a).

2.5. In two separate ligation reactions (one for each sgRNA), ligate the annealed ssODNs with the linearized DR274 plasmid. Use a molar insert:vector ratio of 3:1, calculating the appropriate mass through an online ligation calculator. Mix the required mass of the insert and vector with 1 µL of T4 DNA ligase, 2 µL of ligation buffer, and ddH_2O to 12 µL and incubate at room temperature for 12 h.

2.6. Transform 2 µL of the ligated product into appropriate competent cells (such as 10β cells) using standard approaches, and then amplify and purify the product using a commercially available Miniprep Kit. Optional: create a glycerol stock of this product.

2.7. Transcribe the two sgRNAs by linearizing 2 µg of each guide using a 3' downstream restriction site that is as close to the end of the space sequence as possible. For the DR274 plasmid, linearize with HindIII and then, purify the RNA template using a PCR Purification Kit, resuspending in 30 µL of the elution buffer.

2.8. Transcribe the two guides using an RNA transcription kit. Follow the manufacturer's protocol and purify via lithium chloride precipitation (Sambrook et al., 1989b). Resuspend the two purified sgRNA guides in 10 µL of nuclease-free H_2O, quantify, and store at -20 °C for use in the microinjection mix.

NOTE: The RNA transcription kit cannot incorporate a 5' cap or poly-A tail.

3. Prepare the double-stranded, exogenous HDR reporter template.

NOTE: The template is synthesized in two parts, one upstream and one downstream of the mVenus YFP reporter gene. These two segments are synthetic constructs ordered through a commercial provider and then, ligated into the pKHR5 (which contains mVenus YFP) plasmid sequentially.

3.1. Prepare the pKHR5 plasmid by amplifying bacterial cultures from the commercially available DH5α bacterial strain (see Table 3 for details) in the same way as MLM3613 above (step 1.1 of section 4.2.2.).

NOTE: The pKHR5 plasmid contains the mVenus YFP reporter gene sequence.

3.2. Resuspend the two template segments designed above to 100 µM in TE buffer and then transform into 10β cells.

3.3. Digest the first template segment (the one upstream of the mVenus YFP reporter gene) and the pKHR5 plasmid using the restriction enzymes selected in step 3.3 of section 4.2.1..

NOTE: Sequential digest of pKHR5 is necessary due to close proximity of the selected restriction sites in the MCS.

3.4. To prepare the pKHR5 plasmid for the upstream template segment, digest 4 µg of pKHR5 (as per step 2.4 of section 4.2.2.) with SalI and then purify using a PCR purification kit. Resuspend in 30 µL of ddH_2O and use this as a template for the second digestion reaction with XhoI. Purify the product using the PCR Purification Kit.

3.5. Prepare the first template segment by digesting 2 µg of the template segment (step 2.3.2), 1 µL of XhoI, 1 µL of SalI, 2 µL of appropriate buffer, and ddH_2O to 20 µL for 1 h at 37 °C and gel-purify the product.

3.6. Ligate the upstream template segment from step 3.5 of section 4.2.2 into the prepared pKHR5 using the reaction conditions described in step 2.5 in section 4.2.2. Transform the ligated product into competent 10β cells, amplify, and purify using a miniprep (optional: create a glycerol stock of this product).

3.7. Use the ligated product from step 3.6 in section 4.2.2. (which contains the first template segment ligated into pKHR5 plasmid upstream of the mVenus YFP reporter

gene) and digest to prepare for the second (downstream of the mVenus reporter) template segment. Digest 4 µg of the ligated product (from step 3.6 as in step 2.4 in section 4.2.2.) with BamHI and then, purify using a PCR purification kit. Resuspend in 30 µL of ddH_2O and use this as the template for the second digest reaction with EcoRI. Purify the product using the PCR Purification Kit.

3.8. Prepare the second template segment by digesting 2 µg of the template segment (step 3.2 of section 4.2.2.), 1 µL of BamHI, 1 µL of EcoRI, 2 µL of appropriate buffer, and ddH_2O to 20 µL for 1 h at 37 °C and gel-purify the product.

3.9. Ligate the downstream template segment into the prepared pKHR5 (from step 3.7 of section 4.2.2.) using the reaction conditions described in step 2.5 of section 4.2.2. Transform the ligated product into competent cells, amplify and purify using a miniprep kit. Create a glycerol stock of this final product, which contains both template segments ligated either side of the mVenus YFP reporter gene within pKHR5.

4. Prepare the microinjection mix using the Cas9 mRNA, two sgRNAs, and the exogenous HDR reporter template.

4.1. Mix 200 ng/µL of Cas9 mRNA, 100 ng/µL of each sgRNA, and 200 ng/µL of exogenous HDR reporter template in 1x injection buffer to a final volume of 20 µL.

4.2. Store the microinjection mix at -20 °C and discard the unused mix after three freeze-thaw cycles.

NOTE: Use 4 nL of this microinjection mix for microinjection into the yolk sac of each embryo.

4.2.3. Breeding of zebrafish and embryo microinjection

NOTE: Protocols for zebrafish breeding and microinjection of single cell embryos have been described previously (Rosen et al., 2009; Avdesh et al., 2012; Sorlien et al., 2018).

1. For breeding, use zebrafish of the AB strain and that are 6–12 months of age. Inject the embryos within the 1-cell stage at approximately 40 min post fertilization (see Figure 19).

NOTE: The biologic sex of the injected embryos was not known; sexual dimorphism is not evident until approximately 3 months of age (Kossack and Draper, 2019).

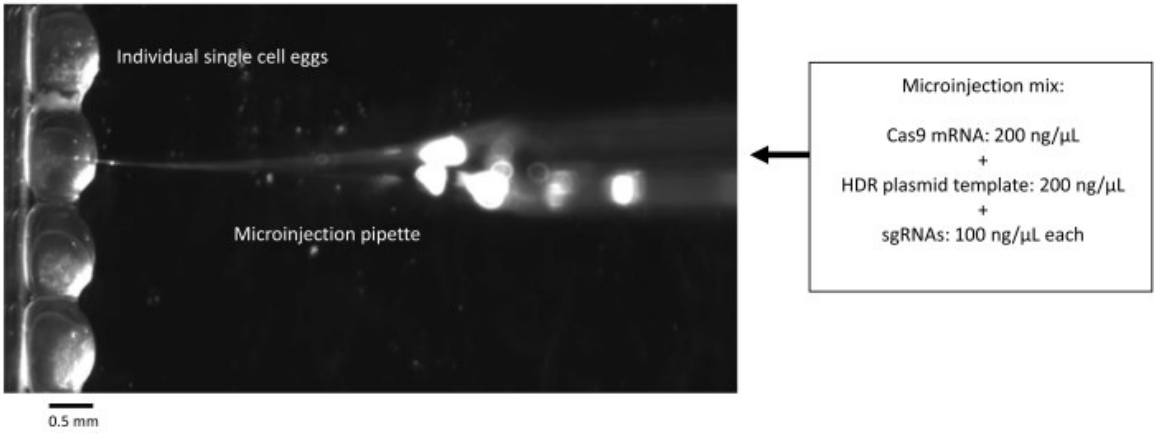


Figure 19: Microinjection of single-cell zebrafish embryos with CRISPR-Cas9 components.
Scale bar = 0.5 mm. Abbreviations: HDR = homology-directed repair; sgRNA = single-guide RNA.

4.2.4. Reporter gene screening of CRISPR-Cas9-edited larval zebrafish

1. Visualize YFP integration in zebrafish larvae following microinjection of CRISPR-Cas9 components to screen for successful HDR edits.

1.1. In a 25 mm Petri dish, anesthetize 24 zebrafish larvae at 3 days post fertilization (dpf) in 0.3% tricaine methane sulfonate (MS-222, buffered to pH 7.0–7.4 with HEPES and sodium hydroxide) until they lose their self-righting reflex (typically 1–2 min). Once anesthetized, transfer each larva into an individual well of a 24-well plate.

1.2. Using a microscope capable of detecting GFP/YFP, screen for reporter gene fluorescence in the eyes of each individual larva.

1.3. Capture images of each larva and document the presence or absence of reporter gene expression.

4.2.5. Phenotyping of CRISPR-Cas9 edited larval zebrafish

1. Following reporter gene screening, perform cardiac phenotyping (heart rate, pericardial dimensions, ECG) on each larvae. Phenotype an equal number of reporter gene-positive and -negative larvae.

1.1. Use a CCD camera (e.g., blackfly USB3) and video and image recording software (e.g., Micromanager for ImageJ) to measure heart rate and pericardial dimensions while the larvae are anesthetized.

1.2. To measure the heart rate, using Micromanager, create a region of interest (ROI) so as to capture the heart and exclude other structures. Import the video into ImageJ as an image sequence, and ensure the correct number of frames is entered under “number of images”. After the file is open, use the “rectangle selection” tool to draw an ROI within the heart but excluding other moving elements, and save the ROI in the ROI manager (analyze -> tools -> ROI manager). Click plugins -> install, and select the heart rate algorithm, installing in the default plugin folder, then select the plugin at the bottom of the plugins tab. Record the beats per minute (bpm) from here.

NOTE: Image detection algorithms were custom-written to detect heart rate by measuring individual pixel density changes associated with ventricular systolic contraction. The code can be found at https://github.com/dpoburko/zFish_HR.

1.3. Measure pericardial dimensions using a free tool such as ImageJ to free-draw ROIs around the pericardial sac and one of the eyes. Open the image in ImageJ and use the “polygon selection” tool to draw an ROI first around the pericardial sac, saving in the ROI manager as done in Step 1.2 of section 4.2.5., and repeat for the eye. Select these two ROIs in the ROI manager, then click “measure”. Record the area for each, to later calculate the area of the pericardial sac normalized to the eye area in each larva.

1.4. Following heart rate and pericardial measurements, record ECG from individual larvae.

NOTE: Protocols for recording zebrafish ECG have been described previously (Yu et al., 2010; Dhillon et al., 2013; Tanaka et al., 2019; Hurst, n.d.).

4.2.6. Genotyping of CRISPR-Cas9-edited larval zebrafish

1. Following phenotypic analyses, conduct on- and potential off-target genotyping to confirm accurate and precise HDR gene editing.

1.1. Anesthetize each 3 dpf larva in 0.3% MS-222 and tail clip to isolate gDNA using the HOTShot method (Meeker et al., 2007). Incubate each excised tail clip in 15 µL of 25 mM

NaOH at 95 °C for 20 min. Then, neutralize with 1.5 µL of Tris-HCl and centrifuge at 13,800 × *g* for 30 s. Retain the supernatant, which contains extracted gDNA.

1.2. Recover the larvae in E3 media and return to the housing system if further development or study is intended.

1.3. Using the extracted gDNA as a template, perform PCR-based Sanger sequencing of on-target and potential off-target sites.

NOTE: Optional: a nested PCR approach may be beneficial for some gene regions.

1.4. Ensure that the on-target primer design captures the mutation site and the closest sgRNA binding site. Design a separate sequencing primer to detect the transition from the inserted homology arm and the target gene to confirm integration into the gene of interest. Design primers to sequence the top three potential off-target sites identified in step 2.3 of section 4.2.1.

NOTE: Guide-design software programs often suggest primers to use, but customization may be necessary to achieve optimal results.

1.5. Compile on- and off-target genotyping, heart rate, pericardial dimension, and ECG phenotyping, and reporter gene data identifiable for each zebrafish.

Table 3: List of materials used in experiments described in Chapter 3.

Software	
Program	**Host Company**
CRISPOR	TEFOR Infrastructure
ENSEMBL	European Bioinformatics Institute
ImageJ	National Institutes of Health (NIH)
Micro-Manager	Open Source (Github)
NEBiocalculator	New England Biolabs (NEB)
Equipment	
Equipment	**Supplier**
24-well Plate	VWR
100 mm Petri Dish	VWR
Blackfly USB3 Camera	Teledyne FLIR
C1000 Thermal Cycler	Bio-Rad
Centrifuge 5415C	Eppendorf
EZNA Gel Extraction Kit	Omega Biotek
MAXIscript T7 Transcription Kit	Invitrogen
MaxQ 5000 Incubator	Barnstead Lab Line
Miniprep Kit	Qiagen
mMessage mMachine T7 Ultra Transcription Kit	Invitrogen

ND1000 Spectrophotometer	Nanodrop
PCR Purification Kit	Qiagen
PLI 100A Picoinjector	Harvard Apparatus
PowerPac Basic Power Supply	Bio-Rad
Stemi 305 Stereoscope	Zeiss
Wide Mini Sub Cell GT Electrophoresis System	Bio-Rad
Zebtec Zebrafish Housing System	Tecniplast
Services	
Service	**Supplier**
Gene Synthesis	Genewiz
Sanger Sequencing	Genewiz
Reagents	
Reagent	**Supplier**
10β Competent Cells	NEB
10X PCR Buffer	Qiagen
100 mM Nucleotide Mixture	ABM
Ampicillin	Sigma
BamHI Endonuclease w/ buffer	NEB
BsaI Endonuclease w/ buffer	NEB
DR274 Plasmid (XL1 Blue bacterial agar stab)	Addgene

EcoRI Endonuclease w/ buffer	NEB
Glycerol	
HEPES	Sigma
HindIII Endonuclease w/ buffer	NEB
Kanamycin	Sigma
Methylene Blue	Sigma
MLM3613 Plasmid (XL1 Blue bacterial agar stab)	Addgene
MS-222 (Tricaine)	Sigma
pKHR5 Plasmid (DH5α bacterial agar stab)	Addgene
PmeI Endonuclease w/ buffer	NEB
SalI Endonuclease w/ buffer	NEB
Sodium Hydroxide	Sigma
T4 Ligase w/ buffer	Sigma
Taq Polymerase	Qiagen
TE Buffer	Sigma
Tris Hydrochloride	Sigma
XhoI Endonuclease w/ buffer	NEB

Recipes

Solution	Component	Supplier
Annealing Buffer (pH 7.5-8.0)	10 mM Tris	Sigma

	50 mM NaCl	Sigma
	1 mM EDTA	Sigma
E3 Media (pH 7.2)	5 mM NaCl	Sigma
	0.17 mM KCl	Sigma
	0.33 mM $CaCl_2$	Sigma
	0.33 mM $MgSO_4$	Sigma
Injection Buffer (pH 7.5)	20 mM HEPES	Sigma
	150 mM KCl	Sigma

4.3. Representative Results

The successful use of this two-sgRNA exon replacement CRISPR approach, is highlighted by the introduction and simple detection of a precise edit to engineer the LQTS-associated variant, R56Q, in the *zkcnh6a* gene in zebrafish. Figure 20 shows a representative 3 dpf larvae injected at the one cell embryo stage with CRISPR components as described above. Figure 20a shows the presence of the YFP mVenus reporter gene expression in the eye lens as a positive reporter of successful template integration. Figure 20b and C show Sanger sequencing chromatograms obtained from genomic DNA isolated from tail clip samples of wild-type and reporter gene-positive fish, respectively. Reporter gene-positive fish were found to have the precise edit, G to A, which introduces the R56Q variant into *zkcnh6a*. Genotyping showed a 100% correlation between YFP reporter gene expression and presence of the precise R56Q gene edit, validating this fluorescence screening tool.

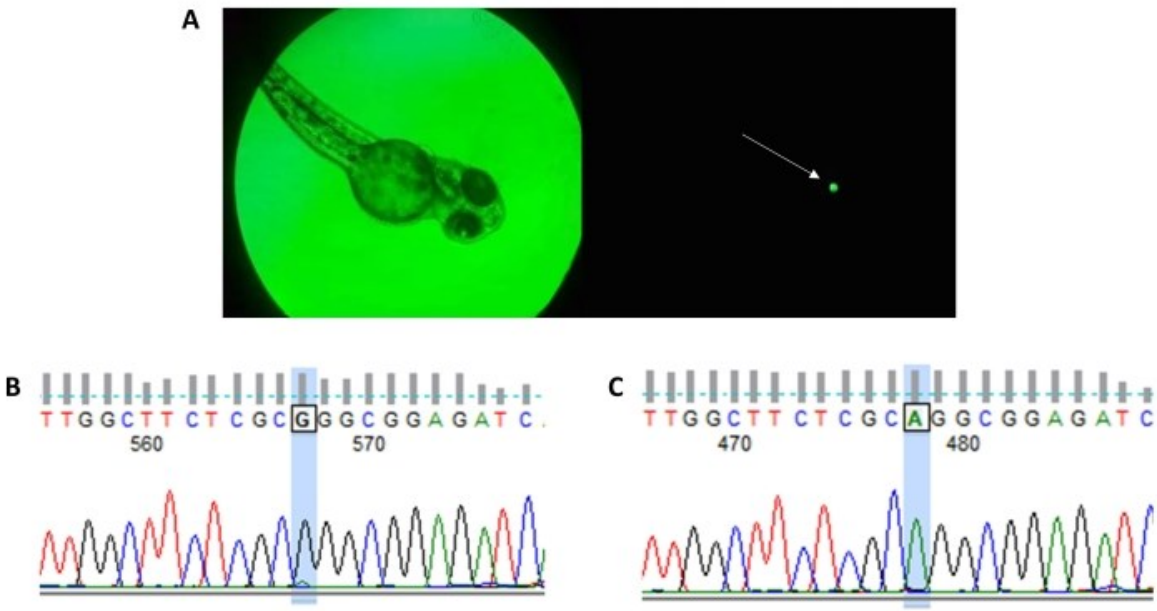


Figure 20: Easy detection of mVenus YFP reporter gene fluorescence indicates positive HDR exogenous template integration into the target gene.
(A) Example of mVenus YFP expression in a zebrafish eye (arrow) in an edited zebrafish larva. (B) Successful edits are confirmed by sequencing chromatograms (left, WT; right, R56Q edit).

Phenotyping of gene-edited zebrafish larvae was conducted at 3 dpf. Figure 21 shows representative results from a wild-type and R56Q gene-edited larvae. Heart rate was detected by video capture as described above. An example of measurement of pericardial dimensions as a ratio of eye area are shown (Figure 21a). Figure 21b plots

heart rate against normalized pericardial dimensions in R56Q zebrafish highlighting a trend of bradycardia with increasing pericardial edema, which is associated with disorders of cardiac repolarization in zebrafish (Langheinrich et al., 2003; Milan et al., 2003; Arnaout et al., 2007; Hoshijima et al., 2016). The R^2 value for this correlation is 0.33, implying a weak correlation. Figure 21c shows a representative example of ECG recordings from 3 dpf larvae. Standard intervals (QT, QRS) were measured from averaged ECG signals. Heart rate was calculated as the average R-R interval, and QTc interval via Bazett's formula: $QTc = QT / \sqrt{RR}$. This correction is due to the fact that QT interval changes with heart rate, as is seen in humans. Recordings for these ECGs were taken using a single lead electrode approach, with an electrode placed over the ventral surface of the fish directly over the heart, and a reference electrode in the surrounding medium. Positioning of the electrode can change the waveform, but has no effect on interval measures (Dhillon et al., 2013).

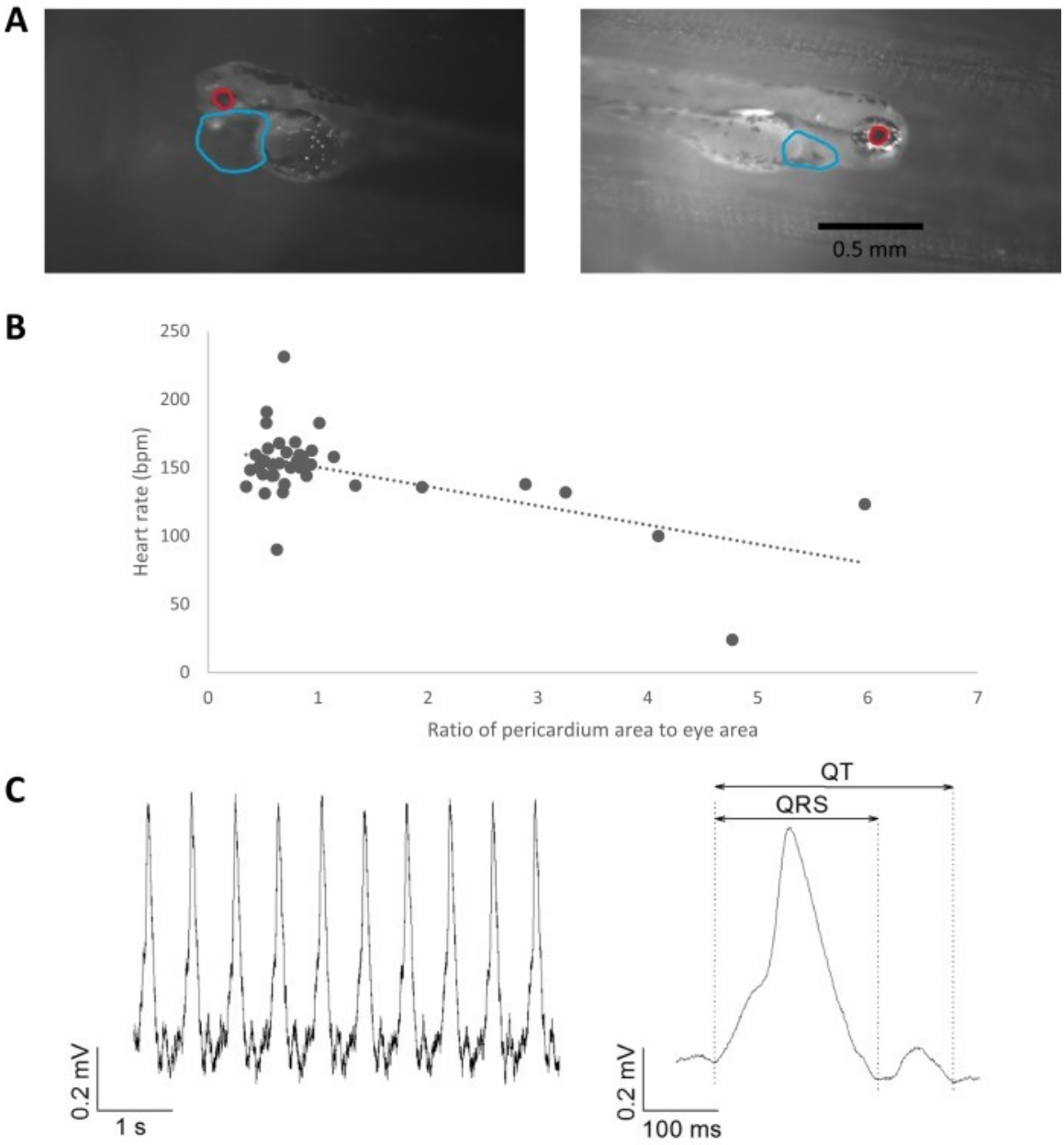


Figure 21: Phenotypic analysis of cardiac consequences in 3 dpf zebrafish following the precise R56Q edit in the zkcnh6a target gene.
(A) Image detection of pericardial dimensions relative to eye size using the polygon tool in ImageJ. The boundaries of the pericardial sac were marked by the user from a single recording frame based on changes in translucency and pigmentation. The fish on the left is an R56Q variant, while the fish on the right is WT. Examples of normal pericardial dimensions, and pericardial effusion are shown. Scale bar = 0.5 mm. (B) Correlation between pericardial dimensions (relative to eye dimension) and heart rate, $R^2 = 0.33$. (C) example of ECG recording from 3 dpf zebrafish larvae heart (left) and averaged complexes (right). Heart rate, 131 bpm; heart rate-corrected QTc interval, 460 ms. Abbreviations: dpf = days post fertilization; ECG = electrocardiogram.

4.4. Discussion

Engineering of precise gene-edits using CRISPR-Cas9 is challenged by low efficiencies of HDR mechanisms and their efficient detection. Here, a CRISPR-Cas9-based two-sgRNA exon replacement approach is described that produces precise edits in zebrafish with straightforward visual detection of positive edits. The efficacy of this approach is demonstrated by generating precise edits in the *zkcnh6a* gene. This paper shows how cardiac function in gene-edited zebrafish larvae may be assessed using non-invasive phenotypic measures of heart rate, pericardial dimensions, and ECG morphology. This approach, from introducing a gene-edit to phenotypic evaluation, can be completed from start to finish within approximately 1 week.

The benefits of the above editing and phenotyping approach are the ease of CRISPR modification design, the wide applicability in multiple physiological systems, the ability to insert large genes or gene fragments, and the ability to track variant effects longitudinally through development and generations. The success of precise edits in this approach may be related to the combination of the large template size (due to the reporter gene insert and long homology arms), which has been shown to increase efficiency of edits in zebrafish (Irion et al., 2014), and the two-sgRNA-guides strategy, which has been used effectively in zebrafish TALEN-induced edits (Hoshijima et al., 2016).

One critical factor in generating precise edits is the possible presence of off-target edits, as these could have unclear implications for the zebrafish and confound phenotyping. With the above design, this is somewhat mitigated due to the two-guide approach, as well as the targeting of introns. With two guides, integration of the template via HDR is dependent on both guides targeting in proximity to each other. There were no off-target sites provided by the CRISPOR design software that were common for both guides, implying off-target HDR events should be of minimal concern. Another potential target are paralogs resulting from genome duplication events, such as the teleost genome duplication. While these do share sequence homology, given the intronic targeting of the guides this should not be a concern. NHEJ events in these intronic sequences would not be expected to cause a change in function for these channels.

One particular strength of the described approach is the ability to insert large genes or gene fragments. This may be useful, for example, to insert human orthologs (MacRae,

2010), allowing for more clinically translatable characterization and comparison between orthologs. Alternatively, genes encoding Cas enzymes could also be inserted, allowing for a line of zebrafish with *in vivo* CRISPR editing mechanisms, providing an inducible system. Similarly, alternative CRISPR mechanisms, such as prime editing, could be integrated and result in a line of zebrafish that are readily edited precisely and efficiently.

Despite the advantages of this approach, there are some limitations. First, only a single gene and locus have been modified, and further testing at other sites or in other genes is necessary to evaluate how broadly applicable this approach is. Due to the long homology arms required the template design costs are higher; however, this may be offset by efficient screening. Another limitation is that the screening approach requires fluorescence detection capability. However, optical requirements are relatively low and can be custom-built or commercially purchased at reasonably low cost. Using a two-sgRNA approach increases the number of potential off-target events; however, this is likely mitigated by the lower probability that the two sgRNA guides will both anneal in a manner that facilitates incorporation of the template to yield reporter gene expression.

Finally, using Cas9 mRNA may lead to mosaicisms as the Cas9 is not active until later developmental stages. As the edit is not integrated in the one-cell stage, the variant in question may not be passed to all cells/tissues via cell division. This could be accounted for by sequencing particular tissue types; however, given the size of the zebrafish larvae this is technically challenging. In summary, this CRISPR-Cas9 two-sgRNA precise editing approach in zebrafish enables simple visual detection of positive edits and may be adapted to incorporate large genes of interest at any locus. Combined with phenotypic measures, this allows for a reliable and high throughput platform for studying clinically-relevant cardiac variants.

Chapter 5. A photoinducible system for precisely controlled CRISPR edits

This chapter is unpublished work. The intent was to develop an inducible CRISPR system to drive forward the genetic model I have developed in Chapter 4. Due to potential difficulties with compensatory effects and embryonic lethality, an inducible CRISPR system would circumvent these problems allowing for a more precisely controlled and robust genetic model platform. Due to difficulties with the zebrafish population, and my own timeline, I was able to design and create the constructs, as well as assemble the light induction system, but I was unable to test the system. In this chapter, I include my designs and plans for a proof-of-concept test, and how this approach might be more widely applicable.

5.1. Introduction

5.1.1. CRISPR in zebrafish

The approach that I have developed to introduce precise edits into the *zkcnh6a* gene in zebrafish described in Chapter 4 is powerful, but there are potential limitations. In this approach, time is a significant factor. CRISPR components are injected into the zebrafish oocyte during the one-cell stage so that edits made via the CRISPR process will be passed down through daughter cell lines. In this way, the variant would, theoretically, be present in every cell in the zebrafish. However, the Cas9 mRNA must be translated before edits can occur. In 2018 Zhang et al. measured the time for Cas9 translation and detected Cas9 protein in embryos starting at the eight-cell stage, approximately 75 min after fertilization, and continuing until the 32-cell stage (the last time point measured) (Zhang et al., 2018). This was measured by extracting protein from 50 pooled embryos and conducting a Western blot analysis. This suggests that there is the potential that cells early in development may not contain all of the components necessary for CRISPR, which could prove a confounding variable. If either the edit does not occur in one of the eight cells, or if the Cas9 mRNA was not divided into each cell, there is a chance of mosaicisms, where cells without edits will result in clusters of un-edited cells. A potential mitigation strategy is to inject directly into the cell cytoplasm, rather than the yolk sac, however this proves a technical challenge as aligning the eggs when injecting to allow for higher throughput (as is possible when injecting the much larger yolk sac) reduces the amount of time available before cell division starts. Beyond these difficulties inherent with mosaicisms, there are also challenges present when all cells in the zebrafish do inherit the variant, as discussed in the next section.

5.1.2. Potential issues with traditional CRISPR approaches

Above, I describe one factor, spatial heterogeneity, that limits the CRISPR approach used in zebrafish. A variant introduced early in development is inherited by every daughter cell, such that all organ systems in the zebrafish will possess the variant in question. From our tail clips and fluorescence screening, we know that the edit is located in at least the tail, eye, and heart of the zebrafish. Leong et al. studied spatial expression patterns of *zkcnh6a* in zebrafish by extracting RNA from eight different organs in adult fish and performing qPCR. They found *zkcnh6a* (at the time named, zerg3) expressed in the

heart and brain, and to a lesser extent in the gills, gut, kidney, liver, muscle tissue, and spleen (Leong et al., 2010a). This suggests that any variants I generate will also be expressed in these locations, with unknown effects that could prove confounding variables to characterizing cardiac effects.

Another factor that may complicate the approach is temporal variability, because CRISPR edits introduced during the embryonic stage are present as the fish grows to the adult stage. There are multiple complications that can arise with this strategy. One is that if the variant is embryonic lethal, then there will be no opportunity to study the variant in adult fish. This can be mitigated if studying cardiac-relevant mutations by phenotyping embryos within the first ten days, since at this stage of development zebrafish larvae are not dependent on a beating heart to circulate oxygen and nutrients. Another potential issue is compensatory effects in response to the generated variant. When the zebrafish is still developing, changes in gene expression can compensate for deleterious effects. In 2015, Rossi et al. studied phenotypic differences between deleterious mutations and knockdowns in zebrafish. The authors created both knockout and knockdown (via morpholinos) zebrafish models for *egfl7*. *Egfl7* was chosen because in mouse knockout models there was no obvious phenotype, while knockdown models in zebrafish, frog, and human cells demonstrated severe vascular tube formation defects. This phenotypic discrepancy between a knockout and knockdown of the same gene made this a prime candidate for studying compensatory effects. The authors generated a mutant zebrafish line, with the *egfl7*^{s981} variant. Using fluorescence imaging, they looked for any morphological defects in the vasculature, angiogenesis, and circulation, but found nothing. To test morpholinos, the authors generated a zebrafish line with Myc-tagged *egfl7* for Western blot analysis, injecting these fish with *egfl7* morpholinos reduced *egfl7* expression by 80%. The authors then injected embryos from in-crossing heterozygous mutants, and found more WT offspring were affected than mutant, indicating that the mutation rendered zebrafish less sensitive to morpholino knockdown. Randomly choosing 10 WT-looking embryos, the authors found that 8 were homozygous and 2 heterozygous for the mutation. This suggested the mutant zebrafish were in some way negating or compensating for the detrimental effect to *egfl7* and were less sensitive to knockdown. The authors finally looked at which molecules may have been upregulated in response to generating this variant: Using mass spectrometry Emilin3a was identified as being significantly upregulated in *egfl7* mutants compared to both MO-knockdowns and WT. The responsible

gene, along with *emilin3b* and *emilin2a* were all upregulated, and when the mRNA was injected into MO-knockdown zebrafish the vascular defects were rescued. This suggests that these three emilin genes were upregulated in a compensatory fashion when *egfl7* was knocked out (Rossi et al., 2015).

5.1.3. Inducible CRISPR

A potential solution to the above problems is to use a version of CRISPR that can be induced by particular triggers. The ability to induce edits in later life stages allows for targeting of edits in particular organs or tissues by providing the inducibility stimulus in particular regions of the body, avoiding potential effects of the variant in other organs where *zkcnh6a* is expressed. An inducible CRISPR system also avoid the temporal difficulties of embryonic lethality and compensatory mechanisms by triggering the edits after the life stage where these effects would occur. These inducible mechanisms can include chemical triggers such as hormones, or light-induction. Chemical triggers have been used to induce protein activity via hormone-binding domains, such as in 2016 when Liu et al. fused Cas9 with the estrogen receptor, ERT2. Comparing multiple constructs, the authors found that fusing ERT2 domains immediately adjacent to nuclear localisation signals provided the most effective control via infusing with 4-HT (afimoxifene). Without the ligand present, the hormone-binding domain physically blocks the nuclear localisation signals, impeding Cas9 entry into the nucleus and interact with genomic DNA. When bound, the hormone-binding domain no longer sterically hinders the nuclear localisation signal and allows passage into the nucleus. This process was found to be reversible, after removing the 4-HT and waiting 72 hours for the Cas9 to exit the nucleus, the authors found that further editing activity was no longer detected (Liu et al., 2016). While this is a functional inducible system, there are associated risks. The chemical inducer used may have developmental effects or other unintended consequences to the zebrafish. Additionally, the timeframe associated with allowing the hormone to bind the receptor and then pass into/out of the nucleus is longer than desired.

Another option is via optogenetics. This is the process of a light-sensitive protein triggering activation of a protein, in this case by bringing a split protein in proximity, allowing functioning as a whole enzyme. Nihongeki et al. in 2015 developed a photoactivatable Cas9 by fusing a pair of proteins called magnet proteins with Cas9 fragments. When exposed to blue light, these heterodimers would bind, carrying

associated proteins in close proximity. If a Cas9 is split and each half fused with either pMag or nMag, binding of the Mag heterodimers upon stimulation by blue light would enable the two halves of Cas9 to associate, and create an active Cas9.

These magnet proteins were developed from a fungal photoreceptor called Vivid (VVD). In 2008, Zoltowski et al. discovered that VVD forms a rapidly exchanging dimer in response to blue light, and deduced that this conformational change could be used as a method of gene expression control (Zoltowski and Crane, 2008). VVD was initially identified as a blue-light photoreceptor in 2003, with excitation peaks at 380 nm and 450 nm (Schwerdtfeger and Linden, 2003). In 2012, Wang et al. used this dimerization property to develop a light-inducible system for gene activation. The authors fused residues 1-65 of Gal4 with the VVD protein, this provided the DNA-binding properties from Gal4 with the light-inducible dimerization of VVD. Gal4 is a transcription factor found in yeast and binds the upstream activating sequence for the *Gal* operon (UASG). After fusing the binding domain of Gal4 with VVD, the authors found light-inducible expression of *Gal* in yeast (Wang et al., 2012).

Based on this VVD-controlled gene expression, Kawano et al. split VVD to create the magnet heterodimerization system for more efficient control of protein activity. When VVD homodimerizes, an alpha-helix termed Ncap is found at the interface, with a stretch of neutral amino acids. The authors designed two version of the VVD photoreceptor: one with positive residues at the Ncap helix and one with negative residues. This ensured that the dimerization could be controlled, and particular proteins or protein fragments could reliably interface. They termed these positive and negative VVD photoswitches nMag and pMag, or magnet proteins (Kawano et al., 2015).

This approach has advantages over the chemical inducible method described above in that the light required for dimerization can be switched off and this will more rapidly split the Cas9 and stop editing activity. Additionally, this method can be used to target gene editing to particular regions of the zebrafish, with a precise light source. In my case this means I could target edits to occur only in the heart. Perhaps most importantly, I could also trigger edits in adult fish rather than in larvae, which would circumvent issues with developmental compensatory effects.

Recently enzymatic control has been utilised for inducible CRISPR activity that is cell-specific. In 2022, Cai et al. developed an enzyme-inducible CRISPR, wherein an sgRNA is self-blocked, that is, an extended portion of the RNA blocks the Cas9-RNA complex from binding target DNA. A deoxyribosome (DNAzyme) is also present that can cleave the sgRNA to allow CRISPR activity. When the DNAzyme is modified to be chemically caged, it requires an enzyme found in particular cell types to activate and then induce CRISPR activity. The authors modified the DNAzyme to require NAD(P)H:quinone oxidoreductase, an enzyme overexpressed in tumour cells, allowing for induction of CRISPR activity specifically in cancer cells (Cai et al., 2022). While allowing for cell-specific CRISPR activation, there is no control for temporal activation, meaning that, in embryonic injections, the CRISPR process would activate as soon as the endogenous protein trigger is expressed. Additionally, an enzyme specific to cardiac tissue would have to be selected, and a DNAzyme modified to activate in its presence.

The option I chose for my approach is optogenetic, for two main reasons. This method allows for temporal control over CRISPR activation; since CRISPR activity in embryonic stages could result in compensatory mechanisms (Rossi et al., 2015) that interfere with our characterisation of zERG variants. Enzymatic control is reliant on expression of a regulatory endogenous protein, which is not temporally controlled and must be identified in the tissue of interest. Chemical-inducible systems can function in this way, however the efficiency of ligand introduction into adult zebrafish is not as effective as using light. The second reason is ease of inactivation; with optogenetic methods turning the light off will revert the Cas9 to a non-functional disassociated state, allowing more precise control over the activity of the Cas9.

5.2. Methods

Studies using zebrafish were conducted in agreement with the policies and procedures of the Simon Fraser University Animal Care Committee and the Canadian Council of Animal Care.

5.2.1. Inducible construct design

Construct design was carried out via sequence information from the Addgene repository, and design inspiration from previous research (Nihongaki et al., 2015).

Plasmids containing magnets were purchased from Addgene (pMag catalog #: 108848; nMag catalog #: 108849), the SpCas9 used was the same as in Chapter 4, in the MLM3613 plasmid (Addgene catalog #: 42251).

5.2.2. Creating the magnet construct

Constructs were designed as described in Chapter 5.3. Qiagen miniprep kits were used for all DNA extractions as described in Table 3. Once cloned into the magnet vectors, these were further digested for ligation into the pKSS plasmid, which allowed for in vitro transcription. Transcriptions were performed using the Mmessage Mmachine T7 Ultra Kit.

5.3. Results

The design of the fusion construct consists of each magnet in frame with a fragment of the Cas9 gene (split site shown in Figure 22). As discussed in Chapter 5.1.3., having the magnet protein fused in frame will allow for a fusion protein that brings the Cas9 fragments together to form a functional nuclease. Gene fusions between magnet proteins and fragments of SpCas9 were synthesized through PCR and restriction digests. I used PCR to split the SpCas9 gene contained within the MLM3613 plasmid into two halves at the RuvC domain. The Cas9 N-terminal fragment had KpnI and EcoRI restriction sites added to each end, and the C-terminal fragment had MluI and BspEI added. This allowed ligation of the N-terminal fragment in frame with the pMag, and the C-terminal fragment with the nMag. See Figure 23 for a construct map.

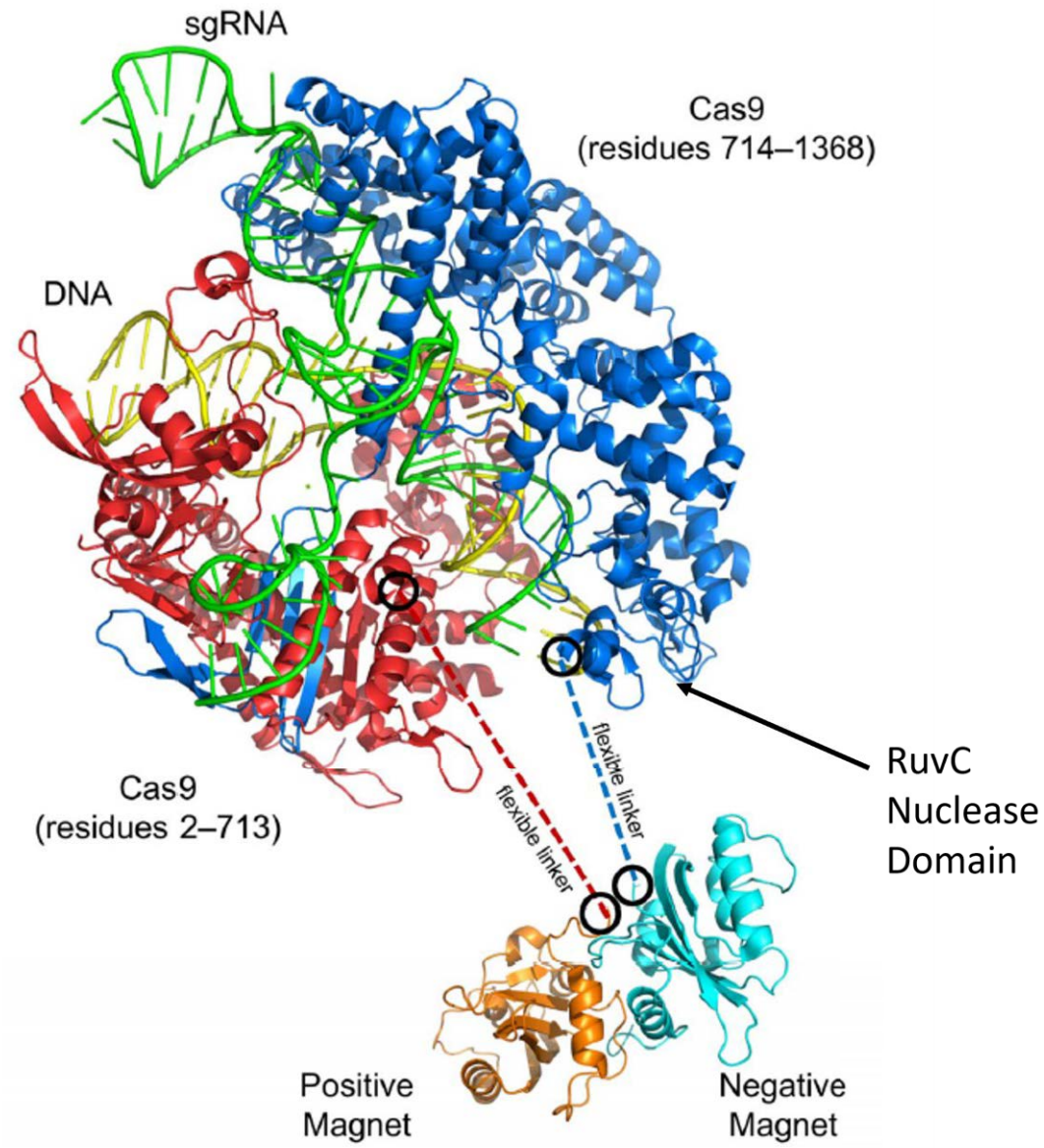


Figure 22: Structure of Cas9 showing the split site and fused magnet proteins.
Cas9 structure showing the two fragments in red and blue. The black circled regions are where the protein was fragmented, and the magnets are fused. The RuvC nuclease domain is labelled as at the split point.

Note: Image obtained and modified from (Nihongaki et al., 2015). Permissions obtained from Springer Nature, license #: 5777980292171

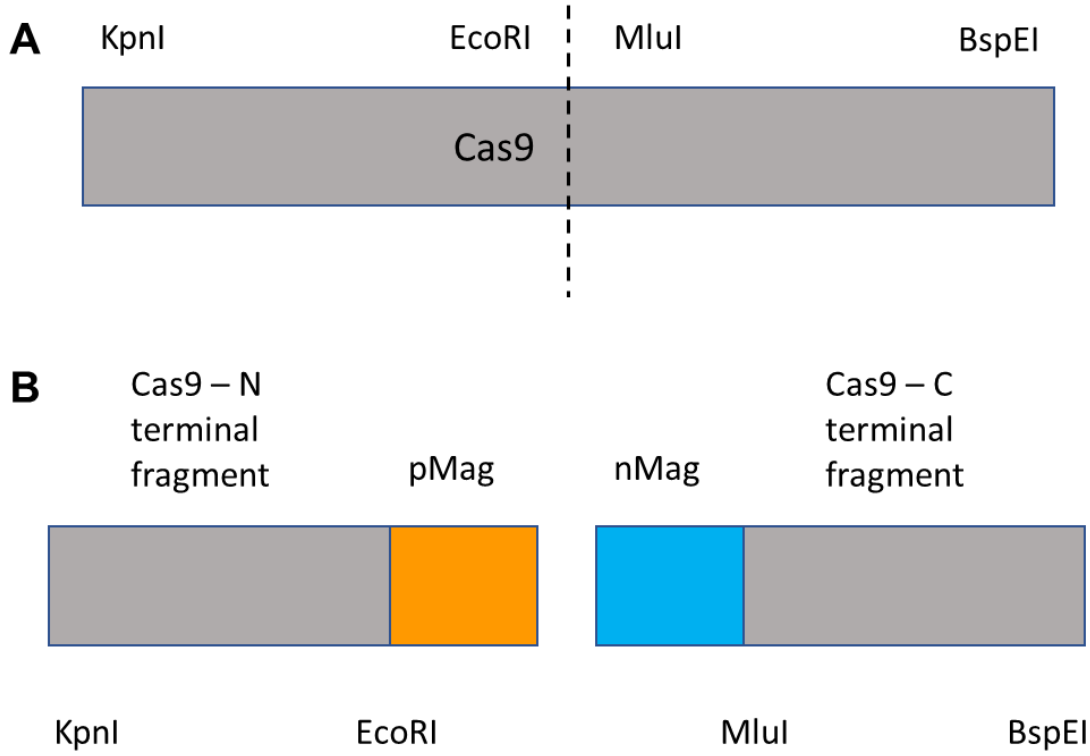


Figure 23: Schematic of the magnet-Cas9 fusions.
A. Cas9 gene from MLM3613, splitting via PCR amplification. In doing so, restriction sites are added on either end of each fragment. The split is occurring at the RuvC site. B. Cas9 halves fused with magnet proteins. The two Cas9 fragments with appropriate restriction sites are cloned into the appropriate magnet vector.

Blue light induction was to be performed via an LED system that I constructed with assistance from the SFU electronics shop, see Figure 24. The LED lights were installed inside the larval fish incubator, on a heat sink which prevented condensation, a necessary consideration in a wet environment. The driver was a 900 mA power source, which provided a constant output.

Figure 24: LED lights and power source.
A. The blue light LED chip, with a wavelength of 450 nm and intensity of 900-1000 LM. B. The driver for our LEDs, with a constant output of 900 mA.

The intended path for this experiment was to inject zebrafish embryos under three sets of conditions, as outlined below:

1. Experimental: Cas9-magnet fusions, guides, HDR template, blue light

2. Control: Cas9-magnet fusions, guides, HDR template

3. Control: Cas9-magnet fusions, HDR template, blue light

My expectation is that in the first trial, I should see evidence of NHEJ or HDR repair through sequencing and potentially YFP-fluorescent eyes. My primary measure would be identifying NHEJ events via sequencing. I would also screen for HDR repair events, which would result in expression of green fluorescence reporter gene in the eyes. The control experiments above would ensure confidence that the fusion approach successfully created an inducible system, since without blue light there should be no dimerization of the magnets (Figure 25). Moving forward I would integrate this photoinducible Cas9 into the zebrafish genome by using the CRISPR approach that I developed in Chapter 4. Inserting both halves of the photoinducible Cas9 into a safe harbour site under a cardiac promoter, such as ventricular myosin heavy chain (*vmhc*), would allow expression of the modified CRISPR system to be localised in the heart.

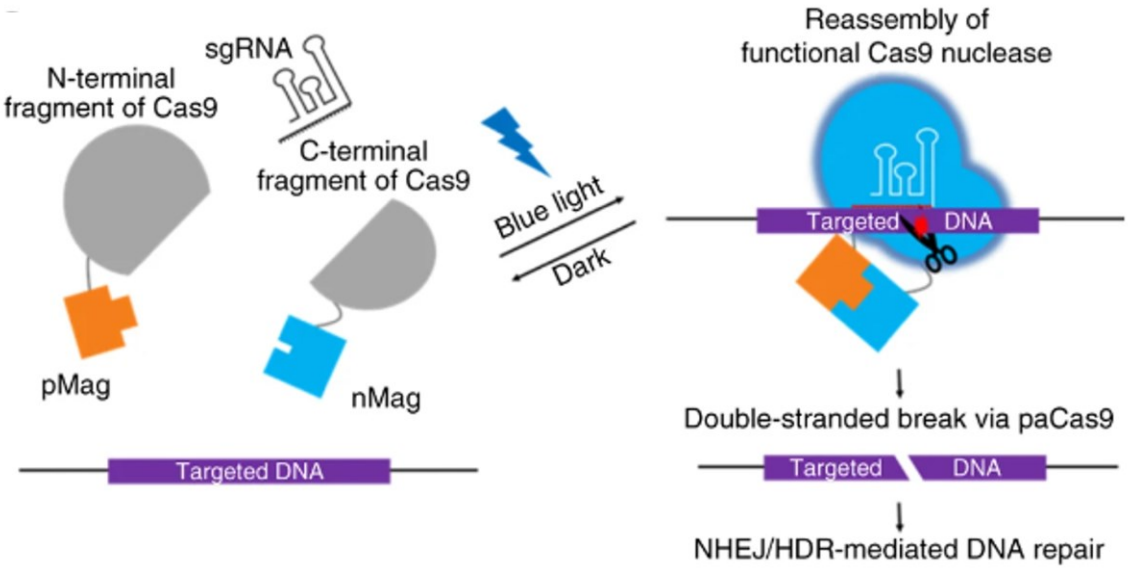


Figure 25: A schematic of the magnet-Cas9 fusions.
The Cas9 is split at the RuvC site, and each half fused to either nMag or pMag. Upon blue-light induction, the magnet proteins heterodimerize and reassemble the Cas9. After the Cas9 performs its editing function, removal of the light stimulus causes dissociation of the magnets and the Cas9 is non-functional again.

Note: Image obtained from (Nihongaki et al., 2015). Permissions obtained from Springer Nature, license #: 5741320818359.

5.4. Discussion

In this chapter I designed and created a Cas9-magnet fusion for inducible control over editing activity. While I was unable to test this system, I successfully designed and created the fusion based on Nihongaki's 2015 research, adapted to zebrafish (Nihongaki et al., 2015). My plan to expand this experiment and platform was to insert this photoinducible Cas9 into the zebrafish genome, so that it is endogenously produced. The intent behind this research is to edit *zkcnh6a* in adult fish; rather than have to introduce the photoinducible Cas9 into adult hearts, the zebrafish can be constantly producing the protein itself. This bypasses issues surrounding introducing the inducible construct into the adult zebrafish heart.

Light induction requires exposure to the blue-light LEDs for approximately 30 minutes, when the photoinducible Cas9 is translated, approximately the 8-cell stage. This test would demonstrate that the photoinducible system only functions in the presence of blue light, and that it does not have any inherent effect without the targeting of guide RNAs. Given the use of light exposure to trigger editing, there is the potential for tissue damage if the light intensity is too high. Additionally, depending on wavelength, tissue penetration by the light may not be high enough to reach cells or tissue layers of interest. Magnets with different sensitivities are available, but this is a consideration that must be made.

With a proof of concept, this system can be carried forward into inclusion in the adult zebrafish genome. Once the photoinducible Cas9 is integrated into the fish genome, it can be used with any variety of mutation, not restricted to the cardiac system. As described in the Results (Chapter 5.3), the long-term approach is to maintain a line of zebrafish that have endogenous expression of the inducible Cas9, which could be used to edit any gene of interest. To do this would, however, require additional components and the delivery of both guides and template are current limitations, since introducing genetic constructs in a targeted fashion in adult fish is a challenge. There are delivery mechanisms through viral infection, or ingestion. But ideally these either also integrate into the genome, or a system such as base editing or prime editing which do not require a template for precise edits. The possibility of integrating these other editing techniques into an inducible system is further discussed in Chapter 7.4.1. This design gives the potential for creating a powerful and versatile model that can be used in virtually any system, by supplying

guides or template and targeting the blue light exposure there are no limits to what this potential zebrafish line is capable of.

Chapter 6. Groups vs Pairs: Ideal first-year learning environments for upper-division topics in molecular biology

Alongside my evidence-based approach in the lab, I'm interested in similar approaches in my teaching. Studying CRISPR in zebrafish, I wanted to learn more impactful ways to teach these same molecular techniques to undergraduate students. I had the opportunity to co-teach BISC101, and decided to undertake a teaching pedagogy research project interrogating whether students learning in groups or pairs would be more effective for evaluating CRISPR guide RNA design criteria. In this chapter I have presented my findings and discuss how these data relate to teaching. As pedagogical data chapters such as this are atypical for a thesis in my field, I include a reflection at the end describing how the pedagogical research data relates to both my field of study, my own development in teaching, and my graduate education.

6.1. Introduction

6.1.1. What is CRISPR and why is it worth teaching to first-year students in general biology?

CRISPR is a gene-editing technique derived from a bacterial innate immunity system, consisting of a targeting guide RNA that forms a complex with an endonuclease to direct it to a target sequence, where the nuclease can make a double-stranded break (Lim and Kim, 2022). The cell can then either use NHEJ repair to connect the two broken ends or use HDR along with a provided template to fill in the break. This allows for targeted sequence edits in any given gene of interest, providing immense utility for multiple applications such as studying gene function, or creating disease models.

The use of CRISPR in research has become widespread and the CRISPR system has become an essential point of understanding for those interested in involving themselves in biomedical research (LaManna and Barrangou, 2018). Therefore providing undergraduate students with relevant background and a fundamental understanding of CRISPR should be beneficial for those seeking experience in research, whether in academia, government, or industry careers (Dahlberg and Groat Carmona, 2018; Sehgal et al., 2018).

When CRISPR is taught in undergraduate courses, it is often in upper-division teaching laboratory classes (Ulbricht, 2019; Pieczynski and Kee, 2021). While this provides rich experiential hands-on opportunities to engage with genetic engineering, undergraduate students often enter these courses with limited understanding of CRISPR fundamentals and applications. I believe that introducing the theoretical basis of CRISPR in undergraduate education at the lower division would better help prepare students for upper-division practical laboratory classes and subsequent entry into the research setting.

Involvement of undergraduate students in research provides a benefit to the student, the institution, the research supervisor, and other research trainees (Adebisi, 2022). Indeed, evidence suggests that directly involving students in research within their formal courses is essential for narrowing the opportunity gap of historically marginalized students (Bangera and Brownell, 2014). A key component of success, however, is sufficient academic preparation such that undergraduate students have relevant background knowledge of techniques that are at the forefront of current research. Given

the constraints (time and format) of the course we needed to select a subcomponent of a CRISPR experiment with which to work towards this goal. In the first-year biology course that I was teaching, we sought to provide experience with the CRISPOR tool, and specifically with how the tool is used in research.

6.1.2. Why guide design?

Experiments that use CRISPR technology have several components. Of these, I decided that guide RNA design and evaluation would be an ideal place to begin an introduction to CRISPR, since it is a core idea and process for any CRISPR experiment, and can easily be made accessible to students in a large first-year course (Mohr et al., 2016). Indeed, guide design is such a critical factor that artificial intelligence (AI) and deep learning is used to optimise design (Lee, 2023). While other components of a CRISPR experiment are also important, such as: the choice of the Cas enzyme (Liu et al., 2020), the type of template and repair system used for modifying the target sequence (Chauhan et al., 2023), and even secondary structures in guide RNA (Riesenberg et al., 2022), these require more in-depth consideration due to their complexity and decision-making can be more nuanced. Additionally, guide RNA design aligned well with the learning outcomes of a general biology course, and was suitable for its constraints and format (tutorials, no laboratory time; and only a short component of the course).

The tasks involved in guide RNA design encompass a number of cognitive skills for students. In order to choose guides, students must evaluate multiple characteristics including specificity, possible off-target sites, efficiency, and PAM site location. Students need to recall these characteristics from earlier lectures, and prioritize which is most important, in order to make an informed expert decision. Students must be able to explain their choices, demonstrating an understanding of the different criteria, and the data that are interpreted within these. When designing the guides from scratch, there is also an element of creation, as students must decide a target location for the guides before evaluating their options. Further, in the course context, students must work collaboratively, which requires skills in communication and collaboration. All of these are skills that are relevant to scientific research, and are valuable transferable skills for undergraduate students to learn (Clemmons et al., 2020; Price et al., 2021).

In addition to learning the cognitive tasks of guide design, focussing teaching on this topic also provides undergraduate students critical experience with software tools that are becoming increasingly important in both CRISPR research and elsewhere (Zhang et al., 2020b). There have been other studies that examine the use of software and technology in group learning environments, including types of technologies and software used, as well as group composition and roles that gender play in use of software (Brown, 2020; Holmes and Kalender, 2020). These factors, while important to consider, are not the focus of this present study.

6.1.3. CRISPR in the classroom

Others have studied and developed lesson plans for teaching CRISPR in undergraduate courses. Published studies have focussed on both practical laboratory classes and upper division courses (Bhatt and Challa, 2018; Pieczynski et al., 2019; Ulbricht, 2019; Pieczynski and Kee, 2021; Sankaran et al., 2021). In addition there are a number of non-refereed resources, such as YouTube, that students access as a standard component of their education (Greeves and Oz, 2024). A cursory search for “crispr lecture” provides a number of videos ranging from two minutes to one hour describing CRISPR in varying levels of detail, but as potentially an unreliable information source rather than an interactive activity developed through peer-reviewed practice.

In 2021, Pieczynski and Kee developed a CRISPR experiment for use in an upper division undergraduate genetics laboratory course, though the authors argue that the approach can be adapted to other course levels. They created a laboratory class module wherein instructors first introduced the process of selecting a mutation target in the CCR5 gene and then designing an HDR repair template before students repeat the workflow with a different gene. The post-activity assessment involved students writing a research proposal outlining their selection of a gene target and their workflow (Pieczynski and Kee, 2021). While this module is aligned with my goal for first-year undergraduate students, it is more in-depth than is suitable for a first-year introductory course and would not fit in the already tight course curriculum.

Also in 2021, Sankaran et al. designed a laboratory module specifically for lower division undergraduate students and high school students, wherein students use a variety of RNA guides to edit multiple targets in the yeast genome (Sankaran et al., 2021). This

approach is simpler than the Pieczynski module, and more appropriate to an introductory course, however it still requires the use of laboratory time and resources. This is not only impractical in an already full laboratory curriculum but not applicable to courses that do not integrate laboratory sections.

Others have published methods of teaching CRISPR in undergraduate courses, but they too overwhelmingly rely on laboratory work which is not suitable for all courses (Bhatt and Challa, 2018; Sehgal et al., 2018; Pieczynski et al., 2019). Therefore, there is need to investigate how teaching of the fundamentals of CRISPR might be introduced within a first-year undergraduate course without the availability of a laboratory section.

6.1.4. Groups or pairs: Optimal learning environments

Evidence suggests that utilising an active learning approach is effective when teaching a topic such as CRISPR, in a way that is useful for an introduction to research. An important aspect of this is creating the opportunity for students to discuss and learn in groups. Several studies demonstrate the efficacy of group over individual learning, both in terms of learning and student satisfaction (Laughlin et al., 2006; Bertucci et al., 2010; Corrégé and Michinov, 2021). This is an important factor in tutorial and lecture settings, as it is a variable that is relatively simple for instructors/facilitators to accommodate that can be impactful. Group size has been examined previously, although in a different context to that which I am interested in exploring (Jensen and Lawson, 2011; Hammar Chiriac, 2014; Brame and Biel, 2015; Corrégé and Michinov, 2021). In 2021, Corrégé and Michinov performed a study involving 102 secondary school students, wherein they had to draw the human respiratory system both before and after group discussion. The four study conditions they used were individual (with no discussion), pairs, three-, and four-member groups. Afterwards, drawings were coded and sorted by 4 independent individuals with a general understanding of biology but not specifically in respiration. They scored the drawings, in comparison to the instructor's copy, from 1-7. The mean difference between post- and pre-discussion drawings were the students' learning score. There was no significant difference between students pre-discussion, but ANOVA statistical testing found that students in four-person groups have a significantly larger learning score than other groups, suggesting four-person groups are optimal for group learning. Additionally, analyses revealed an impact played by gender composition of the groups. While this was not part of the study hypothesis, the authors noted that this is an additional variable that

should be considered (Corrégé and Michinov, 2021). While this provided a convincing argument for larger groups, the setting being high school provides a different environment than a first-year university course, and so is not directly applicable.

The effect of group size on learning has been studied for many years. Alexopoulo *et al*. studied secondary school physics students who were asked to form their own groups, either as pairs or groups of four depending on the class section. Initially students answered six questions on their own, and then discussed in either their pairs or groups. These groups would record consensus answers, or if unable to agree, different possible answers with their reasoning. After 2-3 weeks, students were given the same six questions. The authors found that students in groups of four progressed more than students in pairs, comparing the post- and pre-test answers. Additionally, students in groups of four regressed less in their responses (Alexopoulou and Driver, 1996).

In 2010, Bertucci *et al.* divided seventh grade students who had never experienced cooperative work in school before into pairs, groups of four, or individuals. They answered questions in three units, covering alcohol, tobacco, and drug abuse. Students were placed into groups randomly, accounting for social factors such as gender, ability, and class. In the first unit, individuals and pairs performed better than groups of four. In unit two, groups of four did as well as individuals, both worse than pairs. After the third unit, groups of four were performing as well as pairs, both better than individuals. The authors did find that cooperative group work was more effective than individual study, however did not find a difference in achievement between groups of four and pairs.

There has been evidence of both pairs and larger groups being more productive for student learning, under different circumstances and with different measures. When measuring actual academic success using exam-style assessments or questions, four-person or larger groups tend to be more successful (Laughlin et al., 2006; Sugai et al., 2018; Corrégé and Michinov, 2021). On the other hand, measuring student perception of their learning, pairs or smaller sized groups tend to report as having a better experience even if there was no difference observed in academic outcome (Apedoe et al., 2012; Shaw, 2013; Melero et al., 2015; Amir et al., 2018). In my assessments, students were required to evaluate guide RNA data that was provided, recalling criteria taught earlier in the course to assess which provided guide was most effective. For the in-tutorial activities conducted in either groups or pairs, students performed the same evaluations, but

additionally designed the initial target location, to create the data table that they then evaluated.

Most published lesson plans rely on laboratory settings which restricts their use to particular courses and fields, whereas designs for lectures or tutorials are more universally applicable. This study aims to determine whether group or pair environments more effectively facilitate understanding of a practical research tool, within the time- and resource-constrained educational setting of a first-year general biology lecture or tutorial.

6.2. Methods

6.2.1. Course structure

The study was conducted in the Summer 2022 offering of BISC 101, an introductory biology course covering cellular biology and physiology. There were 117 undergraduate students enrolled, all of whom were at an introductory level regarding biology, which included some biology majors and non-majors. On a weekly basis the course consisted of a three-hour lecture, a two-hour laboratory class, and a one-hour tutorial for each student. Each tutorial had approximately 17-20 students. All instruction and work associated with CRISPR and this study took place in one lecture, one week of tutorials, and a midterm exam. Figure 26 shows the outline of the study, including where data was collected from assessments and where students engaged in group work.

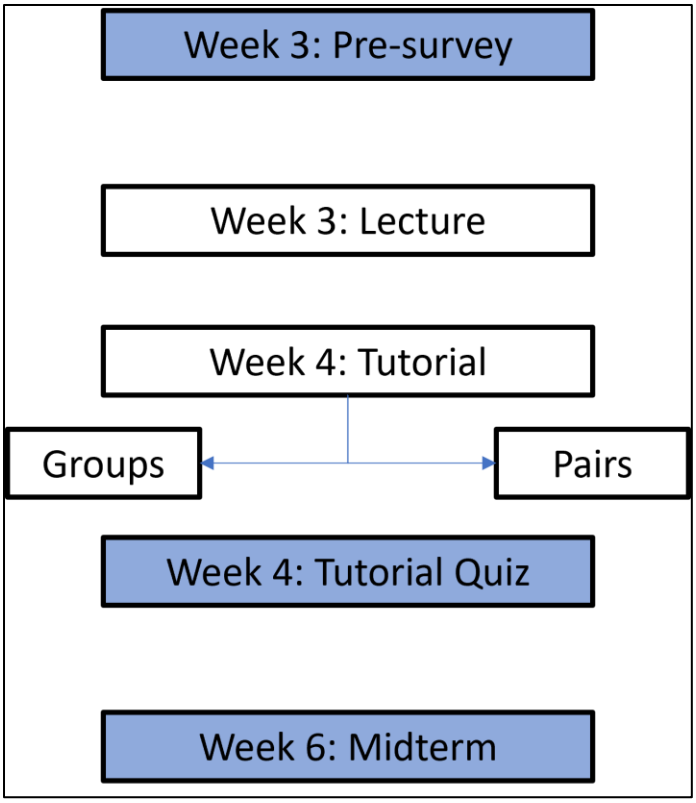


Figure 26: Flowchart outlining the study design. Data was collected at points represented by shaded boxes.

6.2.2. Study design

A 50 min introductory lecture was given to introduce CRISPR and how the different components of gene editing are designed. This lecture included an example of the assessments that would later be provided to the students. It was assumed that this lecture was the first time that most students had encountered these topics.

Each tutorial section was randomly assigned as a group or pair, and students within organised by their lab groups of four. If a tutorial was designated as “pair”, these groups were randomly divided in half. The 50 min tutorials were organised in the following fashion: 10 min preamble about the study, 30 min to perform the exercise as demonstrated in lecture within groups or pairs, and 10 min to individually complete an ungraded quiz. The group/pair activity consisted of being provided a gene to search for on the Ensembl database and identifying an appropriate target in the given sequence. Each group or pair was permitted the use of one electronic device to design potential guides for this sequence and submit these through an ungraded online survey on the course website. A screenshot of the directions provided are in Figure 27. Two tutorials were run by course teaching

assistants, as they were simultaneous with other tutorial sections. The teaching assistants were coached on the instructions they provided to minimize and difference between tutorials.

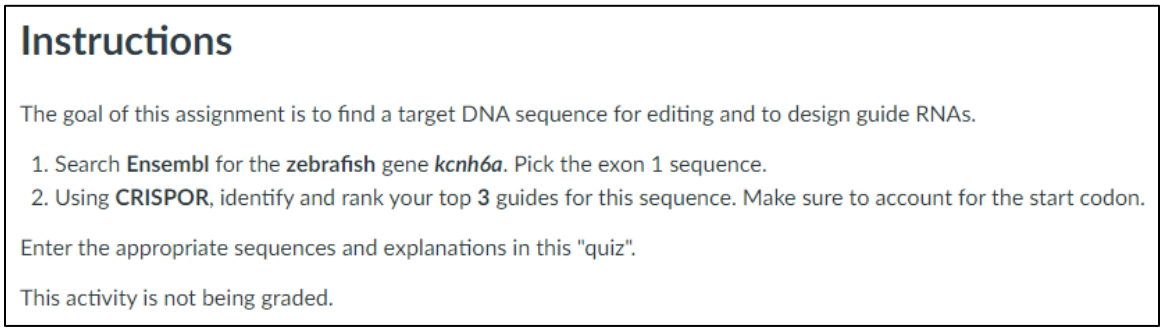


Figure 27: The instructions provided for students during the group/pair assignment in tutorials. Students searched for the specified gene in Ensembl, and using the given sequence designed 3 guides along with explanations for why these are the most appropriate. This was submitted electronically and not graded.

6.2.3. Data collection

To understand the landscape of student perspectives, and to assess student skills, this study used a mixed-methods approach (qualitative and quantitative). One survey and two assessments were completed by students at different points through the semester: a pre-survey provided at the beginning of the semester, a tutorial quiz at the end of the week 3 tutorial, and another assessment integrated into the midterm examination that occurred 2 weeks after the tutorial. This study was approved by the SFU Human Ethics Research Board, reference #: 30001043.

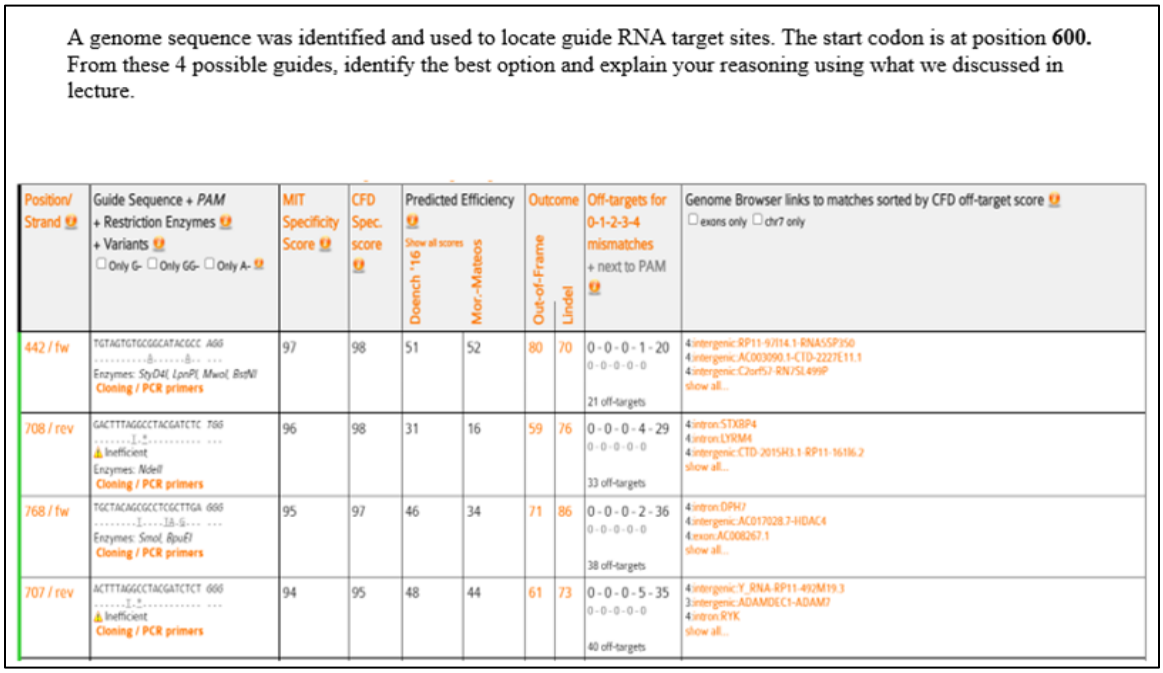
The pre-survey was intended to identify student preconceptions and/or understanding of genetic engineering and CRISPR. The survey was deployed via a class survey over Canvas. The questions asked were:

1. Without looking anything up, what does “genetic engineering” mean to you?

2. Without looking anything up, how familiar are you with the technique called “CRISPR”? (scale of 1 to 5 provided, 1 meaning they never heard of CRISPR and 5 that they are very familiar)

3. Explain your above answer, including your understanding of what CRISPR is used for and/or how it works.

The tutorial quiz took place at the end of the tutorial, after the 30 min group/pair exercise. Students were given 10 min to independently complete the assessment on paper and handed it in before leaving. The assessment consisted of a screenshot of a CRISPOR output showing a collection of 4 potential RNA guides with all related characteristics. Students selected which guide they believed would be most effective and justified their response (Figure 28).

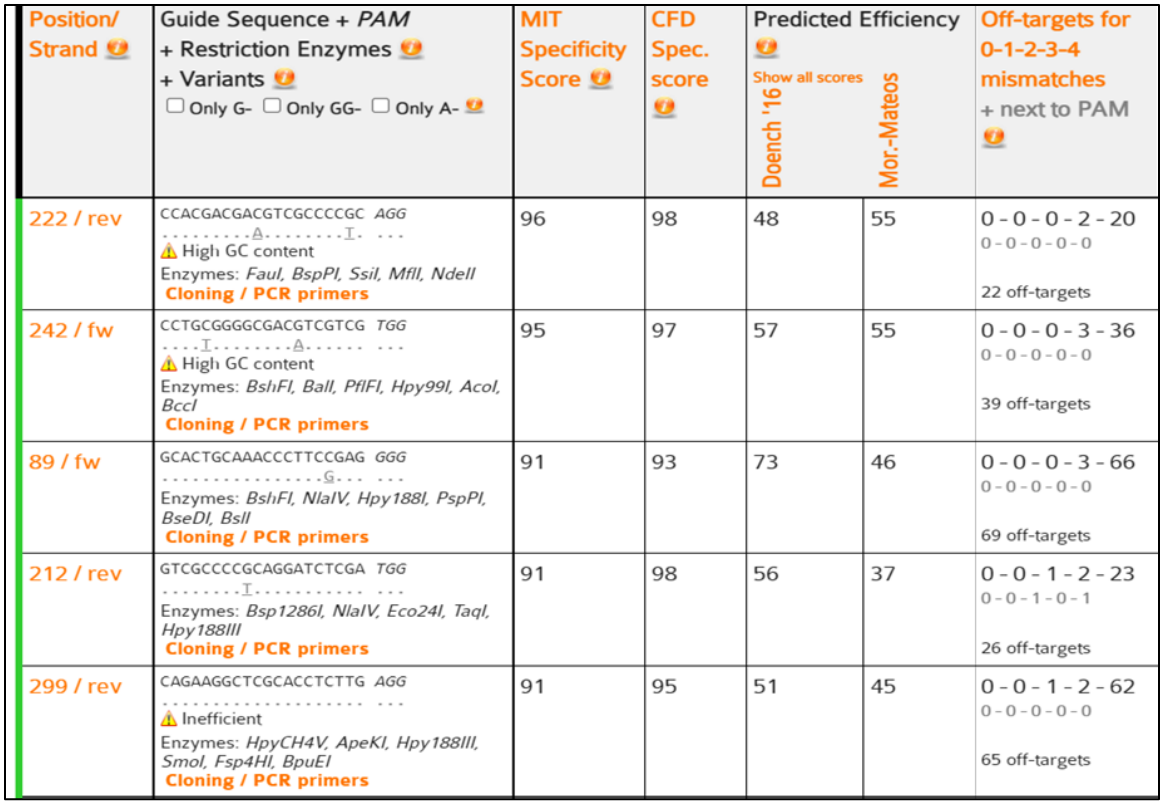


A genome sequence was identified and used to locate guide RNA target sites. The start codon is at position **600.** From these 4 possible guides, identify the best option and explain your reasoning using what we discussed in lecture.

Position/ Strand	Guide Sequence + *PAM* + Restriction Enzymes + Variants	MIT Specificity Score	CFD Spec. score	Predicted Efficiency (Doench '16, Show all scores)	Predicted Efficiency (Mor.-Mateos)	Outcome (Out-of-Frame)	Outcome (Lindel)	Off-targets for 0-1-2-3-4 mismatches + next to PAM	Genome Browser links to matches sorted by CFD off-target score (exons only, chr7 only)
442 / fw	TGTAGTGTGCGGCATACGCC AGG Enzymes: *StyD4I, LpnPI, MwoI, BstNI* Cloning / PCR primers	97	98	51	52	80	70	0 - 0 - 0 - 1 - 20 0 - 0 - 0 - 0 - 0 21 off-targets	4:intergenic:RP11-97I14.1-RNA5SP350 4:intergenic:AC003090.1-CTD-2227I11.1 4:intergenic:C2orf5J-RNJSL499P show all...
708 / rev	GACTTTAGGCCTACGATCTC TGG Inefficient Enzymes: *NdeII* Cloning / PCR primers	96	98	31	16	59	76	0 - 0 - 0 - 4 - 29 0 - 0 - 0 - 0 - 0 33 off-targets	4:intron:STXBP4 4:intron:LYRM4 4:intergenic:CTD-2015H3.1-RP11-1611I6.2 show all...
768 / fw	TGCTACAGCGCCTCGCTTGA GGG Enzymes: *SmoI, BpuEI* Cloning / PCR primers	95	97	46	34	71	86	0 - 0 - 0 - 2 - 36 0 - 0 - 0 - 0 - 0 38 off-targets	4:intron:DPH7 4:intergenic:AC017028.7-HDAC4 4:exon:AC008267.1 show all...
707 / rev	ACTTTAGGCCTACGATCTCT GGG Inefficient Cloning / PCR primers	94	95	48	44	61	73	0 - 0 - 0 - 5 - 35 0 - 0 - 0 - 0 - 0 40 off-targets	4:intergenic:Y_RNA-RP11-492M19.3 3:intergenic:ADAMDEC1-ADAM7 4:intron:RYK show all...

Figure 28: The assessment provided during the last 10 minutes of tutorial in week 3.
Given the potential guides and all the characteristics provided, students picked one best option and justified their choice.

The midterm question on CRISPR was equivalent to the tutorial quiz, asking the same question and providing the same types of information (Figure 29). Five RNA guide options were provided, and in this case the question was graded as a portion of the midterm examination.



Position/ Strand	Guide Sequence + *PAM* + Restriction Enzymes + Variants ☐ Only G- ☐ Only GG- ☐ Only A-	MIT Specificity Score	CFD Spec. score	Predicted Efficiency Show all scores Doench '16	Mor.-Mateos	Off-targets for 0-1-2-3-4 mismatches + next to PAM
222 / rev	CCACGACGACGTCGCCCCGC *AGG* .....A.....T. ... ⚠ High GC content Enzymes: *FauI, BspPI, SsiI, MflI, NdeII* **Cloning / PCR primers**	96	98	48	55	0 - 0 - 0 - 2 - 20 0 - 0 - 0 - 0 - 0 22 off-targets
242 / fw	CCTGCGGGGCGACGTCGTCG *TGG* ....T.....A..... ... ⚠ High GC content Enzymes: *BslFI, BalI, PflFI, Hpy99I, AcoI, BccI* **Cloning / PCR primers**	95	97	57	55	0 - 0 - 0 - 3 - 36 0 - 0 - 0 - 0 - 0 39 off-targets
89 / fw	GCACTGCAAACCCTTCCGAG *GGG* .....G... ... Enzymes: *BslFI, NlaIV, Hpy188I, PspPI, BseDI, BslI* **Cloning / PCR primers**	91	93	73	46	0 - 0 - 0 - 3 - 66 0 - 0 - 0 - 0 - 0 69 off-targets
212 / rev	GTCGCCCCGCAGGATCTCGA *TGG* .....T..... ... Enzymes: *Bsp1286I, NlaIV, Eco24I, TaqI, Hpy188III* **Cloning / PCR primers**	91	98	56	37	0 - 0 - 1 - 2 - 23 0 - 0 - 1 - 0 - 1 26 off-targets
299 / rev	CAGAAGGCTCGCACCTCTTG *AGG* .... ⚠ Inefficient Enzymes: *HpyCH4V, ApeKI, Hpy188III, SmoI, Fsp4HI, BpuEI* **Cloning / PCR primers**	91	95	51	45	0 - 0 - 1 - 2 - 62 0 - 0 - 0 - 0 - 0 65 off-targets

Figure 29: An equivalent assessment provided in the first midterm of the course.
Students had to answer this in a similar fashion to the tutorial assessment, though this was graded as part of their exam.

6.2.4. Qualitative analysis: Coding of student self-reports

The pre-survey questions were intended to provide a descriptive view of student knowledge about CRISPR and genetic engineering overall. To analyze student responses, inductive coding was used to generate categories for student responses (Chandra and Shang, 2019). In this approach, two coders iteratively examined the raw data, defined categories, and discussed to come to consensus on the emergent codes/categories of student responses. Once the codes were formalized with high consensus using the Cohen's kappa statistic (described below), each of the student responses were definitively coded for the presence of each category. All assessments were de-identified by the course instructor prior to analysis. The two coders were myself and the course instructor.

Cohen's kappa statistic is used to test for consistency between two coders (McHugh, 2012). This statistic compares the agreement between coders to the probability

of chance agreement, accounting for the fact that two individuals may give the same code by chance.

For the pre-survey questions, the questions were divided into two groups: question 1, and question 2 + 3. The first question was coded for suggesting a goal to genetic engineering, use of examples or applications, action verbs, a molecular level of detail, and whether they used “genetic” or “gene” as this may indicate repetition of the question. Questions 2 and 3 were coded for blank responses, molecular scale explanations, analogies for CRISPR, and mention of applications. These responses for question 3 were associated with the student’s self-reported level of understanding from question 2. See the codebook in Table 4 below.

Table 4: Codebook providing codes and explanations for qualitative analysis of the pre-survey.

Question	Code	Explanation	Keywords
Question 1: Genetic Engineering	Has a goal	Indicates an intent behind the engineering	Goal, intent
	Uses examples or applications	Provide an example of a genetic engineering system or something this process can be used for.	CRISPR, GMO, disease
	Action verb	Description of an action happening.	Cutting, modify, alter
	Molecular detail	Description of processes on a molecular scale.	DNA, sequence
	Includes "gene" or "genetic"	Potentially giving a molecular scale but just repeating what's in the question.	Gene, genetic
Question 3: CRISPR	Blank or "don't know"	Left blank, or explicitly write they don't know without attempting an answer.	Don't know, don't understand
	Molecular detail	Description of what CRISPR is actually doing in the cell.	Cuts DNA, splice
	Use of analogy	Using any descriptor outside of genetics terminology to represent the process.	Scissors, photo editor
	Application of CRISPR	Providing an example of what CRISPR can be used for.	Disease, GMO, crops

The use of categorization or thematic analysis supports the interpretation of very rich qualitative data, and gives meaningful high-level views of student understanding. For question 1, the proportion of responses that followed each category and provided example responses was calculated. For questions 2 and 3, a stacked bar chart to compare the prevalence of each coding label between self-reported levels of understanding was constructed.

6.2.5. Quantitative analysis: Student performance on a guide design task

The tutorial quiz and midterm questions were evaluated based on the number of arguments in each student response, and categorised by types of evidence that students used in their justifications. These categories included: number of off-targets accounting for mismatches, number of mismatches near the PAM site, warnings provided by the CRISPOR software, efficiency score, specificity score, number of off-targets, and orientation with respect to the start codon. For each category, students were scored a 0 for no response or incorrect use of the argument, and 1 for correct use. This scoring scheme was used for both tutorial and midterm questions.

For analysis, two criteria were chosen for comparison between pairs and groups: specificity score and number of off-targets. These were chosen due to both their fundamental importance in guide design and a noticeable change in their use between the post-tutorial assessment and the midterm. Responses were divided by group and pair, and mean values from the coding were calculated for each of the relevant categories in each group size. Comparisons between groups and pairs were made via Student's two-tailed t-test.

6.3. Results

6.3.1. Pre-survey

Three questions were provided as described in Chapter 6.2.3.: One multiple-choice question asking students to rate their understanding of CRISPR, and two open-response questions asking them to describe what they know about genetic engineering and CRISPR. The responses are summarised in Table 5. The Cohen's kappa statistic was

used to measure level of agreement between the two coders, with the value shown in Table 5. All categories had a kappa value of at least 0.72, which suggests at minimum "substantial agreement" between coders (McHugh, 2012).

Table 5: Student responses to the first question from the pre-survey.

Code	Percentage of responses	Example responses	Cohen's kappa score
Has a goal	41.2%	"Genetic engineering is learning how to change genes and codes in order to change the physical appearance or genetics of an individual for the better."	0.748
Uses examples or applications	22.1%	"My first thought is food and GMOs. Changing genetics to get bigger things like tomatoes or chickens."	0.778
Uses action verbs	81.6%	"Modifying DNA"	0.728
Molecular detail	29.8%	"Having control of the DNA sequence of an organism and being able to edit its DNA sequence in order to produce different results."	0.763
Use "genetics" or "gene"	69.3%	"The editing of genetic information in order to produce a certain result."	0.914

With regards to the first question about genetic engineering, more than 80% of students used an action-oriented verb in their response, some with a molecular focus. It was noted that approximately 69% of students referenced "genetics" or "genes", which could be a reference to the question rather than an understanding of the involvement of

DNA. While students did not tend to use examples to describe genetic engineering (just over 22%), they seemed to understand that it is an active process, based on more than 80% of responses using active language.

For the second and third questions regarding CRISPR, Figure 30 shows a stacked bar graph relating student explanations of CRISPR to their self-reported level of understanding. Unsurprisingly, students reporting an understanding level of 1 (low/none) primarily left their explanation blank or specified they did not know. This proportion decreased as reported level of understanding increased, with zero blank responses at levels of 4 or 5. As level of understanding increased, more students integrated mechanistic explanations into their response, as well as inclusion of an application of CRISPR. An interesting trend was the increase in molecular detail and application references as understanding increased, while analogies only appeared at understanding levels of 2 and 3. This could be due in part to the low number of students identifying with an understanding of 4 or 5, but additional analogy could be used as a way to explain a concept to students with limited prior knowledge. At understanding levels of 4 or 5, students may feel confident enough to give a more detailed account of CRISPR mechanisms, rather than using an analogy. An example of a "5" response is: "*CRISPR, also known as Cas9, is used to alter genetic information. A small enzyme (I believe) is injected into the cell which then cuts a certain part of the DNA (a harmful genetic disease) thus shutting it down and assuring that it does not continue to be replicated*." This response demonstrates an understanding of not only the molecular mechanism underlying CRISPR, but how it can be used. An example of a "3" response is: "*I am aware of the gene editing technology known as CRISPR, which can cut segments of DNA like molecular scissors thereby changing sequences of DNA*." Compared to the level 5 response, this answer uses the analogy of molecular scissors to describe the molecular mechanism without directly stating this.

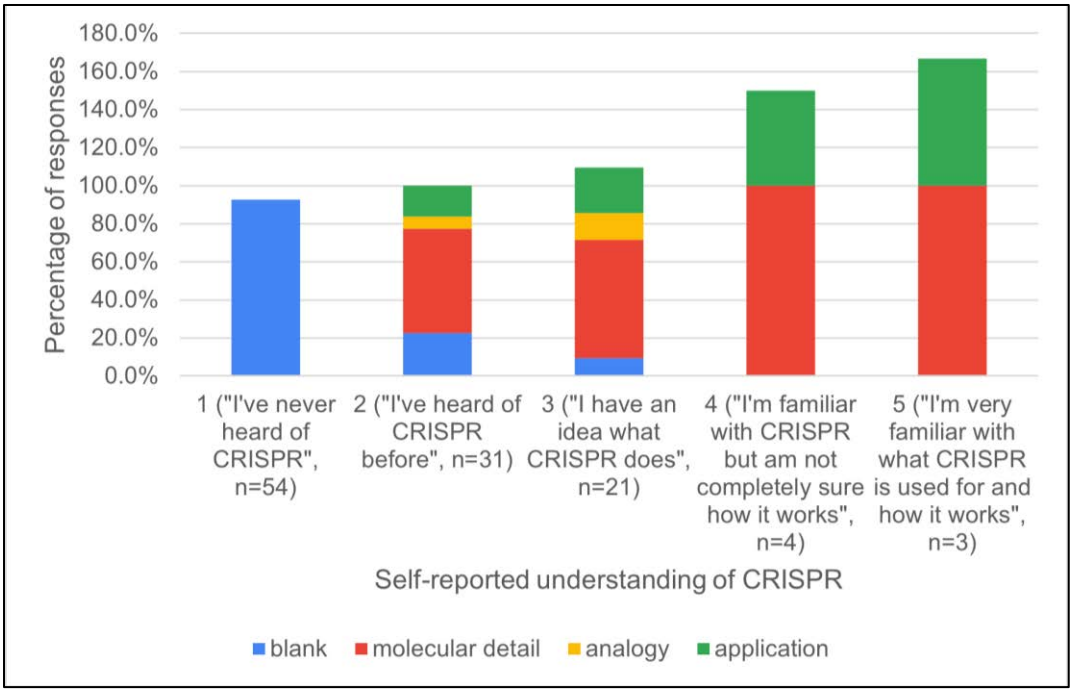


Figure 30: A stacked bar chart showing prevalence of each coding label between self-reported levels of understanding.
The percentage of responses add to more than 100% because the different codes are not mutually exclusive.

6.3.2. Student performance on Tutorial Quiz and Midterm Question

As shown in Figure 31, students scored highly in evaluation criteria regardless of whether they were working in a group or a pair. This indicates that the tutorial instruction was effective at showing students how to properly evaluate CRISPR guide RNA characteristics. The midterm examination that occurred two weeks after the tutorial instruction showed a decrease in retention, in particular in relation to the use of term ‘specificity’, however scores were still high with correct usage of ‘off-target' (>70%) for both pairs and groups.

In the tutorial quiz, the responses from students in pairs were not significantly different from those of students in groups with regards to correctly using ‘specificity’ and ‘off-targets’ to choose guide RNA (Figure 31). In total, 37 out of 50 students who studied in groups used ‘specificity’ correctly, compared with 34 out of 39 students in pairs. For the use of ‘off-targets’, this term was used correctly by 43 out of 50 students in groups, and 30 out of 39 students in pairs. A Student’s t-test evaluating responses from groups versus pairs revealed a value of 0.127 for ‘specificity’, and 0.274 for ‘off-targets’, showing that neither response rate was significantly different.

For the midterm question, students who had worked in pairs and groups in the tutorial were not significantly different in terms of correctly using ‘specificity’ and ‘off-targets’ (Figure 31). In total, 22 out of 50 students who had been in groups and 20 out of 39 students who had been in pairs used the ‘specificity’ characteristic correctly. For ‘off-targets’, 39 out of 50 group students and 30 out of 39 pairs students used the characteristic correctly. The Student’s t-test values were 0.5 and 0.905 respectively, neither of which were significant.

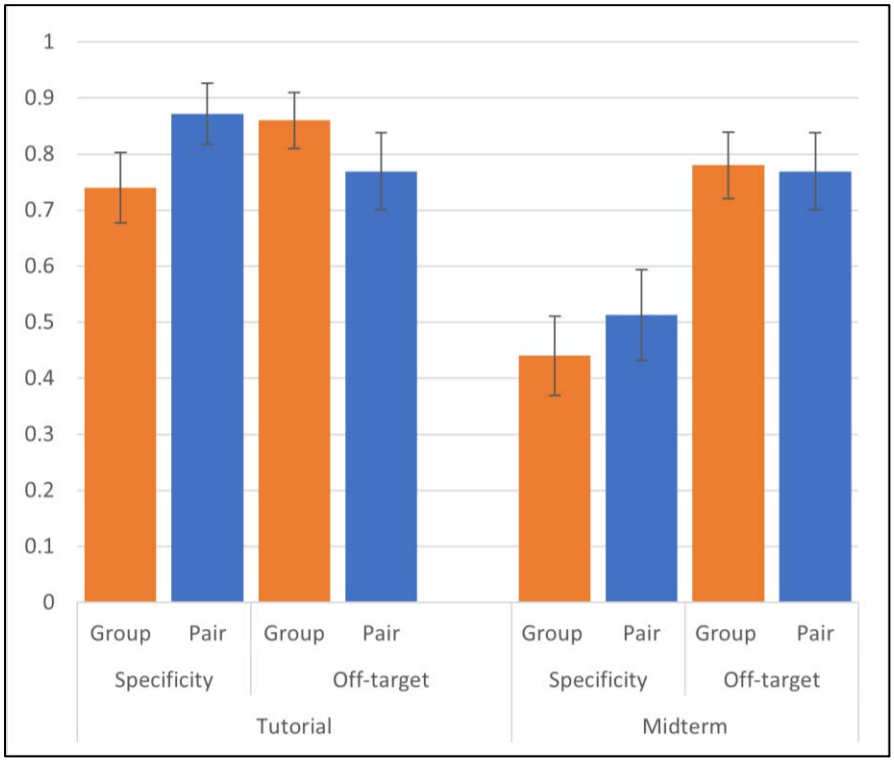


Figure 31: Proportion of correct usage of specificity and off-target number by both groups and pairs, in both the tutorial and midterm assessments.
Using a Student’s t-Test there were no significant differences between groups and pairs in any of the categories.

6.4. Discussion

This research sought to find an effective method for teaching a new research-relevant topic in a first-year undergraduate course. In this goal I was successful: first-year students were able to correctly use important criteria to identify appropriate guides for CRISPR use, based on a short tutorial exercise. The approach was to study the effects of group size on understanding, due to scattered conclusions in the literature. Previous studies used a combination of different study environments and levels of cognitive

understanding in their assessments. My assessments encompassed evaluation, analysis, and recall, providing a range of cognitive tasks rather than focussing primarily on one level as some other reported assessments have. Additionally, demonstrating the use of these assessments and testing the effect of group size in a lecture or tutorial environment rather than relying on laboratory access provides a more applicable context for fields outside of the sciences to examine the use of different group sizes.

Based on the qualitative data collected in the pre-survey, students varied greatly in their prior understanding of genetic engineering and CRISPR. More than 50% of students had heard of CRISPR previously, to the point where they could attempt an explanation of the concept, which was not expected. This variety of prior knowledge is something instructors will need to account for, perhaps with a survey such as this near the beginning of the semester. A number of students had heard about CRISPR in media or from previous teachers, and this informs both instructors and researchers about what preconceptions may exist and how lessons can be structured to account for these. Students appeared to find the more nebulous topic of “genetic engineering” more difficult to describe than CRISPR, with fewer responses describing the molecular context in which genetic engineering acts. This could be due to the prevalence of references to specific tools, such as CRISPR, in the media. Additionally, the use of analogy for students who self-reported a lower level of understanding of CRISPR suggests that this could be an effective method for introducing new topics to students. Our findings suggest that when students don’t have a strong understanding of a topic, analogies may be a more effective way to solidify the concept than purely mechanistic explanations.

Considering student performance, our main variable under study was the group setup: groups versus pairs. Notably, this variable did not appear to influence student learning from the perspective of student performance on the tutorial quiz and midterm examination. There was no significant difference on student performance in correct usage of guide criteria between those in groups and those in pairs, but there were trends that are interesting to consider for follow-up questions. For the term ‘specificity’, those in pairs seemed to do better than those in groups, and this was maintained between both the tutorial and midterm examination questions. On the other hand, those in groups tended to stronger performance on their use of the term ‘off-targets’ than those in pairs, which was also maintained in the midterm examination (though evened out slightly). One possible explanation for these differences is that with more individuals thinking about the problem

and contributing to the solution, less obvious answers are arrived at. The specificity is a single value that students can compare, while off-targets are not as immediately visually obvious when determining guide suitability. Therefore, with more individuals this may be arrived at as an explanation more often.

Additionally, while there was no significant difference between tutorial and midterm questions, there were also patterns. While off-target usage remained consistent, correct use of specificity did experience a drop. In their studying, it is possible that students observed the emphasis placed on the number of off-targets in various study materials, and this may have reinforced their understanding. Comparing the tutorial and midterm questions is complicated however, because we did not directly compare students to themselves, and the variability in academic performance across the student population is not accounted for. This is an important factor as there may have been non-random initial differences in student skills in either the groups or pairs. To account for this there would require correlation with the non-CRISPR components of their midterm.

Several follow-up questions emerge from this work. This study relies on a single course offering and should be expanded to more sections for further comparison. This may reinforce the results from this research, which suggests that learning in groups may not differ from learning in pairs. This is useful for teaching assistants and lecturers as it provides guidance for the optimal learning environment for their students in either lectures or tutorials. Group/pair composition is also important, since both demographics and student capability can inform effective group formation (though care needs to be taken here to engage in an ethical and equitable manner) (Corrégé and Michinov, 2021). More work comparing group size when learning new topics, but with more accounting for these other variables would be valuable. Additionally, while I performed this research within the context of teaching CRISPR, this could and should be done in multiple fields. This study used CRISPR to frame problems where students had to evaluate criteria to make a judgement (in this case, on a guide sequence). As a high-level cognitive task, evaluation-type problems are prevalent outside of molecular lab techniques, and therefore this kind of study has broader relevance. Both the science and social sciences contain research techniques that students early in their education would benefit from learning, and being able to find ways to set these students up for their future should be one of our top priorities.

As discussed earlier, a motivation for this research is to provide undergraduate students the opportunity to learn background skills that are important for entry into research in the life sciences. While the methods of the group vs pair study did not show any significant advantage of one over the other, students did demonstrate their ability to evaluate CRISPR guide design in a way they would have to in a laboratory. From a big picture perspective this demonstrates that this kind of work is viable in first-year courses and can provide the knowledge needed to better facilitate student involvement in research.

6.5. Post-study reflection

This study was the first time I have done qualitative research, as well as experimenting in the field of education and pedagogy. As an initial experience, I found it both enlightening and motivating. Teaching being one of my main passions, having the opportunity to apply my experience as a researcher to improving the learning environment for my students was very fulfilling, and taught me a lot about both teaching styles as well as experimental design in general.

In my other thesis research I have had to complete and consider animal care ethics with regards to working with zebrafish, but filling a human research ethics application forced me to consider my experimental design in ways I have not previously. Considering survey and question design accounting for privacy and identity, as opposed to fish where this idea does not exist, was something I had to pay particular attention to.

While I have always abided by active learning as a core principle of my teaching practices, this study showed me there are many ways I can, even subtly, manipulate these conditions to enhance learning outcomes for my students. Not only group size but accessibility to technology, group composition, how groups are formed, these are all variables that are relatively simple for an instructor or teaching assistant to manipulate that they may not always consider, but could have a significant impact on learning. While this provides many more options for how I can structure assessments and class environments, it also demonstrates a need for instructors to account for these variables, as it is our responsibility to provide students the greatest opportunity to succeed. It is also satisfying to be able to use my scientific and analysis skills in a systematic approach to assessing student learning, whether for course teaching or for rigorous pedagogical research.

Finally, with regards to how I interpret experiments, this study changed part of my outlook. In a lot of my work, an experiment not showing a significant outcome is defeating, and this is perpetuated by how journals select publications. But framing the outcome of this study in terms of its implications for teaching has a grounding effect. Given the applied nature of the work, a negative result is not necessarily emotionally negative: perhaps instructors and teaching assistants do not have to be concerned as much with group size when organising their tutorial activities and can focus on other aspects of their instruction. This perspective, rather than “this study did not work,” improved my interpretation of other “negative results” in studies, both my own and others.

Chapter 7. Discussion

7.1. Zebrafish use in cardiac research

Finding a relevant animal model to study LQTS is challenging; it must recapitulate relevant electrophysiological phenotypes to humans, be relatively simple to maintain, and work with (compared to other commonly used mammalian models) and have acceptance from the wider scientific community.

Zebrafish may not seem like an obvious choice when studying human cardiac disease, but due to a number of advantageous factors, are a powerful model. They have been used to study the cardiac, neurological, and vascular systems, as well as a model for toxicological screening, metabolic disease, and regeneration. Other models exist and are frequently cited as “stronger” or “better” than zebrafish, but the data does not necessarily support this. As demonstrated in Chapter 1.1, there are a plethora of studies using zebrafish, for a wide variety of applications.

Chapter 3 further demonstrates their particular use as a cardiac model, showing that while, structurally, there are significant differences from a mammalian heart, electrically, they are a much more relevant model for human cardiac function and disease than many small mammal models. Even though the heart has only two chambers, zebrafish have a homologous structure for both the SA and A-V nodes, at the junction between sinus venosus and atrium, and atrium and ventricle, respectively. The ventricular action potential is far more similar in morphology to a human ventricular action potential than that of a mouse, a model organism that some will claim to be more relevant or significant, with a similar duration and same phase 2 plateau. Aside from the shape of the action potential, similar channels are involved at different phases, the major difference being the more significant presence of T-type calcium channels in phase 1. Adding on the advantages of larval zebrafish in terms of size, translucence, and non-reliance on a functional heart for the first 10 days makes this a desirable model for study.

When it comes specifically to LQTS, there are even more benefits. The I_{kr} current being the primary driving force behind phase 3 repolarisation allows one to isolate and more readily understand how genetic variants affect this component of the action potential. There is some evidence of an I_{Ks} current but the repolarizing force is primarily I_{Kr}, more-so

relatively speaking than in humans. Combined with available genetic tools, this is a powerful system for studying heritable cardiac electrical disorders such as LQTS.

While powerful tools, zebrafish, as with every animal model, possess traits that hinder the translatability of findings to the human. I_{Kr} being nearly the sole current underlying phase 3 repolarisation does mean that any pharmaceuticals or variants may have an exaggerated effect. While this limits applicability to humans, it does allow for thorough interrogation of these particular phenotypes. Additionally, the small size of the heart may limit propagation of arrhythmia wavefronts, hindering ability to observe these phenomena. The thin cardiac walls also reduce the dispersion of repolarization, which may hinder development of arrhythmias in the same way that occurs in mammalian hearts. This does potentially limit the capacity of zebrafish as an electrical cardiac model. However EADs, as a precursor to arrhythmias, can still be observed and be indicative of zERG dysfunction.

In this thesis I describe a platform for characterizing cardiac variants in zebrafish, following genetic manipulation. Due to the larval fish not requiring a functioning heart for the first 10 days, otherwise lethal variants can be examined in a variety of ways. Direct video and images of the heart allow for interrogation of the variant's effects, in a relatively high throughput manner. Via a fairly simple process of recording video, without requiring any electrophysiological experimentation, characteristics such as heart rate, electrical disconnect between chambers, and structural changes can be directly observed. The non-lethal nature of the embryonic experiments I designed also allow for a wealth of data to be collected, as each phenotyping protocol can be run after the other.

7.2. Zebrafish and CRISPR

7.2.1. Genomic data and human genome homology

One significant aspect of zebrafish models is their genetic toolkit. This includes not only the breadth of genetic engineering tools, but the wealth of information already existing. The zebrafish genome is sequenced and well annotated, with the recent genome assembly (GRCz11) released in 2017 containing over 25,000 coding genes and almost 60,000 transcripts. These can be easily parsed using databases such as the Genome Reference Consortium (GRC, NCBI, and Ensembl). Additionally, there are zebrafish-

specific resource centres such as ZIRC and the closely related ZFIN network. These provide not only key information about zebrafish genes and transcripts, but also the ability to obtain particular fish lines for ease of starting research projects.

As stated in Chapter 1.1.1., there is a strong homology between the human and zebrafish genome. Almost 82% of morbid genes in the human genome have orthologues in zebrafish, including the gene primarily involved in LQTS2. This supports the methods developed in this thesis not only being useful for LQTS research, but many other heritable disorders or traits.

The genome duplication events that ancestral teleost fish have undergone can provide potential problems when using zebrafish as a genetic model. Due to the paralogs resulting from genome duplication, there are potential other transcripts that could compensate for loss of function in *zkcnh6a* or become targeted themselves by my CRISPR approach.

7.2.2. Genetic engineering tools

Genetic engineering is a rapidly developing field, with techniques being designed and updated regularly. From morpholino knockdown, to ZFNs and TALENs, to CRISPR, these techniques have all been developed and used extensively in zebrafish as described in Chapter 1.2. This is another reason zebrafish are such an effective model for any genetic modelling, the prevalence of available tools alongside ease of fish care and manipulation of oocytes.

Ranging from forward to reverse genetics, many advancements have been made and utilized in zebrafish. As discussed in Chapter 1.2.1., Ichino *et al.* used protein trapping in zebrafish to identify approximately 30 genes potentially associated with Sick Sinus Syndrome, and this lead to detecting multiple genes linked to arrhythmogenicity and one with muscular dystrophy (Ichino et al., 2020). This is also an example of a more modern approach to forward genetics, not performed as much, being used in zebrafish to identify more potentially morbidity-related genes.

When it comes to reverse genetics, there have been many approaches. The one addressed in this thesis is CRISPR, but morpholinos, ZFNs, and TALENs have all been used heavily in zebrafish for important discoveries. But the advent of CRISPR, providing

a simpler design and preparation than ZFNs and TALENs, as well as providing more utility than morpholinos, is one of the most important genetic advancements in recent memory.

7.2.3. CRISPR approaches in zebrafish

As described in Chapter 1, CRISPR has been demonstrated as an effective tool for knockouts, point mutations, and gene insertions for zebrafish. While there are constantly advancements being made in CRISPR technology (see Chapter 7.4.1 for discussion on some of these), the ability to insert large genes is inefficient. While base editors and prime editors can perform smaller scale genetic changes efficiently, inserting a large reporter such as the YFP in my template is beyond the ability of these techniques. The genetic engineering platform I have developed is capable of these modifications, at efficiencies of around 10%. This approach of excising an entire exon and replacing with a modified version allows for more customization, and freedom to add reporters, excision sites, and other genetic tools.

This CRISPR approach does have limitations, as explained in Chapter 5. Editing in the embryonic stage allows for compensatory effects to potentially confound characterization of variants of interest. Inducible CRISPR approaches should solve this problem, however there are complications with introduction of guides and templates into the fish. If editing is to happen at adult stages, there is no reliable method for introducing the remaining CRISPR components into particular organs or regions. A potential solution for this is addressed in the Future Directions section, Chapter 7.4.

The combination of an inducible system, with my gene excision approach, has the potential for a high efficiency platform for generating variants of interest. Combined with the phenotyping pipeline I have developed for cardiac electrophysiological studies, moving forward there is the possibility for development of different phenotyping platforms combined with a zebrafish model with endogenous editing capabilities.

7.3. Importance of education

In this thesis I give a lot of consideration to mechanisms and pathology of LQTS, and how zebrafish are a powerful genetic and physiological tool to study this disorder. However, something that is commonly ignored in STEM theses is how to engage new

trainees to continue this type of research. A significant aspect of this is education at the undergraduate level, and finding ways to connect students with what is currently relevant in research.

Undergraduate students are often deprived of learning experiences that are relevant to their involvement in research. Lower division courses tend to focus on introductory level material and laboratory courses that use current techniques are not available until upper division, if at all. Students are encouraged to become involved in research as early as they can, but often lack experience and face the possibility of not being selected into a research group. Introducing what is considered more “complicated” material earlier in the undergraduate education means providing students the resources they need to excel in a laboratory environment. This is especially important when looking at techniques such as those explored in this thesis.

CRISPR, as discussed above, is a rapidly changing and developing toolset that can be difficult to keep pace with. For a new researcher, or individual who wishes to become more involved in research, this can be intimidating. Providing students with the fundamentals to understand the process of CRISPR, or design of its components, enables trainees to better assimilate the ever-increasing volume of published research that is often required to either join or contribute to a research group.

My intent in including Chapter 6 is to demonstrate that, as we are conducting this research, whether it be clinical research, genetic tool development, or otherwise, it is important to not lose sight of how this research will be continued in the future. This is achieved by promoting undergraduate student involvement in research at early stages of their education by reaching out to facilitate student involvement, but also providing the necessary background education.

7.4. Future directions

This thesis research provides a powerful methodology for generating any gene variants of interest, with an efficient system for identification of positive mutants for further sequencing. This system can be used in any system that zebrafish are a relevant model for, which is a broad range. Depending on the application, there is also potential for

integration of this mutation setup into a high throughput characterisation/phenotyping pipeline, allowing for functional characterization of variants of interest.

7.4.1. Inducible CRISPR

A potential issue with my system, as described earlier, is the temporal and spatial variability of CRISPR edits. My solution was to develop an inducible CRISPR for the zebrafish, using pMag and nMag proteins to cause the Cas9 to activate only in the presence of blue-light induction. The system as presented in Chapter 5 serves as proof-of-concept that with future development will be enable inducible editing directly in adults and in just the target organ. I envision the inducible Cas9-magnet fusion integrated into the zebrafish genome, in a safe harbour site. This would allow for expression of the Cas9 fusion throughout the life cycle of the zebrafish, rather than just being present upon injection at embryonic stages before being degraded. Therefore, the CRISPR activity could be induced in adults, bypassing potential compensatory effects in the larvae, as well as specifically in the heart. With the genome duplication effects, there is always the risk that genes are not only expressed elsewhere in the body but that paralogs could be similar enough that they would also be targeted by the guide RNAs. Without spatial specificity, there is potential for *zkcnh6a* to be mutated in multiple organs. If a blue light can be targeted to the heart, this risk is mitigated. Additionally, being able to trigger mutations in adult fish carries multiple benefits. Primary among these is preventing compensatory effects due to variable gene expression; there is evidence that larval zebrafish can modify gene expression to account for deleterious genetic effects. As of now it is possible that the variants I am generating in embryonic zebrafish are causing confounding factors via compensatory gene expression. This is something difficult to account for without doing whole genome sequencing and comparing all known related genes with their wildtype sequence; however, this is not a concern if the variant is generated in adult fish. The other issue, which is particularly relevant for the variant I was studying, is embryonic lethality. The R56Q mutation, along with many other hERG mutations, are homozygous lethal. While in zebrafish there are closely related variants that are viable as homozygotes, this did not seem to be the case for the fish I studied. Edited fish did survive for the first ten days, however as soon as the heart is required for distribution of nutrients and oxygen these fish expired. This allows for study within the first ten days, but this is not necessarily as relevant to human translation and does not account for compensatory effects. However,

if the variants are induced in adult zebrafish, this would allow for rapid characterization with techniques that are lethal regardless. Given enough time for these zERG variants to be expressed, the fish can be euthanized, and their hearts studied through ECG, sharp electrode recordings, or optical mapping techniques.

There are complications regarding introduction of guide RNA and templates into adult zebrafish: either these have to be present in the genome alongside the Cas9-magnet fusions, or they must be introduced into cardiac cells in the adult fish. A potential solution to this is to use either base-editing or prime-editing techniques. These utilize nickase Cas enzymes fused to either deaminases or a reverse transcriptase, respectively. These Cas enzymes function without the need for an HDR template, by either modifying a single base via deamination (base editing) or using the RNA guide as a template with reverse transcription (prime editing). If the magnet fusions are constructed using these base or prime editors, there is no need to add a large HDR template: the one I utilized is a plasmid of almost 7,000 base pairs. This technique won't utilize the YFP in the same way that my CRISPR technique does, however both base- and prime-editing result in higher efficiencies than standard CRISPR approaches (Komor et al., 2016; Anzalone et al., 2019). Integration of guides are still a concern, since these must be introduced to the fish as an adult, or somehow integrated into the genome and transcribed without mRNA processing. Additionally, the split location to create magnet fusions for base editors and prime editors would have to be experimentally determined, because the already fused enzyme domains for deaminase and reverse transcriptase would likely differ sterically.

7.4.2. Future of zebrafish in cardiac studies

With an inducible CRISPR system, there are very few limitations to what zebrafish are capable of as a model system. While cardiac research and disease modelling continue to develop in stem cell models, these approaches do not account for interactions within a whole organ or organism. Arrhythmias are tissue-level events, that propagate differently through different chambers and nodes of the heart. While the zebrafish cardiac structure isn't identical to that of a human, it does contain an analog for the SA and A-V nodes, alongside an atrium and ventricle. iPSC-CMs are not at a point where they can be studied as mature cell types, and possess more homogeneity as opposed to the more heterogeneous zebrafish. While a human iPSC-CM is more translatable on an individual cell level, a whole zebrafish heart allows for organ-wide imaging of arrhythmia

propagation. While stem cell studies are moving into organoid development and eventually printing of whole organs, this is still some distance away and does not account for system-wide effects. Systems such as the Comprehensive in vitro Proarrhythmia Assay (CiPA) advise the use of a complex animal model, alongside heterologous expression systems, iPSC-CM platforms, and *in silico* models. Zebrafish may prove a powerful model to consider to include in this paradigm.

The implications for the studies presented in this thesis do not need to be limited to the cardiac system. As demonstrated in Chapter 1, many other physiological systems and fields of research can take advantage of the zebrafish model. With a robust platform for generating variants-of-interest, this system could be a standard for generation of animal genetic models for a number of fields of study.

7.4.3. Pedagogical approaches to teaching CRISPR

Alongside the advances in CRISPR use described in this thesis, it is imperative that we pair this with education of undergraduate students, as they will be the future researchers continuing our work. In this thesis I described a study analysing the effect of group size on learning CRISPR, and while this did not demonstrate a significant difference in assessments on choosing CRISPR guides, it provided me experience with qualitative and pedagogical research experience and ideas for how this could continue. Further studies should account for group composition, as mentioned in Chapter 6 looking at demographics within groups as well as trying to limit self-selection in groups. I believe this can be studied in a variety of research fields, including outside of the sciences. In order to determine how teaching of topics such as CRISPR in lower division courses affects involvement in research, I would also do longitudinal follow-up studies with students to determine how the early introduction to research techniques potentially affects involvement in research.

References

Abramochkin, D. V., Hassinen, M., and Vornanen, M. (2018). Transcripts of Kv7.1 and MinK channels and slow delayed rectifier K+ current (IKs) are expressed in zebrafish (Danio rerio) heart. *Pflugers Arch - Eur J Physiol* 470, 1753–1764. doi: 10.1007/s00424-018-2193-1

Adebisi, Y. A. (2022). Undergraduate students' involvement in research: Values, benefits, barriers and recommendations. *Ann Med Surg (Lond)* 81, 104384. doi: 10.1016/j.amsu.2022.104384

Aguirre, A., Montserrat, N., Zachiggna, S., Nivet, E., Hishida, T., Krause, M. N., et al. (2014). In vivo activation of a conserved microRNA program induces robust mammalian heart regeneration. *Cell Stem Cell* 15, 589–604. doi: 10.1016/j.stem.2014.10.003

Albadri, S., Del Bene, F., and Revenu, C. (2017). Genome editing using CRISPR/Cas9-based knock-in approaches in zebrafish. *Methods* 121–122, 77–85. doi: 10.1016/j.ymeth.2017.03.005

Alday, A. (2014). Ionic channels underlying the ventricular action potential in zebrafish embryo. *Pharmacological Research*, 6.

Alders, M., Bikker, H., and Christiaans, I. (1993). "Long QT Syndrome," in *GeneReviews®*, eds. M. P. Adam, H. H. Ardinger, R. A. Pagon, S. E. Wallace, L. J. Bean, K. Stephens, et al. (Seattle (WA): University of Washington, Seattle). Available at: http://www.ncbi.nlm.nih.gov/books/NBK1129/ (Accessed October 23, 2020).

Alexopoulou, E., and Driver, R. (1996). Small-group discussion in physics: Peer interaction modes in pairs and fours. *Journal of Research in Science Teaching* 33, 1099–1114. doi: 10.1002/(SICI)1098-2736(199612)33:10<1099::AID-TEA4>3.0.CO;2-N

Al-Moubarak, E., Zhang, Y., Dempsey, C. E., Zhang, H., Harmer, S. C., and Hancox, J. C. (2020). Serine mutation of a conserved threonine in the hERG K+ channel S6-pore region leads to loss-of-function through trafficking impairment. *Biochem Biophys Res Commun* 526, 1085–1091. doi: 10.1016/j.bbrc.2020.04.003

Amir, O., Amir, D., Shahar, Y., Hart, Y., and Gal, K. (2018). The more the merrier? Increasing group size may be detrimental to decision-making performance in nominal groups. *PLoS One* 13, e0192213. doi: 10.1371/journal.pone.0192213

Anzalone, A. V., Randolph, P. B., Davis, J. R., Sousa, A. A., Koblan, L. W., Levy, J. M., et al. (2019). Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* 576, 149–157. doi: 10.1038/s41586-019-1711-4

Apedoe, X. S., Ellefson, M. R., and Schunn, C. D. (2012). Learning Together While Designing: Does Group Size Make a Difference? *J Sci Educ Technol* 21, 83–94. doi: 10.1007/s10956-011-9284-5

Armstrong, G. A. B., Liao, M., You, Z., Lissouba, A., Chen, B. E., and Drapeau, P. (2016). Homology Directed Knockin of Point Mutations in the Zebrafish tardbp and fus Genes in ALS Using the CRISPR/Cas9 System. *PLoS One* 11. doi: 10.1371/journal.pone.0150188

Arnaout, R., Ferrer, T., Huisken, J., Spitzer, K., Stainier, D. Y. R., Tristani-Firouzi, M., et al. (2007). Zebrafish model for human long QT syndrome. *Proc Natl Acad Sci U S A* 104, 11316–11321. doi: 10.1073/pnas.0702724104

Avdesh, A., Chen, M., Martin-Iverson, M. T., Mondal, A., Ong, D., Rainey-Smith, S., et al. (2012). Regular Care and Maintenance of a Zebrafish (Danio rerio) Laboratory: An Introduction. *JoVE (Journal of Visualized Experiments)*, e4196. doi: 10.3791/4196

Bai, H., Liu, L., An, K., Lu, X., Harrison, M., Zhao, Y., et al. (2020). CRISPR/Cas9-mediated precise genome modification by a long ssDNA template in zebrafish. *BMC Genomics* 21, 67. doi: 10.1186/s12864-020-6493-4

Baker, K., Warren, K. S., Yellen, G., and Fishman, M. C. (1997). Defective "pacemaker" current (Ih) in a zebrafish mutant with a slow heart rate. *Proc Natl Acad Sci U S A* 94, 4554–4559.

Bakkers, J. (2011). Zebrafish as a model to study cardiac development and human cardiac disease. *Cardiovasc Res* 91, 279–288. doi: 10.1093/cvr/cvr098

Bandyopadhyay, A., Kancharla, N., Javalkote, V. S., Dasgupta, S., and Brutnell, T. P. (2020). CRISPR-Cas12a (Cpf1): A Versatile Tool in the Plant Genome Editing Tool Box for Agricultural Advancement. *Front Plant Sci* 11, 584151. doi: 10.3389/fpls.2020.584151

Bangera, G., and Brownell, S. E. (2014). Course-Based Undergraduate Research Experiences Can Make Scientific Research More Inclusive. *CBE Life Sci Educ* 13, 602–606. doi: 10.1187/cbe.14-06-0099

Baraban, S. C., Dinday, M. T., and Hortopan, G. A. (2013). Drug screening in Scn1a zebrafish mutant identifies clemizole as a potential Dravet syndrome treatment. *Nat Commun* 4, 2410. doi: 10.1038/ncomms3410

Baraban, S. C., Taylor, M. R., Castro, P. A., and Baier, H. (2005). Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. *Neuroscience* 131, 759–768. doi: 10.1016/j.neuroscience.2004.11.031

Barbazuk, W. B., Korf, I., Kadavi, C., Heyen, J., Tate, S., Wun, E., et al. (2000). The Syntenic Relationship of the Zebrafish and Human Genomes. *Genome Res* 10, 1351–1358.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., et al. (2007). CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. *Science* 315, 1709–1712. doi: 10.1126/science.1138140

Barrio, L. C., Capel, J., Jarillo, J. A., Castro, C., and Revilla, A. (1997). Species-specific voltage-gating properties of connexin-45 junctions expressed in Xenopus oocytes. *Biophysical Journal* 73, 757–769. doi: 10.1016/S0006-3495(97)78108-6

Bass, B. G. (1975). Restitution of the action potential in cat papillary muscle. *Am J Physiol* 228, 1717–1724. doi: 10.1152/ajplegacy.1975.228.6.1717

Bauer, B., Mally, A., and Liedtke, D. (2021). Zebrafish Embryos and Larvae as Alternative Animal Models for Toxicity Testing. *Int J Mol Sci* 22, 13417. doi: 10.3390/ijms222413417

Beffagna, G. (2019). Zebrafish as a Smart Model to Understand Regeneration After Heart Injury: How Fish Could Help Humans. *Front Cardiovasc Med* 6, 107. doi: 10.3389/fcvm.2019.00107

Belardinelli, L., Shryock, J. C., and Fraser, H. (2006). Inhibition of the late sodium current as a potential cardioprotective principle: effects of the late sodium current inhibitor ranolazine. *Heart* 92, iv6–iv14. doi: 10.1136/hrt.2005.078790

Bertucci, A., Conte, S., Johnson, D. W., and Johnson, R. T. (2010). The Impact of Size of Cooperative Group on Achievement, Social Support, and Self-Esteem. *The Journal of General Psychology* 137, 256–272. doi: 10.1080/00221309.2010.484448

Bhatt, J. M., and Challa, A. K. (2018). First Year Course-Based Undergraduate Research Experience (CURE) Using the CRISPR/Cas9 Genome Engineering Technology in Zebrafish. *J Microbiol Biol Educ* 19, 19.1.3. doi: 10.1128/jmbe.v19i1.1245

Black, M. B., Budinsky, R. A., Dombkowski, A., Cukovic, D., LeCluyse, E. L., Ferguson, S. S., et al. (2012). Cross-species Comparisons of Transcriptomic Alterations in Human and Rat Primary Hepatocytes Exposed to 2,3,7,8-Tetrachlorodibenzo-p-dioxin. *Toxicological Sciences* 127, 199–215. doi: 10.1093/toxsci/kfs069

Boel, A., De Saffel, H., Steyaert, W., Callewaert, B., De Paepe, A., Coucke, P. J., et al. (2018). CRISPR/Cas9-mediated homology-directed repair by ssODNs in zebrafish induces complex mutational patterns resulting from genomic integration of repair-template fragments. *Disease Models & Mechanisms* 11, dmm035352. doi: 10.1242/dmm.035352

Brame, C. J., and Biel, R. (2015). Group work: Using cooperative learning groups effectively. *Vanderbilt University*. Available at: https://cft.vanderbilt.edu/guides-sub-pages/setting-up-and-facilitating-group-work-using-cooperative-learning-groups-effectively/ (Accessed November 13, 2023).

Brette, F., Luxan, G., Cros, C., Dixey, H., Wilson, C., and Shiels, H. A. (2008). Characterization of isolated ventricular myocytes from adult zebrafish (Danio rerio). *Biochem Biophys Res Commun* 374, 143–146. doi: 10.1016/j.bbrc.2008.06.109

Brown, G. T. L. (2020). Schooling Beyond COVID-19: An Unevenly Distributed Future. *Frontiers in Education* 5. Available at: https://www.frontiersin.org/articles/10.3389/feduc.2020.00082 (Accessed March 3, 2024).

Burger, A., Lindsay, H., Felker, A., Hess, C., Anders, C., Chiavacci, E., et al. (2016). Maximizing mutagenesis with solubilized CRISPR-Cas9 ribonucleoprotein complexes. *Development* 143, 2025–2037. doi: 10.1242/dev.134809

Burns, C. G., Milan, D. J., Grande, E. J., Rottbauer, W., MacRae, C. A., and Fishman, M. C. (2005). High-throughput assay for small molecules that modulate zebrafish embryonic heart rate. *Nature Chemical Biology* 1, 263–264. doi: 10.1038/nchembio732

Bussmann, J., Bakkers, J., and Schulte-Merker, S. (2007). Early Endocardial Morphogenesis Requires Scl/Tal1. *PLOS Genetics* 3, e140. doi: 10.1371/journal.pgen.0030140

Cai, P., Gao, J., and Zhou, Y. (2019). CRISPR-mediated genome editing in non-conventional yeasts for biotechnological applications. *Microbial Cell Factories* 18, 63. doi: 10.1186/s12934-019-1112-2

Cai, W., Liu, J., Chen, X., Mao, L., and Wang, M. (2022). Orthogonal Chemical Activation of Enzyme-Inducible CRISPR/Cas9 for Cell-Selective Genome Editing. *J. Am. Chem. Soc.* 144, 22272–22280. doi: 10.1021/jacs.2c10545

Chan, P. K., Lin, C. C., and Cheng, S. H. (2009). Noninvasive technique for measurement of heartbeat regularity in zebrafish (Danio rerio) embryos. *BMC Biotechnology* 9, 11. doi: 10.1186/1472-6750-9-11

Chandra, Y., and Shang, L. (2019). "Inductive Coding," in *Qualitative Research Using R: A Systematic Approach*, eds. Y. Chandra and L. Shang (Singapore: Springer Nature), 91–106. doi: 10.1007/978-981-13-3170-1_8

Chang, C.-P., Neilson, J. R., Bayle, J. H., Gestwicki, J. E., Kuo, A., Stankunas, K., et al. (2004). A Field of Myocardial-Endocardial NFAT Signaling Underlies Heart Valve Morphogenesis. *Cell* 118, 649–663. doi: 10.1016/j.cell.2004.08.010

Chang, N., Sun, C., Gao, L., Zhu, D., Xu, X., Zhu, X., et al. (2013). Genome editing with RNA-guided Cas9 nuclease in Zebrafish embryos. *Cell Res* 23, 465–472. doi: 10.1038/cr.2013.45

Chatterjee, B., Chin, A. J., Valdimarsson, G., Finis, C., Sonntag, J. M., Choi, B. Y., et al. (2005). Developmental regulation and expression of the zebrafish connexin43 gene. *Developmental Dynamics* 233, 890–906. doi: 10.1002/dvdy.20426

Chauhan, V. P., Sharp, P. A., and Langer, R. (2023). Altered DNA repair pathway engagement by engineered CRISPR-Cas9 nucleases. *Proceedings of the National Academy of Sciences* 120, e2300605120. doi: 10.1073/pnas.2300605120

Chen, J. N., Haffter, P., Odenthal, J., Vogelsang, E., Brand, M., van Eeden, F. J., et al. (1996). Mutations affecting the cardiovascular system and other internal organs in zebrafish. *Development (Cambridge, England)* 123, 293–302.

Cheng, S., Shakespeare, T., Mui, R., White, T. W., and Valdimarsson, G. (2004). Connexin 48.5 Is Required for Normal Cardiovascular Function and Lens Development in Zebrafish Embryos. *J. Biol. Chem.* 279, 36993–37003. doi: 10.1074/jbc.M401355200

Chi, N. C., Bussen, M., Brand-Arzamendi, K., Ding, C., Olgin, J. E., Shaw, R. M., et al. (2010). Cardiac conduction is required to preserve cardiac chamber morphology. *Proc Natl Acad Sci U S A* 107, 14662–14667. doi: 10.1073/pnas.0909432107

Chi, N. C., Shaw, R. M., Jungblut, B., Huisken, J., Ferrer, T., Arnaout, R., et al. (2008). Genetic and Physiologic Dissection of the Vertebrate Cardiac Conduction System. *PLOS Biology* 6, e109. doi: 10.1371/journal.pbio.0060109

Christian, M., Cermak, T., Doyle, E. L., Schmidt, C., Zhang, F., Hummel, A., et al. (2010). Targeting DNA Double-Strand Breaks with TAL Effector Nucleases. *Genetics* 186, 757–761. doi: 10.1534/genetics.110.120717

Christie, T. L., Mui, R., White, T. W., and Valdimarsson, G. (2004). Molecular cloning, functional analysis, and RNA expression analysis of connexin45.6: a zebrafish cardiovascular connexin. *American Journal of Physiology-Heart and Circulatory Physiology* 286, H1623–H1632. doi: 10.1152/ajpheart.00800.2003

Clark, K. J., Balciunas, D., Pogoda, H.-M., Ding, Y., Westcot, S. E., Bedell, V. M., et al. (2011). In vivo protein trapping produces a functional expression codex of the vertebrate proteome. *Nat Methods* 8, 506–512. doi: 10.1038/nmeth.1606

Clay, H., and Coughlin, S. R. (2015). Mechanical Vessel Injury in Zebrafish Embryos. *J Vis Exp*, 52460. doi: 10.3791/52460

Clemmons, A. W., Timbrook, J., Herron, J. C., and Crowe, A. J. (2020). BioSkills Guide: Development and National Validation of a Tool for Interpreting the Vision and Change Core Competencies. *LSE* 19, ar53. doi: 10.1187/cbe.19-11-0259

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., et al. (2013). Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* 339, 819–823. doi: 10.1126/science.1231143

Cornet, C., Di Donato, V., and Terriente, J. (2018). Combining Zebrafish and CRISPR/Cas9: Toward a More Efficient Drug Discovery Pipeline. *Front Pharmacol* 9. doi: 10.3389/fphar.2018.00703

Corrégé, J.-B., and Michinov, N. (2021). Group Size and Peer Learning: Peer Discussions in Different Group Size Influence Learning in a Biology Exercise Performed on a Tablet With Stylus. *Frontiers in Education* 6. Available at: https://www.frontiersin.org/articles/10.3389/feduc.2021.733663 (Accessed November 13, 2023).

Cui, Y., Xu, J., Cheng, M., Liao, X., and Peng, S. (2018). Review of CRISPR/Cas9 sgRNA Design Tools. *Interdiscip Sci* 10, 455–465. doi: 10.1007/s12539-018-0298-z

Dahlberg, L., and Groat Carmona, A. M. (2018). CRISPR-Cas Technology In and Out of the Classroom. *The CRISPR Journal* 1, 107–114. doi: 10.1089/crispr.2018.0007

D'Costa, A., and Shepherd, I. T. (2009). Zebrafish development and genetics: introducing undergraduates to developmental biology and genetics in a large introductory laboratory class. *Zebrafish* 6, 169–177. doi: 10.1089/zeb.2008.0562

De Silva, A. O., Allard, C. N., Spencer, C., Webster, G. M., and Shoeib, M. (2012). Phosphorus-Containing Fluorinated Organics: Polyfluoroalkyl Phosphoric Acid Diesters (diPAPs), Perfluorophosphonates (PFPAs), and Perfluorophosphinates (PFPIAs) in Residential Indoor Dust. *Environ. Sci. Technol.* 46, 12575–12582. doi: 10.1021/es303172p

de Vrieze, E., de Bruijn, S. E., Reurink, J., Broekman, S., van de Riet, V., Aben, M., et al. (2021). Efficient Generation of Knock-In Zebrafish Models for Inherited Disorders Using CRISPR-Cas9 Ribonucleoprotein Complexes. *Int J Mol Sci* 22, 9429. doi: 10.3390/ijms22179429

Del Vecchio, G., Mazzei, A., Schiavone, R., Gomes, A. S., Frangelli, G., Sala, T., et al. (2022). Rearing Conditions and Automated Feed Distribution Systems for Zebrafish (Danio rerio). *Applied Sciences* 12, 10961. doi: 10.3390/app122110961

Desplantez, T., Marics, I., Jarry-Guichard, T., Veteikis, R., Briand, J.-P., Weingart, R., et al. (2003). Characterization of Zebrafish Cx43.4 Connexin and its Channels. *Experimental Physiology* 88, 681–690. doi: 10.1113/eph8802584

Dhillon, S. S., Dóró, É., Magyary, I., Egginton, S., Sík, A., and Müller, F. (2013). Optimisation of Embryonic and Larval ECG Measurement in Zebrafish for Quantifying the Effect of QT Prolonging Drugs. *PLoS ONE* 8, e60552. doi: 10.1371/journal.pone.0060552

Ding, Y., Lang, D., Yan, J., Bu, H., Li, H., Jiao, K., et al. (2022). A phenotype-based forward genetic screen identifies Dnajb6 as a sick sinus syndrome gene. *eLife* 11, e77327. doi: 10.7554/eLife.77327

Doench, J. G., Hartenian, E., Graham, D. B., Tothova, Z., Hegde, M., Smith, I., et al. (2014). Rational design of highly active sgRNAs for CRISPR-Cas9–mediated gene inactivation. *Nature Biotechnology* 32, 1262–1267. doi: 10.1038/nbt.3026

Doyon, Y., McCammon, J. M., Miller, J. C., Faraji, F., Ngo, C., Katibah, G. E., et al. (2008). Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat Biotechnol* 26, 702–708. doi: 10.1038/nbt1409

Echeazarra, L., Hortigón-Vinagre, M. P., Casis, O., and Gallego, M. (2021). Adult and Developing Zebrafish as Suitable Models for Cardiac Electrophysiology and Pathology in Research and Industry. *Front Physiol* 11, 607860. doi: 10.3389/fphys.2020.607860

Eliceiri, B. P., Gonzalez, A. M., and Baird, A. (2011). Zebrafish Model of the Blood-Brain Barrier: Morphological and Permeability Studies. *Methods Mol Biol* 686, 371–378. doi: 10.1007/978-1-60761-938-3_18

El-Sherif, N., Turitto, G., and Boutjdir, M. (2019). Acquired Long QT Syndrome and Electrophysiology of Torsade de Pointes. *Arrhythm Electrophysiol Rev* 8, 122–130. doi: 10.15420/aer.2019.8.3

Eschstruth, A., Schneider-Maunoury, S., and Giudicelli, F. (2020). Creation of zebrafish knock-in reporter lines in the nefma gene by Cas9-mediated homologous recombination. *genesis* 58, e23340. doi: 10.1002/dvg.23340

Essner, J. J., Laing, J. G., Beyer, E. C., Johnson, R. G., and Hackett Jr., P. B. (1996). Expression of Zebrafish connexin43.4 in the Notochord and Tail Bud of Wild-Type and Mutant no tail Embryos. doi: 10.1006/dbio.1996.0177

Fang, M., Guo, J., Chen, D., Li, A., Hinton, D. E., and Dong, W. (2016). Halogenated carbazoles induce cardiotoxicity in developing zebrafish (Danio rerio) embryos. *Environmental Toxicology and Chemistry* 35, 2523–2529. doi: 10.1002/etc.3416

Fedorov, V. V., Glukhov, A. V., Sudharshan, S., Egorov, Y., Rosenshtraukh, L. V., and Efimov, I. R. (2008). Electrophysiological mechanisms of antiarrhythmic protection during hypothermia in winter hibernating versus nonhibernating mammals. *Heart Rhythm* 5, 1587–1596. doi: 10.1016/j.hrthm.2008.08.030

Fenichel, R. R., Malik, M., Antzelevitch, C., Sanguinetti, M., Roden, D. M., Priori, S. G., et al. (2004). Drug-induced torsades de pointes and implications for drug development. *J Cardiovasc Electrophysiol* 15, 475–495. doi: 10.1046/j.1540-8167.2004.03534.x

Findlay, A. R., Paing, M. M., Daw, J. A., Haller, M., Bengoechea, R., Pittman, S. K., et al. (2023). DNAJB6 isoform specific knockdown: Therapeutic potential for limb girdle muscular dystrophy D1. *Mol Ther Nucleic Acids* 32, 937–948. doi: 10.1016/j.omtn.2023.05.017

Fossa, A. A., DePasquale, M. J., Raunig, D. L., Avery, M. J., and Leishman, D. J. (2002). The relationship of clinical QT prolongation to outcome in the conscious dog using a beat-to-beat QT-RR interval assessment. *J Pharmacol Exp Ther* 302, 828–833. doi: 10.1124/jpet.102.035220

Franz, M. R. (2003). The electrical restitution curve revisited: steep or flat slope--which is better? *J Cardiovasc Electrophysiol* 14, S140-147. doi: 10.1046/j.1540.8167.90303.x

Franz, M. R., Gray, R. A., Karasik, P., Moore, H. J., and Singh, S. N. (2014). Drug-induced post-repolarization refractoriness as an antiarrhythmic principle and its underlying mechanism. *Europace* 16 Suppl 4, iv39–iv45. doi: 10.1093/europace/euu274

Frommeyer, G., Milberg, P., Witte, P., Stypmann, J., Koopmann, M., Lücke, M., et al. (2011). A new mechanism preventing proarrhythmia in chronic heart failure: rapid phase-III repolarization explains the low proarrhythmic potential of amiodarone in contrast to sotalol in a model of pacing-induced heart failure. *Eur J Heart Fail* 13, 1060–1069. doi: 10.1093/eurjhf/hfr107

Garfinkel, A., Kim, Y. H., Voroshilovsky, O., Qu, Z., Kil, J. R., Lee, M. H., et al. (2000). Preventing ventricular fibrillation by flattening cardiac restitution. *Proc Natl Acad Sci U S A* 97, 6061–6066. doi: 10.1073/pnas.090492697

Garrity, D. M., Childs, S., and Fishman, M. C. (2002). The heartstrings mutation in zebrafish causes heart/fin Tbx5 deficiency syndrome. *Development* 129, 4635–4645.

Gemberling, M., Bailey, T. J., Hyde, D. R., and Poss, K. D. (2013). The zebrafish as a model for complex tissue regeneration. *Trends Genet* 29, 10.1016/j.tig.2013.07.003. doi: 10.1016/j.tig.2013.07.003

Genge, C. E., Lin, E., Lee, L., Sheng, X., Rayani, K., Gunawan, M., et al. (2016). "The Zebrafish Heart as a Model of Mammalian Cardiac Function," in *Reviews of Physiology, Biochemistry and Pharmacology, Vol. 171*, eds. B. Nilius, P. de Tombe, T. Gudermann, R. Jahn, R. Lill, and O. H. Petersen (Cham: Springer International Publishing), 99–136. doi: 10.1007/112_2016_5

Genge, C. E., Muralidharan, P., Kemp, J., Hull, C. M., Yip, M., Simpson, K., et al. (2024). Zebrafish cardiac repolarization does not functionally depend on the expression of the hERG1b-like transcript. *Pflugers Arch - Eur J Physiol* 476, 87–99. doi: 10.1007/s00424-023-02875-z

Ghosh, S., and Hui, S. P. (2016). Regeneration of Zebrafish CNS: Adult Neurogenesis. *Neural Plast* 2016, 5815439. doi: 10.1155/2016/5815439

Gintant, G. A., Su, Z., Martin, R. L., and Cox, B. F. (2016). Utility of hERG Assays as Surrogate Markers of Delayed Cardiac Repolarization and QT Safety: *Toxicologic Pathology*. doi: 10.1080/01926230500431376

Giudicessi, J. R., and Ackerman, M. J. (2013). Genotype- and Phenotype-Guided Management of Congenital Long QT Syndrome. *Curr Probl Cardiol* 38, 417–455. doi: 10.1016/j.cpcardiol.2013.08.001

Goldenberg, I., Moss, A. J., and Zareba, W. (2006). QT Interval: How to Measure It and What Is "Normal." *Journal of Cardiovascular Electrophysiology* 17, 333–336. doi: 10.1111/j.1540-8167.2006.00408.x

González-Rosa, J. M. (2022). Zebrafish Models of Cardiac Disease: From Fortuitous Mutants to Precision Medicine. *Circulation Research* 130, 1803–1826. doi: 10.1161/CIRCRESAHA.122.320396

Granato, M., Eeden, F. J. M. van, Schach, U., Trowe, T., Brand, M., Furutani-Seiki, M., et al. (1996). Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva. *Development* 123, 399–413. doi: 10.1242/dev.123.1.399

Greeves, S., and Oz, M. (2024). YouTube in higher education: comparing student and instructor perceptions and practices. *Frontiers in Education* 8. Available at: https://www.frontiersin.org/articles/10.3389/feduc.2023.1330405 (Accessed March 5, 2024).

Griffin, A., Hamling, K. R., Knupp, K., Hong, S., Lee, L. P., and Baraban, S. C. (2017). Clemizole and modulators of serotonin signalling suppress seizures in Dravet syndrome. *Brain* 140, 669–683. doi: 10.1093/brain/aww342

Guettler, N., Rajappan, K., and Nicol, E. (2019). The impact of age on long QT syndrome. *Aging (Albany NY)* 11, 11795–11796. doi: 10.18632/aging.102623

Gupta, R. M., and Musunuru, K. (2014). Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9. *J Clin Invest* 124, 4154–4161. doi: 10.1172/JCI72992

Gut, P., Reischauer, S., Stainier, D. Y. R., and Arnaout, R. (2017). Little Fish, Big Data: Zebrafish as a Model for Cardiovascular and Metabolic Disease. *Physiol Rev* 97, 889–938. doi: 10.1152/physrev.00038.2016

Haeussler, M., Schönig, K., Eckert, H., Eschstruth, A., Mianné, J., Renaud, J.-B., et al. (2016). Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. *Genome Biology* 17, 148. doi: 10.1186/s13059-016-1012-2

Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., et al. (1996). The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. *Development* 123, 1–36. doi: 10.1242/dev.123.1.1

Hammar Chiriac, E. (2014). Group work as an incentive for learning – students' experiences of group work. *Frontiers in Psychology* 5. Available at: https://www.frontiersin.org/articles/10.3389/fpsyg.2014.00558 (Accessed November 13, 2023).

Hammond, T. G., and Pollard, C. E. (2005). Use of in vitro methods to predict QT prolongation. *Toxicology and Applied Pharmacology* 207, 446–450. doi: 10.1016/j.taap.2005.03.022

Hassel, D., Scholz, E. P., Trano, N., Friedrich, O., Just, S., Meder, B., et al. (2008). Deficient zebrafish ether-à-go-go-related gene channel gating causes short-QT syndrome in zebrafish reggae mutants. *Circulation* 117, 866–875. doi: 10.1161/CIRCULATIONAHA.107.752220

Hassinen, M., Haverinen, J., Hardy, M. E., Shiels, H. A., and Vornanen, M. (2015). Inward rectifier potassium current (IK1) and Kir2 composition of the zebrafish (Danio rerio) heart. *Pflugers Arch - Eur J Physiol* 467, 2437–2446. doi: 10.1007/s00424-015-1710-8

Haverinen, J., Hassinen, M., Dash, S. N., and Vornanen, M. (2018). Expression of calcium channel transcripts in the zebrafish heart: dominance of T-type channels. *Journal of Experimental Biology*, 10.

Holmes, N. G., and Kalender, Z. Y. (2020). Preliminary evidence for available roles in mixed-gender and all-women lab groups. doi: 10.48550/arXiv.2007.14833

Hondeghem, L. M., Dujardin, K., and De Clerck, F. (2001). Phase 2 prolongation, in the absence of instability and triangulation, antagonizes class III proarrhythmia. *Cardiovasc Res* 50, 345–353. doi: 10.1016/S0008-6363(01)00259-0

Hoshijima, K., Jurynec, M. J., and Grunwald, D. J. (2016). Precise Editing of the Zebrafish Genome Made Simple and Efficient. *Dev Cell* 36, 654–667. doi: 10.1016/j.devcel.2016.02.015

Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., et al. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 496, 498–503. doi: 10.1038/nature12111

Hruscha, A., Krawitz, P., Rechenberg, A., Heinrich, V., Hecht, J., Haass, C., et al. (2013). Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish. *Development* 140, 4982–4987. doi: 10.1242/dev.099085

Hu, J. H., Miller, S. M., Geurts, M. H., Tang, W., Chen, L., Sun, N., et al. (2018). Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature* 556, 57–63. doi: 10.1038/nature26155

Huang, C.-J., Tu, C.-T., Hsiao, C.-D., Hsieh, F.-J., and Tsai, H.-J. (2003). Germ-line transmission of a myocardium-specific GFP transgene reveals critical regulatory elements in the cardiac myosin light chain 2 promoter of zebrafish. *Developmental Dynamics* 228, 30–40. doi: 10.1002/dvdy.10356

Huang, P., Xiao, A., Zhou, M., Zhu, Z., Lin, S., and Zhang, B. (2011). Heritable gene targeting in zebrafish using customized TALENs. *Nat Biotechnol* 29, 699–700. doi: 10.1038/nbt.1939

Hull, C. M., Genge, C. E., Hobbs, Y., Rayani, K., Lin, E., Gunawan, M., et al. (2019). Investigating the utility of adult zebrafish ex vivo whole hearts to pharmacologically screen hERG channel activator compounds. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 317, R921–R931. doi: 10.1152/ajpregu.00190.2019

Huo, J., Zhang, Y., Huang, N., Liu, P., Huang, C., Guo, X., et al. (2008). The G604S-hERG mutation alters the biophysical properties and exerts a dominant-negative effect on expression of hERG channels in HEK293 cells. *Pflugers Arch - Eur J Physiol* 456, 917–928. doi: 10.1007/s00424-008-0454-0

Hurst, R. M. (n.d.). Development and optimization of tools for embryonic electrocardiograph recording for heart dysfunction in zebrafish. 252.

Hussman, J. P. (2001). Letters to the Editor: Suppressed GABAergic Inhibition as a Common Factor in Suspected Etiologies of Autism. *J Autism Dev Disord* 31, 247–248. doi: 10.1023/A:1010715619091

Hwang, W. Y., Fu, Y., Reyon, D., Maeder, M. L., Tsai, S. Q., Sander, J. D., et al. (2013). Efficient In Vivo Genome Editing Using RNA-Guided Nucleases. *Nat Biotechnol* 31, 227–229. doi: 10.1038/nbt.2501

Hwang, W. Y., Peterson, R. T., and Yeh, J.-R. J. (2014). Methods for targeted mutagenesis in zebrafish using TALENs. *Methods* 69, 76–84. doi: 10.1016/j.ymeth.2014.04.009

Ichino, N., Serres, M. R., Urban, R. M., Urban, M. D., Treichel, A. J., Schaefbauer, K. J., et al. (2020). Building the vertebrate codex using the gene breaking protein trap library. *eLife* 9, e54572. doi: 10.7554/eLife.54572

Iovine, M. K., Higgins, E. P., Hindes, A., Coblitz, B., and Johnson, S. L. (2005). Mutations in connexin43 (GJA1) perturb bone growth in zebrafish fins. *Developmental Biology* 278, 208–219. doi: 10.1016/j.ydbio.2004.11.005

Irion, U., Krauss, J., and Nüsslein-Volhard, C. (2014). Precise and efficient genome editing in zebrafish using the CRISPR/Cas9 system. *Development* 141, 4827–4830. doi: 10.1242/dev.115584

Ishino, Y., Shinagawa, H., Makino, K., Amemura, M., and Nakata, A. (1987). Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *J Bacteriol* 169, 5429–5433.

Isogai, S., Horiguchi, M., and Weinstein, B. M. (2001). The Vascular Anatomy of the Developing Zebrafish: An Atlas of Embryonic and Early Larval Development. *Developmental Biology* 230, 278–301. doi: 10.1006/dbio.2000.9995

Jensen, J. L., and Lawson, A. (2011). Effects of Collaborative Group Composition and Inquiry Instruction on Reasoning Gains and Achievement in Undergraduate Biology. *LSE* 10, 64–73. doi: 10.1187/cbe.10-07-0089

Jeong, J.-Y., Kwon, H.-B., Ahn, J.-C., Kang, D., Kwon, S.-H., Park, J. A., et al. (2008). Functional and developmental analysis of the blood–brain barrier in zebrafish. *Brain Research Bulletin* 75, 619–628. doi: 10.1016/j.brainresbull.2007.10.043

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., and Charpentier, E. (2012). A programmable dual RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821. doi: 10.1126/science.1225829

Johnson, S. L., and Weston, J. A. (1995). Temperature-Sensitive Mutations That Cause Stage-Specific Defects in Zebrafish Fin Regeneration. *Genetics* 141, 1583–1595.

Jost, N., Virág, L., Bitay, M., Takács, J., Lengyel, C., Biliczki, P., et al. (2005). Restricting Excessive Cardiac Action Potential and QT Prolongation. *Circulation* 112, 1392–1399. doi: 10.1161/CIRCULATIONAHA.105.550111

Jou, C. J., Barnett, S. M., Bian, J.-T., Weng, H. C., Sheng, X., and Tristani-Firouzi, M. (2013). An In Vivo Cardiac Assay to Determine the Functional Consequences of Putative Long QT Syndrome Mutations. *Circ Res* 112, 826–830. doi: 10.1161/CIRCRESAHA.112.300664

Kang, J. H., Han, J., Lee, H., Lim, M. H., Kim, K.-T., and Kim, C. (2018). A water-soluble fluorescence chemosensor for the sequential detection of Zn2+ and pyrophosphate in living cells and zebrafish. *Dyes and Pigments* 152, 131–138. doi: 10.1016/j.dyepig.2018.01.039

Kannankeril, P., Roden, D. M., and Darbar, D. (2010). Drug-Induced Long QT Syndrome. *Pharmacol Rev* 62, 760–781. doi: 10.1124/pr.110.003723

Kawano, F., Suzuki, H., Furuya, A., and Sato, M. (2015). Engineered pairs of distinct photoswitches for optogenetic control of cellular proteins. *Nat Commun* 6, 6256. doi: 10.1038/ncomms7256

Keegan, B. R., Meyer, D., and Yelon, D. (2004). Organization of cardiac chamber progenitors in the zebrafish blastula. *Development* 131, 3081–3091. doi: 10.1242/dev.01185

Kegel, L., Rubio, M., Almeida, R. G., Benito, S., Klingseisen, A., and Lyons, D. A. (2019). "Forward Genetic Screen Using Zebrafish to Identify New Genes Involved in Myelination," in *Oligodendrocytes: Methods and Protocols*, eds. D. A. Lyons and L. Kegel (New York, NY: Springer), 185–209. doi: 10.1007/978-1-4939-9072-6_11

Kemmler, C. L., Riemslagh, F. W., Moran, H. R., and Mosimann, C. (2021). From Stripes to a Beating Heart: Early Cardiac Development in Zebrafish. *Journal of Cardiovascular Development and Disease* 8, 17. doi: 10.3390/jcdd8020017

Kemp, J. M., Whittaker, D. G., Venkateshappa, R., Pang, Z., Johal, R., Sergeev, V., et al. (2021). Electrophysiological characterization of the hERG R56Q LQTS variant and targeted rescue by the activator RPR260243. *Journal of General Physiology* 153, e202112923. doi: 10.1085/jgp.202112923

Kim, Y. G., Cha, J., and Chandrasegaran, S. (1996). Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proc Natl Acad Sci U S A* 93, 1156–1160.

Kimura, Y., Hisano, Y., Kawahara, A., and Higashijima, S. (2014). Efficient generation of knock-in transgenic zebrafish carrying reporter/driver genes by CRISPR/Cas9-mediated genome engineering. *Sci Rep* 4, 6545. doi: 10.1038/srep06545

Klaauw, A. A. van der, Croizier, S., Oliveira, E. M. de, Stadler, L. K. J., Park, S., Kong, Y., et al. (2019). Human Semaphorin 3 Variants Link Melanocortin Circuit Development and Energy Balance. *Cell* 176, 729-742.e18. doi: 10.1016/j.cell.2018.12.009

Kleinstiver, B. P., Prew, M. S., Tsai, S. Q., Topkar, V. V., Nguyen, N. T., Zheng, Z., et al. (2015). Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature* 523, 481–485. doi: 10.1038/nature14592

Kocak, D. D., Josephs, E. A., Bhandarkar, V., Adkar, S. S., Kwon, J. B., and Gersbach, C. A. (2019). Increasing the specificity of CRISPR systems with engineered RNA secondary structures. *Nat Biotechnol* 37, 657–666. doi: 10.1038/s41587-019-0095-1

Koller, B. S., Karasik, P. E., Solomon, A. J., and Franz, M. R. (1995). Relation between repolarization and refractoriness during programmed electrical stimulation in the human right ventricle. Implications for ventricular tachycardia induction. *Circulation* 91, 2378–2384. doi: 10.1161/01.cir.91.9.2378

Koller, M. L., Riccio, M. L., and Gilmour, R. F. (1998). Dynamic restitution of action potential duration during electrical alternans and ventricular fibrillation. *Am J Physiol* 275, H1635-1642. doi: 10.1152/ajpheart.1998.275.5.H1635

Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A., and Liu, D. R. (2016). Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* 533, 420–424. doi: 10.1038/nature17946

Kopp, R., Schwerte, T., and Pelster, B. (2005). Cardiac performance in the zebrafish breakdance mutant. *Journal of Experimental Biology* 208, 2123–2134. doi: 10.1242/jeb.01620

Kossack, M. E., and Draper, B. W. (2019). Genetic regulation of sex determination and maintenance in zebrafish (Danio rerio). *Curr Top Dev Biol* 134, 119–149. doi: 10.1016/bs.ctdb.2019.02.004

Kozol, R. A., Cukier, H. N., Zou, B., Mayo, V., De Rubeis, S., Cai, G., et al. (2015). Two knockdown models of the autism genes SYNGAP1 and SHANK3 in zebrafish produce similar behavioral phenotypes associated with embryonic disruptions of brain morphogenesis. *Hum Mol Genet* 24, 4006–4023. doi: 10.1093/hmg/ddv138

LaManna, C. M., and Barrangou, R. (2018). Enabling the Rise of a CRISPR World. *CRISPR J* 1, 205–208. doi: 10.1089/crispr.2018.0022

Langheinrich, U., Vacun, G., and Wagner, T. (2003). Zebrafish embryos express an orthologue of HERG and are sensitive toward a range of QT-prolonging drugs inducing severe arrhythmia☆. *Toxicology and Applied Pharmacology* 193, 370–382. doi: 10.1016/j.taap.2003.07.012

Laughlin, P. R., Hatch, E. C., Silver, J. S., and Boh, L. (2006). Groups perform better than the best individuals on letters-to-numbers problems: effects of group size. *J Pers Soc Psychol* 90, 644–651. doi: 10.1037/0022-3514.90.4.644

Lawrence, C. L., Pollard, C. E., Hammond, T. G., and Valentin, J.-P. (2005). Nonclinical proarrhythmia models: Predicting Torsades de Pointes. *Journal of Pharmacological and Toxicological Methods* 52, 46–59. doi: 10.1016/j.vascn.2005.04.011

Lee, H., and Mabury, S. A. (2011). A Pilot Survey of Legacy and Current Commercial Fluorinated Chemicals in Human Sera from United States Donors in 2009. *Environ. Sci. Technol.* 45, 8067–8074. doi: 10.1021/es200167q

Lee, H., Yoon, D. E., and Kim, K. (2020). Genome editing methods in animal models. *Anim Cells Syst (Seoul)* 24, 8–16. doi: 10.1080/19768354.2020.1726462

Lee, Y. M., Cope, J. J., Ackermann, G. E., Goishi, K., Armstrong, E. J., Paw, B. H., et al. (2006). Vascular endothelial growth factor receptor signaling is required for cardiac valve formation in zebrafish. *Developmental Dynamics* 235, 29–37. doi: 10.1002/dvdy.20559

Leibold, S., Lakshminarasimha, A. B., Gremse, F., Hammerschmidt, M., and Michel, M. (2022). Long-term obesogenic diet leads to metabolic phenotypes which are not exacerbated by catch-up growth in zebrafish. *PLOS ONE* 17, e0267933. doi: 10.1371/journal.pone.0267933

Leong, I. U. S., Skinner, J. R., Shelling, A. N., and Love, D. R. (2010a). Identification and expression analysis of kcnh2 genes in the zebrafish. *Biochemical and Biophysical Research Communications* 396, 817–824. doi: 10.1016/j.bbrc.2010.04.157

Leong, I. U. S., Skinner, J. R., Shelling, A. N., and Love, D. R. (2010b). Zebrafish as a model for long QT syndrome: the evidence and the means of manipulating zebrafish gene expression. *Acta Physiologica* 199, 257–276. doi: 10.1111/j.1748-1716.2010.02111.x

Letamendia, A., Quevedo, C., Ibarbia, I., Virto, J. M., Holgado, O., Diez, M., et al. (2012). Development and Validation of an Automated High-Throughput System for Zebrafish In Vivo Screenings. *PLOS ONE* 7, e36690. doi: 10.1371/journal.pone.0036690

Levic, D. S., Yamaguchi, N., Wang, S., Knaut, H., and Bagnat, M. (2021). Knock-in tagging in zebrafish facilitated by insertion into non-coding regions. *Development* 148, dev199994. doi: 10.1242/dev.199994

Li, L., Wu, L. P., and Chandrasegaran, S. (1992). Functional domains in Fok I restriction endonuclease. *Proc Natl Acad Sci U S A* 89, 4275–4279.

Li, Q., Qin, Z., Wang, Q., Xu, T., Yang, Y., and He, Z. (2019). Applications of Genome Editing Technology in Animal Disease Modeling and Gene Therapy. *Comput Struct Biotechnol J* 17, 689–698. doi: 10.1016/j.csbj.2019.05.006

Liang, P., Lan, F., Lee, A. S., Gong, T., Sanchez-Freire, V., Wang, Y., et al. (2013). Drug Screening Using a Library of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes Reveals Disease Specific Patterns of Cardiotoxicity. *Circulation* 127. doi: 10.1161/CIRCULATIONAHA.113.001883

Liang, X., Potter, J., Kumar, S., Ravinder, N., and Chesnut, J. D. (2017). Enhanced CRISPR/Cas9-mediated precise genome editing by improved design and delivery of gRNA, Cas9 nuclease, and donor DNA. *Journal of Biotechnology* 241, 136–146. doi: 10.1016/j.jbiotec.2016.11.011

Lim, J. M., and Kim, H. H. (2022). Basic Principles and Clinical Applications of CRISPR-Based Genome Editing. *Yonsei Med J* 63, 105–113. doi: 10.3349/ymj.2022.63.2.105

Liu, C., Zhang, L., Liu, H., and Cheng, K. (2017a). Delivery Strategies of the CRISPR-Cas9 Gene-Editing System for Therapeutic Applications. *J Control Release* 266, 17–26. doi: 10.1016/j.jconrel.2017.09.012

Liu, J., Zhou, Y., Qi, X., Chen, J., Chen, W., Qiu, G., et al. (2017b). CRISPR/Cas9 in zebrafish: an efficient combination for human genetic diseases modeling. *Hum Genet* 136, 1–12. doi: 10.1007/s00439-016-1739-6

Liu, K. I., Ramli, M. N. B., Woo, C. W. A., Wang, Y., Zhao, T., Zhang, X., et al. (2016). A chemical-inducible CRISPR–Cas9 system for rapid control of genome editing. *Nat Chem Biol* 12, 980–987. doi: 10.1038/nchembio.2179

Liu, Y., Asnani, A., Zou, L., Bentley, V. L., Yu, M., Wang, Y., et al. (2014). Visnagin protects against doxorubicin-induced cardiomyopathy through modulation of mitochondrial malate dehydrogenase. *Sci Transl Med* 6, 266ra170. doi: 10.1126/scitranslmed.3010189

Liu, Z., Dong, H., Cui, Y., Cong, L., and Zhang, D. (2020). Application of different types of CRISPR/Cas-based systems in bacteria. *Microbial Cell Factories* 19, 172. doi: 10.1186/s12934-020-01431-z

Lu, H. R., Vlaminckx, E., Hermans, A. N., Rohrbacher, J., Van Ammel, K., Towart, R., et al. (2008). Predicting drug-induced changes in QT interval and arrhythmias: QT-shortening drugs point to gaps in the ICHS7B Guidelines. *Br J Pharmacol* 154, 1427–1438. doi: 10.1038/bjp.2008.191

Ma, X., Ding, Y., Wang, Y., and Xu, X. (2018). A Doxorubicin-induced Cardiomyopathy Model in Adult Zebrafish. *J Vis Exp*, 57567. doi: 10.3791/57567

MacRae, C. A. (2010). Cardiac Arrhythmia: In vivo screening in the zebrafish to overcome complexity in drug discovery. *Expert Opin Drug Discov* 5, 619–632. doi: 10.1517/17460441.2010.492826

Makarova, K. S., Grishin, N. V., Shabalina, S. A., Wolf, Y. I., and Koonin, E. V. (2006). A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. *Biol Direct* 1, 7. doi: 10.1186/1745-6150-1-7

Mandenius, C.-F., Steel, D., Noor, F., Meyer, T., Heinzle, E., Asp, J., et al. (2011). Cardiotoxicity testing using pluripotent stem cell-derived human cardiomyocytes and state-of-the-art bioanalytics: a review. *Journal of Applied Toxicology* 31, 191–205. doi: 10.1002/jat.1663

Massoz, L., Dupont, M. A., and Manfroid, I. (2021). Zebra-Fishing for Regenerative Awakening in Mammals. *Biomedicines* 9, 65. doi: 10.3390/biomedicines9010065

Mattioni, T. A., Zheutlin, T. A., Sarmiento, J. J., Parker, M., Lesch, M., and Kehoe, R. F. (1989). Amiodarone in patients with previous drug-mediated torsade de pointes. Long-term safety and efficacy. *Ann Intern Med* 111, 574–580. doi: 10.7326/0003-4819-111-7-574

McHugh, M. L. (2012). Interrater reliability: the kappa statistic. *Biochem Med (Zagreb)* 22, 276–282.

McNeish, J. (2004). Embryonic stem cells in drug discovery. *Nature Reviews Drug Discovery* 3, 70–80. doi: 10.1038/nrd1281

Meeker, N. D., Hutchinson, S. A., Ho, L., and Trede, N. S. (2007). Method for isolation of PCR-ready genomic DNA from zebrafish tissues. *BioTechniques* 43, 610–614. doi: 10.2144/000112619

Melby, A. E., Warga, R. M., and Kimmel, C. B. (1996). Specification of cell fates at the dorsal margin of the zebrafish gastrula. *Development* 122, 2225–2237. doi: 10.1242/dev.122.7.2225

Melero, J., Hernández-Leo, D., and Manatunga, K. (2015). Group-based mobile learning: Do group size and sharing mobile devices matter? *Computers in Human Behavior* 44, 377–385. doi: 10.1016/j.chb.2014.11.078

Michlits, G., Jude, J., Hinterndorfer, M., de Almeida, M., Vainorius, G., Hubmann, M., et al. (2020). Multilayered VBC score predicts sgRNAs that efficiently generate loss-of-function alleles. *Nat. Methods* 17, 708–716. doi: 10.1038/s41592-020-0850-8

Milan, D. J., Giokas, A. C., Serluca, F. C., Peterson, R. T., and MacRae, C. A. (2006a). Notch1b and neuregulin are required for specification of central cardiac conduction tissue. *Development* 133, 1125–1132. doi: 10.1242/dev.02279

Milan, D. J., Jones, I. L., Ellinor, P. T., and MacRae, C. A. (2006b). In vivo recording of adult zebrafish electrocardiogram and assessment of drug-induced QT prolongation. *Am. J. Physiol. Heart Circ. Physiol.* 291, H269-273. doi: 10.1152/ajpheart.00960.2005

Milan, D. J., Kim, A. M., Winterfield, J. R., Jones, I. L., Pfeufer, A., Sanna, S., et al. (2009). A Drug-Sensitized Zebrafish Screen Identifies Multiple Genes, Including GINS3, as Regulators of Myocardial Repolarization. *Circulation* 120, 553–559. doi: 10.1161/CIRCULATIONAHA.108.821082

Milan, D. J., Peterson, T. A., Ruskin, J. N., Peterson, R. T., and MacRae, C. A. (2003). Drugs That Induce Repolarization Abnormalities Cause Bradycardia in Zebrafish. *Circulation* 107, 1355–1358. doi: 10.1161/01.CIR.0000061912.88753.87

Miller, J. C., Tan, S., Qiao, G., Barlow, K. A., Wang, J., Xia, D. F., et al. (2011). A TALE nuclease architecture for efficient genome editing. *Nat Biotechnol* 29, 143–148. doi: 10.1038/nbt.1755

Mittelstadt, S. W., Hemenway, C. L., Craig, M. P., and Hove, J. R. (2008). Evaluation of zebrafish embryos as a model for assessing inhibition of hERG. *Journal of Pharmacological and Toxicological Methods* 57, 100–105. doi: 10.1016/j.vascn.2007.10.004

Mohr, S. E., Hu, Y., Ewen-Campen, B., Housden, B. E., Viswanatha, R., and Perrimon, N. (2016). CRISPR guide RNA design for research applications. *FEBS J* 283, 3232–3238. doi: 10.1111/febs.13777

Mojica, F. J. M., Díez-Villaseñor, C., García-Martínez, J., and Soria, E. (2005). Intervening Sequences of Regularly Spaced Prokaryotic Repeats Derive from Foreign Genetic Elements. *J Mol Evol* 60, 174–182. doi: 10.1007/s00239-004-0046-3

Mojica, F. J. M., Díez-Villaseñor, C., Soria, E., and Juez, G. (2000). Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. *Molecular Microbiology* 36, 244–246. doi: 10.1046/j.1365-2958.2000.01838.x

Moreno-Mateos, M. A., Vejnar, C. E., Beaudoin, J.-D., Fernandez, J. P., Mis, E. K., Khokha, M. K., et al. (2015). CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo. *Nature Methods* 12, 982–988. doi: 10.1038/nmeth.3543

Motoike, T., Loughna, S., Perens, E., Roman, B. L., Liao, W., Chau, T. C., et al. (2000). Universal GFP reporter for the study of vascular development. *genesis* 28, 75–81. doi: 10.1002/1526-968X(200010)28:2<75::AID-GENE50>3.0.CO;2-S

Moulton, J. D. (2006). Using Morpholinos to Control Gene Expression. *Curr Protoc Nucleic Acid Chem* 27, 4301–43024. doi: 10.1002/0471142700.nc0430s27

Nasevicius, A., and Ekker, S. C. (2000). Effective targeted gene “knockdown” in zebrafish. *Nat Genet* 26, 216–220. doi: 10.1038/79951

Nemtsas, P., Wettwer, E., Christ, T., Weidinger, G., and Ravens, U. (2010a). Adult zebrafish heart as a model for human heart? An electrophysiological study. *Journal of Molecular and Cellular Cardiology* 48, 161–171. doi: 10.1016/j.yjmcc.2009.08.034

Nemudryi, A. A., Valetdinova, K. R., Medvedev, S. P., and Zakian, S. M. (2014). TALEN and CRISPR/Cas Genome Editing Systems: Tools of Discovery. *Acta Naturae* 6, 19–40.

Ng, G. A., Brack, K. E., Patel, V. H., and Coote, J. H. (2007). Autonomic modulation of electrical restitution, alternans and ventricular fibrillation initiation in the isolated heart. *Cardiovasc Res* 73, 750–760. doi: 10.1016/j.cardiores.2006.12.001

Nihongaki, Y., Kawano, F., Nakajima, T., and Sato, M. (2015). Photoactivatable CRISPR-Cas9 for optogenetic genome editing. *Nat Biotechnol* 33, 755–760. doi: 10.1038/nbt.3245

Oliveira-Mendes, B., Feliciangeli, S., Ménard, M., Chatelain, F., Alameh, M., Montnach, J., et al. (2021). A standardised hERG phenotyping pipeline to evaluate KCNH2 genetic variant pathogenicity. *Clin Transl Med* 11, e609. doi: 10.1002/ctm2.609

Parichy, D. M. (2015). Advancing biology through a deeper understanding of zebrafish ecology and evolution. *eLife* 4, e05635. doi: 10.7554/eLife.05635

Pavletich, N. P., and Pabo, C. O. (1991). Zinc Finger-DNA Recognition: Crystal Structure of a Zif268-DNA Complex at 2.1 Å. *Science* 252, 809–817. doi: 10.1126/science.2028256

Peal, D. S., Lynch, S. N., and Milan, D. J. (2011). Patterning and Development of the Atrioventricular Canal in Zebrafish. *J. of Cardiovasc. Trans. Res.* 4, 720–726. doi: 10.1007/s12265-011-9313-z

Pelka, K. E., Henn, K., Keck, A., Sapel, B., and Braunbeck, T. (2017). Size does matter – Determination of the critical molecular size for the uptake of chemicals across the chorion of zebrafish (*Danio rerio*) embryos. *Aquatic Toxicology* 185, 1–10. doi: 10.1016/j.aquatox.2016.12.015

Pérez-Riera, A. R., Barbosa-Barros, R., Daminello Raimundo, R., da Costa de Rezende Barbosa, M. P., Esposito Sorpreso, I. C., and de Abreu, L. C. (2017). The congenital long QT syndrome Type 3: An update. *Indian Pacing Electrophysiol J* 18, 25–35. doi: 10.1016/j.ipej.2017.10.011

Perissinotti, L. L., De Biase, P. M., Guo, J., Yang, P.-C., Lee, M. C., Clancy, C. E., et al. (2018). Determinants of Isoform-Specific Gating Kinetics of hERG1 Channel: Combined Experimental and Simulation Study. *Front. Physiol.* 9. doi: 10.3389/fphys.2018.00207

Pieczynski, J. N., Deets, A., McDuffee, A., and Lynn Kee, H. (2019). An undergraduate laboratory experience using CRISPR-cas9 technology to deactivate green fluorescent protein expression in Escherichia coli. *Biochemistry and Molecular Biology Education* 47, 145–155. doi: 10.1002/bmb.21206

Pieczynski, J. N., and Kee, H. L. (2021). “Designer babies?!” A CRISPR-based learning module for undergraduates built around the CCR5 gene. *Biochemistry and Molecular Biology Education* 49, 80–93. doi: 10.1002/bmb.21395

Poon, K.-L., Liebling, M., Kondrychyn, I., Brand, T., and Korzh, V. (2016). Development of the cardiac conduction system in zebrafish. *Gene Expression Patterns* 21, 89–96. doi: 10.1016/j.gep.2016.08.003

Poss, K. D., Wilson, L. G., and Keating, M. T. (2002). Heart Regeneration in Zebrafish. doi: 10.1126/science.1077857

Postema, P. G., and Wilde, A. A. M. (2014). The Measurement of the QT Interval. *Curr Cardiol Rev* 10, 287–294. doi: 10.2174/1573403X10666140514103612

Powell, E. M., Campbell, D. B., Stanwood, G. D., Davis, C., Noebels, J. L., and Levitt, P. (2003). Genetic Disruption of Cortical Interneuron Development Causes Region- and GABA Cell Type-Specific Deficits, Epilepsy, and Behavioral Dysfunction. *J. Neurosci.* 23, 622–631. doi: 10.1523/JNEUROSCI.23-02-00622.2003

Price, A. M., Kim, C. J., Burkholder, E. W., Fritz, A. V., and Wieman, C. E. (2021). A Detailed Characterization of the Expert Problem-Solving Process in Science and Engineering: Guidance for Teaching and Assessment. *LSE* 20, ar43. doi: 10.1187/cbe.20-12-0276

Printemps, R., Salvetat, C., Faivre, J., Grand, M. L., Bois, P., and Maati, H. M. ou (2019). Role of Cardiac IKs Current in Repolarization Reserve Process During Late Sodium Current (INaL) Activation. *Cardiology and Cardiovascular Medicine* 3, 168–185.

Priori, S. G., Schwartz, P. J., Napolitano, C., Bloise, R., Ronchetti, E., Grillo, M., et al. (2003). Risk Stratification in the Long-QT Syndrome. *New England Journal of Medicine* 348, 1866–1874. doi: 10.1056/NEJMoa022147

Prykhozhij, S. V., Fuller, C., Steele, S. L., Veinotte, C. J., Razaghi, B., Robitaille, J. M., et al. (2018). Optimized knock-in of point mutations in zebrafish using CRISPR/Cas9. *Nucleic Acids Research* 46, e102. doi: 10.1093/nar/gky512

Quiñonez-Silvero, C., Hübner, K., and Herzog, W. (2020). Development of the brain vasculature and the blood-brain barrier in zebrafish. *Developmental Biology* 457, 181–190. doi: 10.1016/j.ydbio.2019.03.005

Ramirez, C. L., Foley, J. E., Wright, D. A., Müller-Lerch, F., Rahman, S. H., Cornu, T. I., et al. (2008). Unexpected failure rates for modular assembly of engineered zinc fingers. *Nat Methods* 5, 374–375. doi: 10.1038/nmeth0508-374

Rappe, C., Andersson, R., Bergqvist, P.-A., Brohede, C., Hansson, M., Kjeller, L.-O., et al. (1987). Sources and Relative Importance of PCDD and PCDF Emissions. *Waste Manag Res* 5, 225–237. doi: 10.1177/0734242X8700500135

Rawat, P. S., Jaiswal, A., Khurana, A., Bhatti, J. S., and Navik, U. (2021). Doxorubicin-induced cardiotoxicity: An update on the molecular mechanism and novel therapeutic strategies

for effective management. *Biomedicine & Pharmacotherapy* 139, 111708. doi: 10.1016/j.biopha.2021.111708

Rayani, K., Lin, E., Craig, C., Lamothe, M., Shafaattalab, S., Gunawan, M., et al. (2018). Zebrafish as a model of mammalian cardiac function: Optically mapping the interplay of temperature and rate on voltage and calcium dynamics. *Prog Biophys Mol Biol* 138, 69–90. doi: 10.1016/j.pbiomolbio.2018.07.006

Reijnders, M. R. F., Kousi, M., van Woerden, G. M., Klein, M., Bralten, J., Mancini, G. M. S., et al. (2017). Variation in a range of mTOR-related genes associates with intracranial volume and intellectual disability. *Nat Commun* 8, 1052. doi: 10.1038/s41467-017-00933-6

Richardson, C. D., Ray, G. J., DeWitt, M. A., Curie, G. L., and Corn, J. E. (2016). Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. *Nat Biotechnol* 34, 339–344. doi: 10.1038/nbt.3481

Riesenberg, S., Helmbrecht, N., Kanis, P., Maricic, T., and Pääbo, S. (2022). Improved gRNA secondary structures allow editing of target sites resistant to CRISPR-Cas9 cleavage. *Nat Commun* 13, 489. doi: 10.1038/s41467-022-28137-7

Roden, D. M. (2008). Long-QT Syndrome. Available at: https://www-nejm-org.proxy.lib.sfu.ca/doi/10.1056/NEJMcp0706513 (Accessed October 23, 2020).

Roder, K., Kabakov, A., Moshal, K. S., Murphy, K. R., Xie, A., Dudley, S., et al. (2019). Trafficking of the human ether-a-go-go-related gene (hERG) potassium channel is regulated by the ubiquitin ligase rififylin (RFFL). *Journal of Biological Chemistry* 294, 351–360. doi: 10.1074/jbc.RA118.003852

Rosen, J. N., Sweeney, M. F., and Mably, J. D. (2009). Microinjection of Zebrafish Embryos to Analyze Gene Function. *JoVE (Journal of Visualized Experiments)*, e1115. doi: 10.3791/1115

Rossi, A., Kontarakis, Z., Gerri, C., Nolte, H., Hölper, S., Krüger, M., et al. (2015). Genetic compensation induced by deleterious mutations but not gene knockdowns. *Nature* 524, 230–233. doi: 10.1038/nature14580

Sacconi, L., Silvestri, L., Rodríguez, E. C., Armstrong, G. a. B., Pavone, F. S., Shrier, A., et al. (2020). KHz-rate volumetric voltage imaging of the whole zebrafish heart. *bioRxiv*, 2020.07.13.196063. doi: 10.1101/2020.07.13.196063

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989a). “Agarose Gel Electrophoresis,” in *Molecular Cloning: A Laboratory Manual - Book 1*, (Cold Spring Harbor Laboratory Press), 6.3-6.20.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989b). “Precipitation of Large RNAs with Lithium Chloride,” in *Molecular Cloning: A Laboratory Manual - Book 3*, (Cold Spring Harbor Laboratory Press), E.15.

Sankaran, S. M., Smith, J. D., and Roy, K. R. (2021). CRISPR-Cas9 Gene Editing in Yeast: A Molecular Biology and Bioinformatics Laboratory Module for Undergraduate and High School Students. *J Microbiol Biol Educ* 22, e00106-21. doi: 10.1128/jmbe.00106-21

Sarmah, S., and Marrs, J. A. (2016). Zebrafish as a Vertebrate Model System to Evaluate Effects of Environmental Toxicants on Cardiac Development and Function. *Int J Mol Sci* 17. doi: 10.3390/ijms17122123

Scholz, E. P., Niemer, N., Hassel, D., Zitron, E., Bürgers, H. F., Bloehs, R., et al. (2009). Biophysical properties of zebrafish ether-à-go-go related gene potassium channels. *Biochemical and Biophysical Research Communications* 381, 159–164. doi: 10.1016/j.bbrc.2009.02.042

Schwartz, P. J., Crotti, L., and Insolia, R. (2012a). Long-QT syndrome from genetics to management. *Circulation: Arrhythmia and Electrophysiology* 5, 868–877. doi: 10.1161/CIRCEP.111.962019

Schwartz, P. J., MD, L. C., and Insolia, R. (2012b). Long QT Syndrome: From Genetics to Management. *Circ Arrhythm Electrophysiol* 5, 868–877. doi: 10.1161/CIRCEP.111.962019

Schwartz, P. J., Stramba-Badiale, M., Crotti, L., Pedrazzini, M., Besana, A., Bosi, G., et al. (2009a). Prevalence of the Congenital Long QT Syndrome. *Circulation* 120, 1761–1767. doi: 10.1161/CIRCULATIONAHA.109.863209

Schwartz, P. J., Stramba-Badiale, M., Crotti, L., Pedrazzini, M., Besana, A., Bosi, G., et al. (2009b). Prevalence of the Congenital Long-QT Syndrome. *Circulation* 120, 1761–1767. doi: 10.1161/CIRCULATIONAHA.109.863209

Schwerdtfeger, C., and Linden, H. (2003). VIVID is a flavoprotein and serves as a fungal blue light photoreceptor for photoadaptation. *EMBO J* 22, 4846–4855. doi: 10.1093/emboj/cdg451

Sedmera, D., Reckova, M., deAlmeida, A., Sedmerova, M., Biermann, M., Volejnik, J., et al. (2003). Functional and morphological evidence for a ventricular conduction system in zebrafish and *Xenopus* hearts. *American Journal of Physiology-Heart and Circulatory Physiology* 284, H1152–H1160. doi: 10.1152/ajpheart.00870.2002

Sehgal, N., Sylves, M. E., Sahoo, A., Chow, J., Walker, S. E., Cullen, P. J., et al. (2018). CRISPR Gene Editing in Yeast: An Experimental Protocol for an Upper-Division Undergraduate Laboratory Course. *Biochemistry and Molecular Biology Education* 46, 592–601. doi: 10.1002/bmb.21175

Shah, A. N., Davey, C. F., Whitebirch, A. C., Miller, A. C., and Moens, C. B. (2015). Rapid reverse genetic screening using CRISPR in zebrafish. *Nat Methods* 12, 535–540. doi: 10.1038/nmeth.3360

Shah, M., and Carter, C. (2008). Long QT syndrome: A therapeutic challenge. *Ann Pediatr Cardiol* 1, 18–26. doi: 10.4103/0974-2069.41051

Shah, S. R., Park, K., and Alweis, R. (2019). Long QT Syndrome: A Comprehensive Review of the Literature and Current Evidence. *Current Problems in Cardiology* 44, 92–106. doi: 10.1016/j.cpcardiol.2018.04.002

Shaw, R.-S. (2013). The relationships among group size, participation, and performance of programming language learning supported with online forums. *Computers & Education* 62, 196–207. doi: 10.1016/j.compedu.2012.11.001

Shi, Y. P., Pang, Z., Venkateshappa, R., Gunawan, M., Kemp, J., Truong, E., et al. (2020). The hERG channel activator, RPR260243, enhances protective I_{Kr} current early in the refractory period reducing arrhythmogenicity in zebrafish hearts. *American Journal of Physiology-Heart and Circulatory Physiology* 319, H251–H261. doi: 10.1152/ajpheart.00038.2020

Shimizu, W., Ohe, T., Kurita, T., Kawade, M., Arakaki, Y., Aihara, N., et al. (1995). Effects of verapamil and propranolol on early afterdepolarizations and ventricular arrhythmias induced by epinephrine in congenital long QT syndrome. *J Am Coll Cardiol* 26, 1299–1309. doi: 10.1016/0735-1097(95)00313-4

Singleman, C., and Holtzman, N. G. (2014). Growth and Maturation in the Zebrafish, Danio Rerio: A Staging Tool for Teaching and Research. *Zebrafish* 11, 396–406. doi: 10.1089/zeb.2014.0976

Smith, P. L., Baukrowitz, T., and Yellen, G. (1996). The inward rectification mechanism of the HERG cardiac potassium channel. *Nature* 379, 833–836. doi: 10.1038/379833a0

Song, Y., and Cone, R. D. (2007). Creation of a genetic model of obesity in a teleost. *The FASEB Journal* 21, 2042–2049. doi: 10.1096/fj.06-7503com

Sorlien, E. L., Witucki, M. A., and Ogas, J. (2018). Efficient Production and Identification of CRISPR/Cas9-generated Gene Knockouts in the Model System Danio rerio. *JoVE (Journal of Visualized Experiments)*, e56969. doi: 10.3791/56969

Sourbron, J., Schneider, H., Kecskés, A., Liu, Y., Buening, E. M., Lagae, L., et al. (2016). Serotonergic Modulation as Effective Treatment for Dravet Syndrome in a Zebrafish Mutant Model. *ACS Chem. Neurosci.* 7, 588–598. doi: 10.1021/acschemneuro.5b00342

Stoyek, M. R., Quinn, T. A., Croll, R. P., and Smith, F. M. (2016). Zebrafish heart as a model to study the integrative autonomic control of pacemaker function. *Am J Physiol Heart Circ Physiol* 311, H676–H688. doi: 10.1152/ajpheart.00330.2016

Sugai, M., Horita, T., and Wada, Y. (2018). Identifying Optimal Group Size for Collaborative Argumentation Using SNS for Educational Purposes., in *2018 7th International Congress on Advanced Applied Informatics (IIAI-AAI)*, 226–231. doi: 10.1109/IIAI-AAI.2018.00051

Tanaka, Y., Hayashi, K., Fujino, N., Konno, T., Tada, H., Nakanishi, C., et al. (2019). Functional analysis of KCNH2 gene mutations of type 2 long QT syndrome in larval zebrafish using microscopy and electrocardiography. *Heart Vessels* 34, 159–166. doi: 10.1007/s00380-018-1231-4

Teame, T., Zhang, Z., Ran, C., Zhang, H., Yang, Y., Ding, Q., et al. (2019). The use of zebrafish (Danio rerio) as biomedical models. *Anim Front* 9, 68–77. doi: 10.1093/af/vfz020

Tessadori, F., Roessler, H. I., Savelberg, S. M. C., Chocron, S., Kamel, S. M., Duran, K. J., et al. (2018). Effective CRISPR/Cas9-based nucleotide editing in zebrafish to model human genetic cardiovascular disorders. *Disease Models & Mechanisms* 11, dmm035469. doi: 10.1242/dmm.035469

Tessadori, F., van Weerd, J. H., Burkhard, S. B., Verkerk, A. O., de Pater, E., Boukens, B. J., et al. (2012). Identification and Functional Characterization of Cardiac Pacemaker Cells in Zebrafish. *PLoS ONE* 7, e47644. doi: 10.1371/journal.pone.0047644

Tsai, C.-T., Wu, C.-K., Chiang, F.-T., Tseng, C.-D., Lee, J.-K., Yu, C.-C., et al. (2011). In-vitro recording of adult zebrafish heart electrocardiogram — A platform for pharmacological testing. *Clinica Chimica Acta* 412, 1963–1967. doi: 10.1016/j.cca.2011.07.002

Tseng, G.-N. (2001). IKr: The hERG Channel. *Journal of Molecular and Cellular Cardiology* 33, 835–849. doi: 10.1006/jmcc.2000.1317

Tveito, A., Jæger, K. H., Maleckar, M. M., Giles, W. R., and Wall, S. (2020). Computational translation of drug effects from animal experiments to human ventricular myocytes. *Scientific Reports* 10, 10537. doi: 10.1038/s41598-020-66910-0

Ulbricht, R. (2019). Resources: CRISPR/Cas9 in yeast: a multi-week laboratory exercise for undergraduate students. Available at: https://qubeshub.org/community/groups/coursesource/publications?id=2635&tab_active=about&v=1 (Accessed November 13, 2023).

van Opbergen, C. J. M., Koopman, C. D., Kok, B. J. M., Knöpfel, T., Renninger, S. L., Orger, M. B., et al. (2018a). Optogenetic sensors in the zebrafish heart: a novel in vivo electrophysiological tool to study cardiac arrhythmogenesis. *Theranostics* 8, 4750–4764. doi: 10.7150/thno.26108

van Opbergen, C. J. M., van der Voorn, S. M., Vos, M. A., de Boer, T. P., and van Veen, T. A. B. (2018b). Cardiac Ca2+ signalling in zebrafish: Translation of findings to man. *Progress in Biophysics and Molecular Biology* 138, 45–58. doi: 10.1016/j.pbiomolbio.2018.05.002

Vandenberg, J. I., Perry, M. D., Perrin, M. J., Mann, S. A., Ke, Y., and Hill, A. P. (2012). hERG K+ Channels: Structure, Function, and Clinical Significance. *Physiological Reviews* 92, 1393–1478. doi: 10.1152/physrev.00036.2011

Varshney, G. K., Pei, W., LaFave, M. C., Idol, J., Xu, L., Gallardo, V., et al. (2015). High-throughput gene targeting and phenotyping in zebrafish using CRISPR/Cas9. *Genome Res* 25, 1030–1042. doi: 10.1101/gr.186379.114

Verheule, S., and Kaese, S. (2013). Connexin diversity in the heart: insights from transgenic mouse models. *Front. Pharmacol.* 4. doi: 10.3389/fphar.2013.00081

Vornanen, M., and Hassinen, M. (2016). Zebrafish heart as a model for human cardiac electrophysiology. *Channels* 10, 101–110. doi: 10.1080/19336950.2015.1121335

Wang, J., Panáková, D., Kikuchi, K., Holdway, J. E., Gemberling, M., Burris, J. S., et al. (2011). The regenerative capacity of zebrafish reverses cardiac failure caused by genetic cardiomyocyte depletion. *Development* 138, 3421–3430. doi: 10.1242/dev.068601

Wang, X., Chen, X., and Yang, Y. (2012). Spatiotemporal control of gene expression by a light-switchable transgene system. *Nat Methods* 9, 266–269. doi: 10.1038/nmeth.1892

Warren, K. S., Baker, K., and Fishman, M. C. (2001). The *slow mo* mutation reduces pacemaker current and heart rate in adult zebrafish. *American Journal of Physiology-Heart and Circulatory Physiology* 281, H1711–H1719. doi: 10.1152/ajpheart.2001.281.4.H1711

Watts, S. A., Lawrence, C., Powell, M., and D'Abramo, L. R. (2016). The Vital Relationship Between Nutrition and Health in Zebrafish. *Zebrafish* 13, S-72-S-76. doi: 10.1089/zeb.2016.1299

Wierson, W. A., Welker, J. M., Almeida, M. P., Mann, C. M., Webster, D. A., Torrie, M. E., et al. (2020). Efficient targeted integration directed by short homology in zebrafish and mammalian cells. *eLife* 9, e53968. doi: 10.7554/eLife.53968

Wu, C., Sharma, K., Laster, K., Hersi, M., Torres, C., Lukas, T. J., et al. (2014). Kcnq1-5 (Kv7.1-5) potassium channel expression in the adult zebrafish. *BMC Physiol* 14, 1. doi: 10.1186/1472-6793-14-1

Xu, H., Li, C., Li, Y., Ng, G. H. B., Liu, C., Zhang, X., et al. (2015). Generation of Tg(cyp1a:gfp) Transgenic Zebrafish for Development of a Convenient and Sensitive In Vivo Assay for Aryl Hydrocarbon Receptor Activity. *Mar Biotechnol* 17, 831–840. doi: 10.1007/s10126-015-9669-1

Yu, F., Huang, J., Adlerz, K., Jadvar, H., Hamdan, M. H., Chi, N., et al. (2010). Evolving Cardiac Conduction Phenotypes in Developing Zebrafish Larvae: Implications to Drug Sensitivity. *Zebrafish* 7, 325–331. doi: 10.1089/zeb.2010.0658

Zarei, A., Razban, V., Hosseini, S. E., and Tabei, S. M. B. (2019). Creating cell and animal models of human disease by genome editing using CRISPR/Cas9. *The Journal of Gene Medicine* 21, e3082. doi: 10.1002/jgm.3082

Zhang, G., Dai, Z., and Dai, X. (2020a). C-RNNCrispr: Prediction of CRISPR/Cas9 sgRNA activity using convolutional and recurrent neural networks. *Comput Struct Biotechnol J* 18, 344–354. doi: 10.1016/j.csbj.2020.01.013

Zhang, P.-C., Llach, A., Sheng, X., Hove-Madsen, L., and Tibbits, G. (2010). Calcium handling in zebrafish ventricular myocytes. *American journal of physiology. Regulatory, integrative and comparative physiology* 300, R56-66. doi: 10.1152/ajpregu.00377.2010

Zhang, T., Zhao, S., Dong, F., Jia, Y., Chen, X., Sun, Y., et al. (2023). Novel Insight into the Mechanisms of Neurotoxicity Induced by 6:6 PFPiA through Disturbing the Gut–Brain Axis. *Environ. Sci. Technol.* 57, 1028–1038. doi: 10.1021/acs.est.2c04765

Zhang, Y., Kecskés, A., Copmans, D., Langlois, M., Crawford, A. D., Ceulemans, B., et al. (2015). Pharmacological Characterization of an Antisense Knockdown Zebrafish Model of Dravet Syndrome: Inhibition of Epileptic Seizures by the Serotonin Agonist Fenfluramine. *PLoS One* 10, e0125898. doi: 10.1371/journal.pone.0125898

Zhang, Y., Zhang, Z., and Ge, W. (2018). An efficient platform for generating somatic point mutations with germline transmission in the zebrafish by CRISPR/Cas9-mediated gene editing. *J Biol Chem* 293, 6611–6622. doi: 10.1074/jbc.RA117.001080

Zhang, Y., Zhao, G., Ahmed, F. Y. H., Yi, T., Hu, S., Cai, T., et al. (2020b). In silico Method in CRISPR/Cas System: An Expedite and Powerful Booster. *Frontiers in Oncology* 10. Available at: https://www.frontiersin.org/articles/10.3389/fonc.2020.584404 (Accessed December 17, 2023).

Zhao, Y., James, N. A., Beshay, A. R., Chang, E. E., Lin, A., Bashar, F., et al. (2020). Adult zebrafish ventricular electrical gradients as tissue mechanisms of ECG patterns under baseline vs. oxidative stress. *Cardiovascular Research*, cvaa238. doi: 10.1093/cvr/cvaa238

Zheng, Y., Zhao, J., Li, J., Guo, Z., Sheng, J., Ye, X., et al. (2021). SARS-CoV-2 spike protein causes blood coagulation and thrombosis by competitive binding to heparan sulfate. *Int J Biol Macromol* 193, 1124–1129. doi: 10.1016/j.ijbiomac.2021.10.112

Zoltowski, B. D., and Crane, B. R. (2008). Light activation of the LOV protein Vivid generates a rapidly exchanging dimer. *Biochemistry* 47, 7012–7019. doi: 10.1021/bi8007017

Zu, Y., Tong, X., Wang, Z., Liu, D., Pan, R., Li, Z., et al. (2013). TALEN-mediated precise genome modification by homologous recombination in zebrafish. *Nat Methods* 10, 329–331. doi: 10.1038/nmeth.2374