Dissecting the direct downstream targets of Hand2 during zebrafish cardiac development

A Thesis

submitted to

Indian Institute of Science Education and Research Pune in partial fulfillment of the requirements for the BS-MS Dual Degree Programme

by

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April, 2023

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Certificate

This is to certify that this dissertation entitled “Dissecting the direct downstream targets of Hand2 during zebrafish cardiac development” towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Rupal Gehlot at the Max Planck Institute for Heart and Lung Research under the supervision of Prof. Dr. Didier Stainier, Director and Scientific Member at the Max Planck Institute for Heart and Lung Research, during the academic year 2022-2023.

Supervisor’s signature

Committee:

Prof. Dr. Didier Stainier

Prof. Richa Rikhy

Declaration

I hereby declare that the matter embodied in the report entitled “Dissecting the direct downstream targets of Hand2 during zebrafish cardiac development” are the results of the work carried out by me at the Department of Developmental Genetics, Max Planck Institute for Heart and Lung Research, Germany, under the supervision of Prof. Dr. Didier Stainier and the same has not been submitted elsewhere for any other degree.

Student’s Signature

Table of Contents

List of Figures

List of Tables

Abstract

Defects in the development of the heart lead to Congenital Heart Diseases (CHD), making it important to study the developmental mechanisms and signaling pathways. Hand2 is one of the transcription factors that is found to be important in cardiac development. Hand2 is a basic helix-loop-helix (bHLH) transcription factor that is expressed in the lateral plate mesoderm (LPM) starting at the completion of gastrulation, and is expressed in the LPM of zebrafish, chick, frog, and mouse embryos. Its function is essential for many processes including precardiac cell differentiation into *clmc2* expressing myocardial precursors; it is important for the generation of *vmhc* expressing ventricular cells; for maintenance of myocardial *tbx5* expression and formation of the midline heart tube. Hand2 is also important for the early transition of the cardiogenic LPM, and in *hand2* mutants the progenitor cells residing in the anterior LPM fail to generate differentiated cardiomyocytes. Here, in this study we focused on generating a zebrafish transgenic line with an HA epitope tag inserted into the endogenous Hand2 protein to determine the downstream targets of Hand2 using ChIP-sequencing. We also looked at two possible downstream targets of Hand2, which are *pdgfra* and *timp2a,* and performed functional studies on both genes in the endocardium and myocardium.

Keywords

Cardiac development, Hand2, zebrafish, *pdgfra, timp2a*

Acknowledgments

This project would not have been possible without the help of many people involved. Prof. Didier Stainier who gave me the opportunity to work in his lab at the Max Planck Institute for Heart and Lung Research and for giving me his guidance at the important stages of my project. A very big thank you to my PhD mentor Yanli Xu, who constantly guided me throughout the project and for teaching me all the new techniques in the lab, and most importantly for being my biggest moral support in these months and for teaching me to work and think independently. Jonas Gretz, a bachelor student working with Yanli who helped me in some of the experiments at times, and now I see him as a good friend. I would like to thank all the lab technicians for their help in the lab as well as in the fish facility, and especially Sarah and Denis for performing some of my injections, and Carmen for all the fin clips. Our imaging specialist Dr. Radhan Ramadass for teaching me how to use the different microscopes and helping me with them whenever needed. All of the lab members who were there for help and discussions whenever needed. I would also like to thank my supervisor Prof. Richa Rikhy for being my advisor from IISER Pune and for her valuable feedback during mid-year, and Prof. Sanjeev Galande, Prof. Saikrishnan Kayarat, and Prof. Chaitanya Athale whose labs I have worked in during my undergraduate studies for providing me with all the knowledge. In the end, I would like to thank Max Planck Institute for providing me with all the resources and Indian Institute of Science Education and Research, Pune for giving me this platform to participate in such exciting research.

Contributions

Contributor name	Contributor role
Yanli Xu, Didier Stainier	Conceptualization Ideas
Yanli Xu	Methodology
-	Software
Rupal Gehlot	Validation
Yanli Xu, Rupal Gehlot	Formal analysis
Rupal Gehlot	Investigation
Didier Stainier	Resources
Rupal Gehlot	Data Curation
Rupal Gehlot	Writing - original draft preparation
Yanli Xu, Didier Stainier	Writing - review and editing
Rupal Gehlot	Visualization
Yanli Xu	Supervision
Didier Stainier	Project administration
Didier Stainier	Funding acquisition

Chapter 1 Introduction

1.1 Vertebrate heart development

Heart is the first body organ that is formed and functions during the vertebrate embryogenesis. Defects in the structure and function of the heart leads to Congenital Heart Disease (CHD), which is reported as one of the most common types of birth defect (Fahed et al., 2013). This makes it important to study the developmental processes and signaling pathways involved in the development of the heart.

The initiation of cardiac development starts during gastrulation at the end of second week of human development. During the process of gastrulation, the heart transforms from a two-layered heart to three-layered heart comprising of ectoderm, endoderm and mesoderm. First the mesoderm migrates from the site of gastrulation towards the anteriolateral border of the trilaminar embryonic disc. Upon migration these cells differentiate into their cell lineages. Wnt signalling plays an important role in preventing this differentiation to take place during gastrulation. Other factors like Bone morphogenic protein (BMP) growth factors are secreted by the endoderm and ectoderm are involved here in the differentiation of cardiomyocytes. As the development progresses, the structure forms a 3-dimensional structure because of looping which further transforms into an inverted Y shape. This looping of the heart begins at the beginning of the fourth week of development. When differentiated cardiomyocytes are added to the heart tube, this tube transforms into an S-shaped structure (Buijtendijk et al., 2020). The development of heart is a very complex process, which proceeds to formation of the four chambers of the heart and the valves. This process is quite similar in the mouse embryos as well, where the migration of precursor cells takes place at E7.5. At around E8.5, three regions can be seen which is the bulbus cordis (future right ventricle), primitive left ventricle and the common atrial chamber (Savolainen et al., 2009).

In zebrafish, the development of the heart begins as the cardiac progenitor cells specify within the anterior lateral plate mesoderm. The organ arises from two regions of dorsolateral mesoderm and these cardiogenic cells then migrate medially where they undergo differentiation. This gives rise to formation of a tube like structure which upon

further proliferation, differentiation and looping to form an S-shaped heart. The looping occurs as a result of cells from the second heart field being added to the inflow and outflow poles. This tube is made up of inner endocardial layer and outer myocardial layer (Stainier et al., 1993). With time, the structure develops into one ventricle and one atrium, where the ventricle is characterized by more rapid myocardiocyte proliferation as compared to the atrium (MARKWALD et al., 1997). After cardiac looping, the endocardial cushions begin to form in the outflow tract (OFT) and the atrioventricular canal (AVC) to prevent retrograde blood flow. This is responsible for forming the chambers of the heart (McCulley & Black, 2012) (Figure 1).

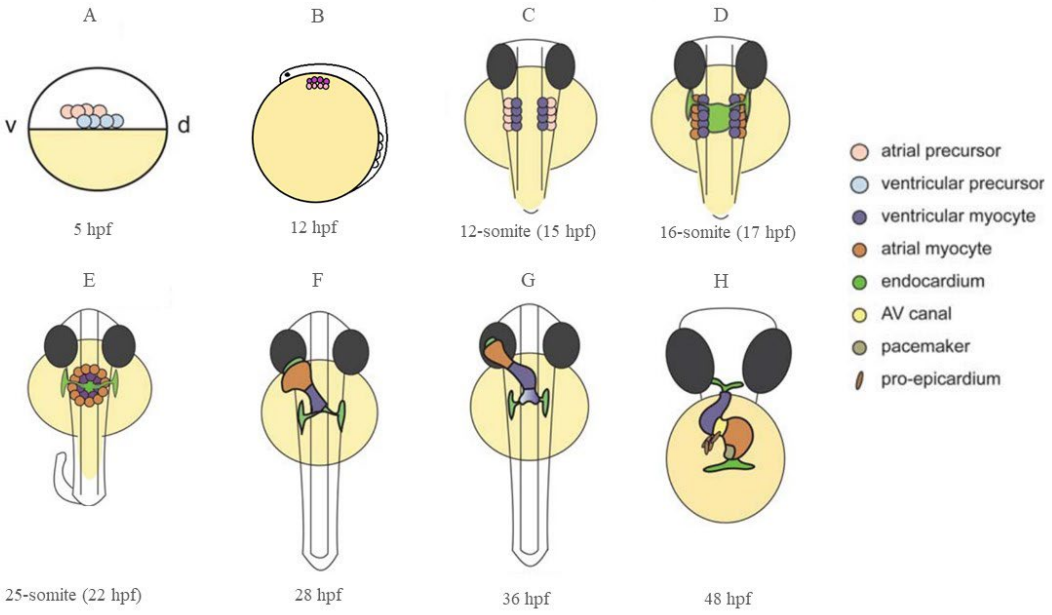


Figure 1 Different stages of heart development in zebrafish. (Bakkers, 2011; Stainier, 2001)

The cardiac muscle cells arise from the lateral plate mesoderm (LPM), and it has been found that there are several transcription factors that play a role in the differentiation of the myocardial precursors. One such homeobox-containing transcription factor is Nkx2.5 which is found to be expressed in the differentiating myocardial cells in vertebrates. The homeobox is an evolutionarily conserved DNA sequence motif which

was first found in Drosophila melanogaster (Hayashi & Scott, 1990). In addition to this, the other group of transcription factors include GATA family zinc finger proteins, MEF2 factors, MADS box proteins, T-box proteins etc. are important for heart development in processes of cardiac progenitor specification and proliferation (McCulley & Black, 2012). These transcription factors, mainly Nkx2.5, GATA4 and Tbx5 are well characterized and studied in patients suffering from the congenital heart disease and looking at these transcription factor pathways and studying them in depth becomes important. Nkx2.5 is found to work together with a basic helix loop helix (bHLH) protein Hand2 to activate Irx4, which plays a very important role for determining ventricular identity (Figure 2).

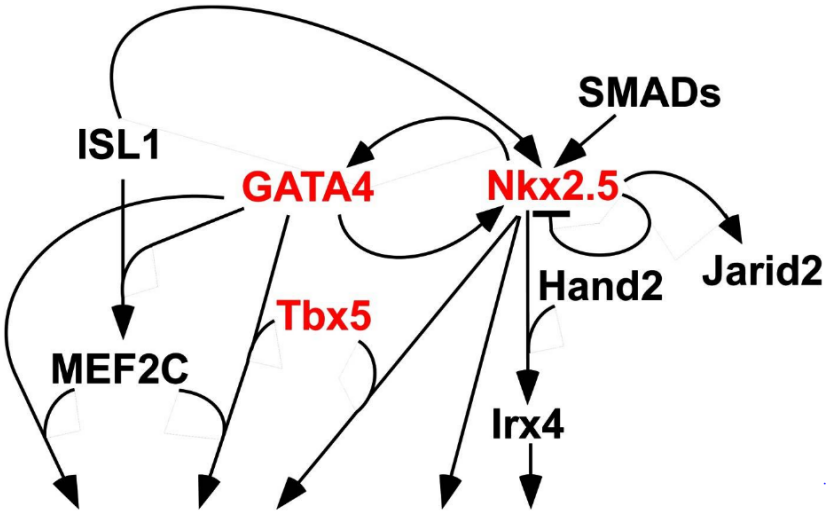


Figure 2 Different transcription factor pathways. (McCulley & Black, 2012)

1.2 Zebrafish as a model organism to study the vertebrate cardiac development

In the past few years, using zebrafish (*Danio rerio*) as a model organism to study heart development and defects has increased to a great extent. Its use as a model organism to study modern biological functions was first done by George Streesinger and his colleagues in the year 1981, where they used it to study the production of clones of the homozygous diploid zebrafish (Streisinger et al., 1981).

They serve as a great model organism as they take only 3-4 months to grow to adulthood, and further on, the females can lay eggs in weekly intervals. The size of the

fish is very small i.e. approximately 3 cm, so it becomes easy to maintain them. The fertilization is external and the development occurs outside the embryos, which are transparent during development, which makes studying the different developmental stages very easy (Streisinger et al., 1981). Each fish can lay a few hundreds of eggs at a time and one can easily screen for mutants because even at a very early stage the fish shows many behavioral and morphological traits of the parent.

The embryos are transparent until at least 5 days post fertilization (dpf), to the extent that one can see the living cells and developing organs just under a microscope without any staining. Using different techniques, even cellular and subcellular organelles can be visualized with much detail. To observe the pattern of gene expression one can perform whole mount *in situ* of the embryo up to 72 hpf. Techniques using fluorescent probes can be used for studying different processes including the cell lineage tracking (Kimmel & Law, 1985; Kozlowski & Weinberg, 2000). In addition, zebrafish development in the early stages is not dependent on a functional cardiovascular system because of which looking at the heart defects in the initial phases of vertebrate development is very easy (Bakkers, 2011).

1.3 Hand2 plays important role during cardiac development

In vertebrates, a lot of different tissue types originate from the Lateral Plate Mesoderm (LPM) which includes the heart, smooth muscles, connective tissue, endothelium etc. Now, when we look at zebrafish, it is found that the *hands off* (*han*) locus plays an important role in the development of two structures that originate from the LPM. A mutation in *han* can lead to severe defects in development, especially seen in myocardial differentiation, patterning and morphogenesis (Yelon et al., 2000). The *han* locus encodes for Hand2 which is a basic helix-loop-helix (bHLH) transcription factor. It possesses three functional domains: the transcriptional activation domain, bHLH and a basic domain (Schindler et al., 2014). The transcriptional activation domain, short TAD, is essential for downstream target gene transcription and the basic domain promotes

DNA binding. In contrast the bHLH domain is responsible for binding to a target DNA motif and for protein–protein interactions (Wang et al., 2017).

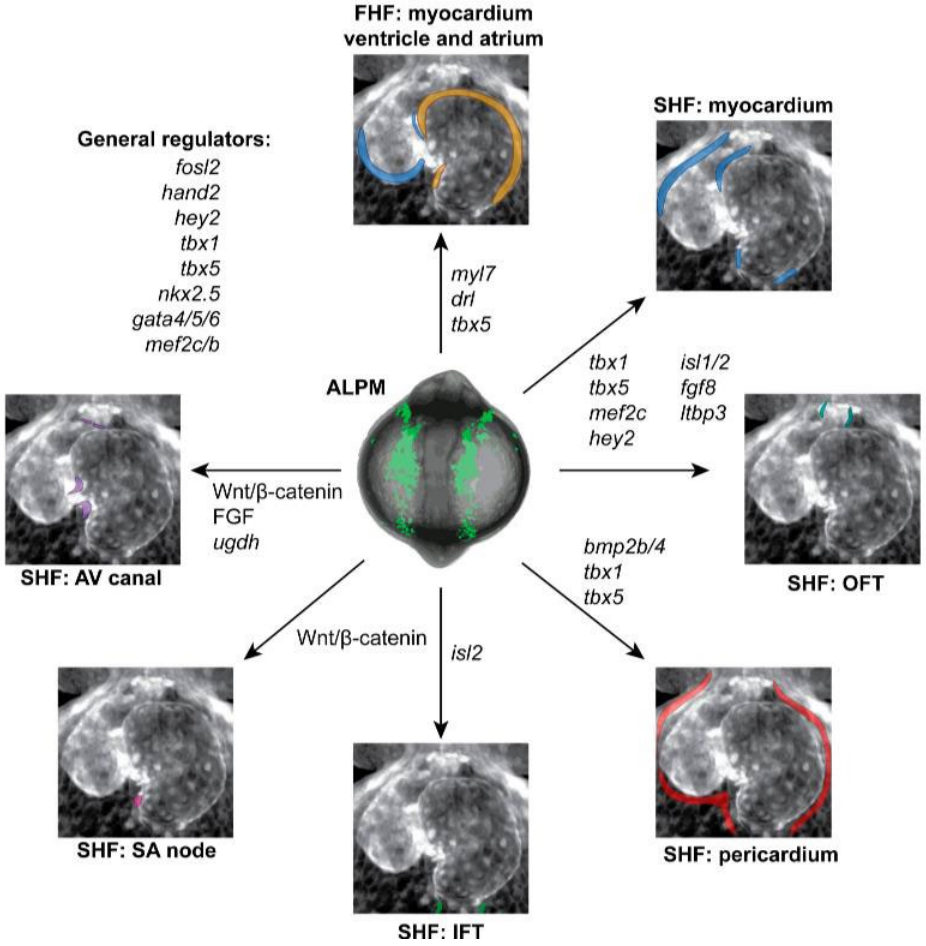


Figure 3 Genes contributing to early heart development in zebrafish (Kemmler et al., 2021).

Studies have shown that overexpression (OE) of *hand2* can enhance the production of cardiomyocytes resulting in an enlarged heart. This confirms that Hand2 plays an important role in heart development, but the mechanism via which Hand2 acts is believed to be through binding of Hand2 to multimeric complexes (Schindler et al., 2014). The *cardiac myosin light chain 2* (*clmc2*), which marks differentiating cardiomyocytes is used to understand these mechanisms. The few major discoveries in this field suggest that Hand2 function is essential for the differentiation of pre cardiac

cells to *clmc2* expressing myocardial precursors, maintenance of myocardial *tbx5* expression, formation of midline heart tube and in generation of *ventricular myosin heavy chain* (*vmhc*) expressing ventricular cells (Yelon et al., 2000).

By fate mapping it is found that though the complete ALPM possess the ability of forming the heart region, only the *hand2* expressing portion produces the myocardium (Schoenebeck et al., 2007). However, the downstream targets of Hand2 and how it influences the differentiation process of different cardiac cell types are still unclear. To investigate this question, we would insert a 3xHA and 1XHA epitope tag into the endogenous Hand2 protein to first determine the range of genomic regions enriched in endogenous Hand2 chromatin complexes (Laurent et al., 2017).

1.4 Crispr/Cas9 mediate KI techniques

HA tag is derived from the human influenza virus HA protein, and is used extensively as a general antibody epitope tag. HA tag antibodies provide a method for detection and purification of tagged protein without a protein-specific antibody or probe. Here, we would generate endogenous protein tagging allele of *hand2* by oligonucleotide-mediated knock-in approach (Burg et al., 2018) and simplified CRISPR / Cas9 knock- in approach in zebrafish (Nath et al., 2022).

We can study the role a gene plays in an organism by deleting the gene and checking the phenotype, which we call as the reverse genetics. However, the gene that we want to study in one specific cell type may also have an important role in other cell types or tissues. Therefore, for studying the role of a gene in one specific cell type we can create very specific edits in the genome. The recently discovered method by using CRISPR/Cas9 makes it possible to perform such an editing.

To dissect the directly downstream targets of hand2 during zebrafish cardiac development by Hand2-CHIP-seq dataset, we tried to generate an endogenous HA KI hand2 allele. There are 2 methods to do so:

1. Insertion of HA in between ATG and *hand2* coding sequence (Figure 4).

Figure 4 Schematic representation of the HA insertion. The yellow and green box indicates *hand2* exons and line between the two exons is intron, before the first exon is the 5 UTR and after the second one is 3 UTR. Here, the Kozak sequence functions as the protein translation initiation site.

2. Insertion of the complete sequence – loxP – 3 x HA – *hand2* CDS – p2a BFP – stop –loxP – 2a mCherry – (as shown in Figure 5). In this we want our inserted *hand2* coding sequence to be transcribed and translated along with the HA tag. The stop sign in the end will ensure that the already existing *hand2* coding sequence is not transcribed, but to be sure we can generate some mutations in the organism's *hand2* coding sequence or insert this cassette in the first exon of hand2.

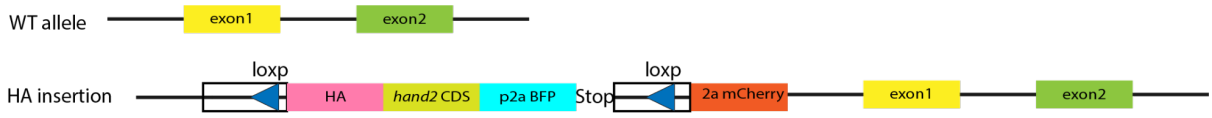


Figure 5 Schematic representation of the HA insertion along with *hand2* CDS. The lines represent the introns and the insertion sequence will consist of start sequence, HA tag, *hand2* coding sequence and stop sequence.

1.5 Platelet derived growth factor receptor type alpha (Pdgfra) is important for cardiac fusion

Platelet derived growth factors (Pdgf) and their receptors (Pdgfr) are known to perform their functions as growth factors and tyrosine kinase receptors for a long time. Pdgf affect a variety of cellular responses including proliferation, migration, differentiation,

deposition of extracellular factors, etc. The Pdgfr-alpha signaling plays an important role in processes like gastrulation and development. Pdgfr-beta signaling on the other hand plays a role in blood vessel formation and early hematopoiesis (Andrae et al., 2008; Chong et al., 2013). Each receptor in the tyrosine kinase receptors has five immunoglobulin repeats in the extracellular ligand binding domain and a split tyrosine kinase domain in the cytoplasmic region. The binding of the Pdgf to Pdgfr is responsible for stimulating the tyrosine kinase activity (Hoch & Soriano, 2003). There have been several studies done where they knock-out (KO) of Pdgfr-alpha has been found to be lethal for the mouse due to some defects observed in the heart. One such study showed that the loss of *pdgfra* function gives defects in the endocardial and myocardial migration to the midline (El-Rass et al., 2017). During cardiac fusion there are some factors secreted by the endoderm that give signal to the cardiomyocytes to move towards the midline. Time lapse analysis of individual cardiomyocyte trajectories reveals misdirected cells in zebrafish *pdgfrα* mutants, which suggests that the Pdgf that is produced by the endoderm fails to interact with the Pdgf receptors on the myocardial cells, leading to failure of these cells to move towards the middle of the embryo (Figure 6) (Bloomekatz et al., 2017).

Yelon and his colleagues showed that global KO of *pdgfra* phenocopies the *hand2* mutant phenotype, i.e. the myocardium fails to fuse in the midline. Combining this observation with the RNA-seq analysis by Yanli Xu which shows *pdgfra* is downregulated in the *hand2 FLD* mutant cardiomyocytes, we hypothesized that *pdgfra* might be a downstream target of Hand2.

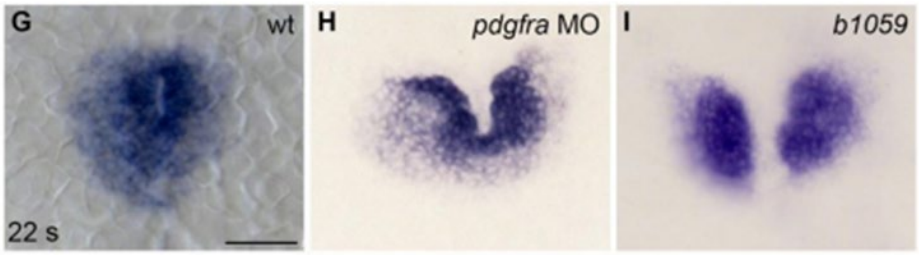


Figure 6 The cardiac fusion defects in the loss-of-function mutation in *pdgfra.* Left most block shows the wildtype, in which we can observe a disc like structure. Right

most block showing the mutant phenotype in the homozygous embryos and the one in center showing migration (Bloomekatz et al., 2017).

1.6 Tissue Inhibitor of Metalloproteinase-2a (*timp-2a*) as a potential downstream target of Hand2.

Matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of MMPs (TIMPs) are known to play an important role in degradation of extracellular matrix (ECM) components. Recently studies have shown that this inflammation can lead to valve defects (Fondard et al., 2005). In the RNA-seq data of the *hand2* endocardial mutants it was found that *timp-2a* was upregulated. There is not much knowledge available in literature regarding this but one of the previous lab member Alessandra Gentile has seen in her experiments that OE (over expression) of *timp2a* in endocardium gives rise to a phenotype that is similar to *hand2* endocardial mutant phenotype. For this reason, this is a potential candidate to study as the downstream targets of Hand2 in the endocardium.

Chapter 2 Materials and Methods

2.1 Zebrafish Husbandry

Zebrafish husbandry was performed in accordance with the Max Planck Gesellschaft institutional and German national ethical and animal welfare regulations. All zebrafish used in the study were derived from the wild-type line derived from line A and line B (AB). Females and males both were used for the experiments. Rearing systems and incubators were maintained at 28°C with a light cycle of 10 hour dark/14 hour light. The following lines were used in this study: *Tg(myl7: pdgfrα-p2a-GFP)*, *hand2 FLD, Tg(fli1a: pdgfrα, GFP), Tg(myl7:EGFP)*^{twu26}*, Tg(myl7: hand2); hand2 FLD, Tg(fli1a: hand2); hand2 FLD, Tg(eh22: hand2); hand2 FLD, Tg(eh22: pdgfra); hand2 FLD, Tg(myl7: EGFP); hand2 FLD, Tg(kdrl:Cre); hand2 FLD, Tg(kdrl:Cre-mCherry); hand2*$^{floxed/floxed}$, *Tg(myl7:EGFP-Has.HRAS)*^{883} *and Tg(kdrl:NLS-mCherry)*^{is4}*.*

2.2 Agarose gel electrophoresis

Agarose gels were prepared by adding 0.8 to 2% agarose (depending on the band size to visualize) in 1 X TBE buffer and 2.5 µl SyBr Safe per 100 ml TBE buffer. The gel was run for around 30-45 minutes. Gels were visualized with a blue light transilluminator and a picture of the gel was taking using a UV light in a gel imager system.

2.3 PCR product purification from agarose gel

The band of interest from the PCR loaded on the gel was purified using a GeneJET gel extraction kit and following manufacturer´s instructions. The purified DNA was eluted in 7 to 15 µL of nuclease-free water.

2.4 Measurement of nucleic acid concentrations

The concentration of nucleic acids was measured using 1 µL of sample loaded on a spectrophotometer. Absorption at 230 nm, 260 nm, and 280 nm was measured the concentration was then calculated according to Lamber-Beer law. The quality of RNA and DNA was evaluated based on 260/280 and 260/230 ratios: > 2.0 for RNA, > 1.8 for DNA for the former case; around 2.0-2.2 for both nucleic acids for the latter.

2.5 Plasmid generation

The backbone ptol2--0.2myl7:-desmb-p2a-gfp was cut by restriction enzymes KpnI and Afl II to obtain a 2949 bp backbone. This digested plasmid was then run on a 1% agarose gel to verify the correct digestion and the band of the correct site was extracted using GeneJET PCR Purification Kit (Thermo Scientific). Primers were designed to amplify the insert, to generate PCR products containing ends homologous to those of the vector. The desired target DNA consisted of *hand2* CDS, *t2a-TagBFP*, *mCherry-BGH*. [Note: The orientation of insert in the final product will depend on the homology sequence that we include in the forward and reverse primer.]

Reaction Set up (From In-Fusion HD Cloning Kit User Manual)

Reaction Component	Cloning Reaction
Purified PCR fragment	10-200 ng
Linearized vector	50-200 ng
5X In-Fusion HD Enzyme Premix	2 µl
Deionized Water	To 10 µl

This reaction was incubated at 50℃ for 15 minutes and then a transformation procedure was performed.

2.5.1 Transformation

Thaw the competent cells on ice just before use and then add the In-Fusion reaction mix to the competent cells (approx. 20 µl). This was then placed on ice for 30 minutes. We then heat-shock these cells for exactly 60 seconds at 42℃, and then place them on ice for 1-2 minutes. SOC medium (2% triptone, 0.5% yeast extract, 0.05% Sodium chloride, 0.0186% Potassium chloride rest MilliQ to adjust volume, pH adjusted to 7.0 with Sodium hydroxide) is then added to the tube to make the final volume to 100 µl. The tubes were incubated at 37℃, 300 rpm for 45 minutes. Spread each reaction on LB plates with appropriate antibiotics for the cloning vector. All the plates were incubated at 37℃ overnight (O/N). Plasmid was then isolated using GeneJET Plasmid Miniprep

Kit (Thermo Scientific). Upon purification the plasmid was sent for sequencing to check if the infusion was successful.

2.6 CRISPR/Cas9 mediated genome editing of desired sequence

2.6.1 Preparation of PCR product to be injected

A PCR reaction (using primeSTAR Max Premix) was run with primers with 5´ AmC6 end-protections for generating the knock-in. The correct band was selected and purified using GeneJET PCR Purification Kit (Thermo Scientific).

[Note: This new technique with the modified primers was used to ensure that the new primers generated improve the PCR amplicons harboring the short or long homologous arms (Mi & Andersson, 2023).]

2.6.2 Designing and synthesizing the single guide RNA (sgRNA) (based on protocol by James Gagnon-Schier Lab and further modified by Nana Fukuda and Kenny Mattnonet- Stainier Lab)

The sgRNA site is identified for targeting. Primers are then designed to amplify the target exon. Then the gRNA sites can be either selected manually or CRISPRScan or CHOPCHOP can be used for that purpose. The synthesis of sgRNA was performed using sgRNA oligo and constant oligo.

sgRNA oligo (100μm stock concentration)	1 μl
Constant oligo (100μm stock concentration)	1 μl
H_2O	8 μl

These oligos were annealed in the cycler at 95℃ for 5 min, 95℃ →85℃ for -2℃/sec, 85℃→25℃ for -0.1℃/sec and 4℃ hold. T4 polymerase mix (dNTPs, 10x NEB Buffer 2.1[2], T4 NEB DNA Polymerase and H2O) was then added to the tube and put at 12℃ for 20 minutes. This template was purified using the QIAquick nucleotide removal kit (QIAGEN Ref. 28304) and was eluted in 20 μl H2O. To verify the correct size 1 μl was run on a gel. [Note: these oligos can be stored at 4℃]

The Ambion MEGAscript™ T7 Kit was used to set the reaction for sgRNA synthesis.

ATP	1 µl
GTP	1 µl
CTP	1 µl
UTP	1 µl
10X buffer	1 µl
T7 enzyme mix	1 µl
H_2O	2 µl
Template	2 µl

This was incubated at 37℃ for 4 hrs. 1 µl DNase was added and incubated at 37℃ for 15 minutes. The RNA was extracted using RNA clean up kit (Zymo) and eluted in 12 µl nuclease free water.

2.6.3 Microinjection

The synthesized sgRNAs were injected into the wildtype embryos; around 4-8 for each, and checked which sgRNAs out of all synthesized work with a good efficiency using HRM. These sgRNAs are then selected for the injection.

	Sequence	**GC content**	**T_m(°C)**
1XHA 5#3 Donor	cgtactccgtctgtggttcgccgtagggtatagacaagtctgtcgccGCCACCatgtacccatacgatgttcctgactatgcgggaagtttagttggagggtttccccaccaccctgtgatgcaTcatgacggctattccttcgctgctgcc	55%	81
3XHA 5#3 Donor	ggtatagacaagtctgtcgccatgtacccatacgatgttcctgactatgcgggctatccctatgacgtcccggactatgcagcatcctatccatatgacgttccagattacgctggaagtttagttggagggtttccccaccaccctgtgatgcaTcatgacggctattcctcgctgct	51%	80

Genotyping Primer Forward (#79)	cgtcaagtaaaagtacgcgcaa	45%	59
Genotyping Primer Reverse (#86)	agccacccatgaaaatagggg	52%	59
5 UTR sgRNA 3 ki loxp	GGCACGTTGTGGAATCAATG	50%	54

Table 1 Sequences for generating HA KI line.

HRM Primers	**Sequence**	**GC content**	**T_m(°C)**
1 F	CATAATCCTGCTCGATGCAAAAGTGT	42%	60
1 F1	CTGCTCGATGCAAAAGTGTCCA	50%	59
1 R	CATTCGATTTGATAACTTGT	30%	48
2/3 F	CAAGAACACATTTAATTAGTCT	27%	50
2/3 R	GGTATTGTATGTTTCAATTAG	29%	47
4/5/6 F	CTCTAATAAGCCCAAGGGTCCTCA	50%	58
4/5/6 R	GAATCAATTACAGGTTGCTGTTGCA	40%	58
7/8 F	GACCATATGTTTTTGTAAAGTCTA	29%	50
7/8 R	CACTTTTAATGGGTCACTTGAC	41%	53
9 F	CAGGCTTAGGTAGGAAAGAAATCA	42%	55
9 R	GAGCGTGAAGAATGGAGCGTGAT	52%	63
10 F	GCACACTAAATATAGGCTATGTG	39%	53
10 R	CAGAAACACACTGTACTCGAC	48%	56
11/12 F1	CTGAAATAGCTCATTCAGCAGA	41%	55
11/12 F	GCTCATTCAGCAGAAAAAAATTG	35%	53
11/12 R1	CGAGAACCACTAAATGTGTTTCTTGTG	41%	58

11/12 R	gaTTAAAACGAGAACCACTAAATGTG	35%	55

Table 2 *pdgfra* enhancer KO HRM primers.

sgRNA	Sequence	GC content	T_m(°C)
#1	GGGTCCATTGTGAGCATTGA	50%	54
#2	GGAAGTGCAATTATGTTCAG	40%	49
#3	GGAGGTTCAACCGTTTTCGA	50%	54
#4	GGGAGCGCAGCACAAGGGAA	65%	61
#5	GGCGGAGGAGCGCAGCACAA	70%	61
#6	GGGGCCGTGGAGCAGGACGG	80%	66
#7	GGGGGCCCTTTTGGAATAAA	50%	53
#8	GGGGCCCTTTTGGAATAAA	47%	53
#9	GGGGAATGCACAATTACATT	40%	53
#10	GGAGATAATAATGGATAGTA	30%	43
#11	GGCACGCGTATTTACGCAGG	60%	59
#12	GGGCACGCGTATTTACGCAG	60%	57

Table 3 sgRNAs for *pdgfra* enhancer KO

HRM Primers	Sequence	GC content	T_m(°C)
1/2/3 F1	gtcagatactgaagtgaggcga	50%	58
1/2/3 F	gaagtgaggcgaaaatgaagagcgt	48%	63
1/2/3 R1	gaaccggggaacaactgcacgcttct	58%	68
1/2/3 R	gttgaggatgaaccggggaacaact	52%	64
4 F	gatcgcagaagcgtgcagttgtt	52%	62
4 R	cattgtgacgtagcatcgttcgga	50%	61
5 F	cgtctacctgcctgcttttattgct	48%	61
5 R	gaaacagtaccagcacaaccagt	48%	59

Table 4 *timp2a* KO HRM primers.

sgRNA	Sequence	GC content	T_m(°C)
#1	GGTGCTGGTACTGTTTCGGG	60%	59
#2	GGGTTGTGCTGGTACTGTTT	50%	57
#3	GGGCTGGTACTGTTTCGGGT	60%	58
#4	GGATTGCAGAACGCCTGTTG	55%	57
#5	GGGCGAAAATGAAGAGCGTC	55%	56

Table 5 sgRNAs for *timp2a* KO.

Injection mix (100ng/µl Cas9, 20-50 ng/µl PCR product, 50-75 ng/µl targeted sgRNA, 1% PhenoRed and H_2O), volume 1nl per embryo was injected into cell of the 1 cell stage wildtype embryos.

2.7 Founder Screening

Zebrafish that you want to screen is set up for crossing with a wildtype fish of opposite gender, keeping a divider in the tank a day before collecting the eggs. Next day, the divider is removed and approximately 30 minutes after, you can collect the embryos. These collected embryos are cleaned (removing the unfertilized and poorly developed embryos), and kept in a 28.5℃ incubator. On day three of the experiment i.e. at 24 hpf you can already put the embryos in a tube and extract the genomic DNA and set up PCR with correct primers to screen for founders. The correct band from the potential founder is purified and sent for sequencing after T-vector insertion.

T-vector ligation

2X Rapid Ligation Buffer	5 µl
T-easy vector	1 µl
PCR product	X µl
T4 DNA ligase	1 µl
H_2O	To 10 µl

Calculate for X using the following equation,

$$\left(\frac{ng\ of\ vector\ *\ kb\ size\ of\ insert}{kb\ size\ of\ vector}\right) * insert{:}\ vector\ molar\ ratio\ = ng\ of\ insert$$

You can also use the NEB calculator to calculate the ng of insert. The ratio of insert: vector used was 3:1. The reaction was then mixed by pipetting and incubated at room temperature for 1 hour or O/N at 4℃. Transformation was performed and the reaction mix was spread on a LB/ampicillin/IPTG/X-Gal plate (50 µl X-Gal on one plate) and incubated at 37°C for 30 minutes. Pick up white colonies from the blue-white colonies and perform miniprep using the GeneJET Plasmid Miniprep Kit (Thermo Scientific).

2.8 Whole mount *in situ* hybridization Antisense probe synthesis

A selected part of the gene of interest was amplified by PCR using a couple of primers where the reverse primer has a T7 promoter sequence in the front. The product was purified from a gel as described above in section 2.3. The probe synthesis was performed as follows:

3 µL of Template DNA (100-200 ng)

2 µL of 10X transcription buffer

1 µL of DIG-RNA labelling mix

2 µL of RNAsin (40 U/µL)

2 µL of T7 RNA polymerase (20 U/µL)

20 µL of nuclease-free H_2O

The mix was incubated for 2 hours at 37° C. Then, 1µL of DNase was added and incubated for 30 minutes at 37° C. The probe was purified using RNA clean and concentrator kit according to manufacturer's instructions. The RNA probe was eluted in 11 µL of nuclease-free water. The probe was stored as stock solution of 1 µg/mL of hybridization buffer at -20° C, while the rest of the RNA was stored at -80° C.

2.8.1 Embryo preparation

Embryos were treated with PTU and dechorionated as described before. At the desired stage, the embryos were fixed with 4% PFA overnight at 4° C or 2 hours at room temperature. Next, the samples were washed in 0.1% PBST 3 times of 15 minutes each and dehydrated with sequential dilution of methanol in 0.1% PBST, in the end wash in 100% methanol for 5 minutes at room temperature. The samples were stored at -20° C for at least one overnight.

2.8.2 Day 1

The embryos were rehydrated with dilution of methanol in 0.1% PBST for 5 minutes each. After 3 washes of 5 minutes each with 0.1% PBST, the samples were permeabilized using proteinase K (10 µg/mL) in 0.1% PBST at room temperature for 10 minutes (24 hpf stage) or 15 minutes (36 and 48 hpf stages). To stop the digestion, fixation in 4% PFA was performed, followed by 4 washes in 0.1% PBST for 5 minutes. The samples were then prehybridized with 250 µL of HM+ (Hybridization Mix) for 2-5 hours at 70° C. the HM+ was discarded and replaced with 250 µL of the RNA probe stock solution and left overnight at 70° C.

2.8.3 Day 2

The RNA probe stock solution was collected in a 2 mL tube and stored at -20° C for further use. The HM+ was gradually changed with 2X SSC with series of washes of 15 minutes at 70° C. Next, the embryos were washed twice with 0.2X SSC. The SSC was gradually replaced with 0.1% PBST through a series of washes of 10 minutes at room temperature. The samples were then incubated with blocking buffer for 3-4 hours at room temperature in slow agitation. The blocking buffer was replaced by blocking buffer with anti-DIG antibody diluted 1:10,000 and incubated at 4° C overnight.

2.8.4 Day 3

The antibody was discarded and the embryos were washed 6 times of 15 minutes each at room temperature with 0.1% PBST. The samples were then washed with alkaline Tris buffer 3 times for 5 minutes each. The solution was replaced with 500 µL BM

purple AP substrate solution and kept in dark at room temperature in slow agitation. When the desired staining is reached, the reaction was stopped with 0.1% PBST and washed 3 times for 15 minutes at room temperature.

2.8.5 Imaging *in situ* samples

The images were captured on Nikon SMZ25 (High-end Stereoscopic Microscope). After switching on the PC, open the app Nis-Elements BR 4.30.60 64 bit. Go to live and set the exposure and other things like zoom and focus. The fish was mounted on the injection plate and eyelash was used for perfecting the orientation of the embryo. The images were saved as tif as well as nd2 file format.

[Note: Capture all images at the same zoom]

For genotyping these imaged embryos, they were placed in a tube and let to completely air dry. Add DNA extraction buffer (1M KCl, 0.5M Tris pH 8.0, 0.5 M EDTA, 10% IGEPAL, 10% Tween20, MQW, 10mg/ml proteinase K) to this. This was incubated for 2 hours at 55°C and 99°C for 5 minutes.

In the second method, which proved to be more efficient for genotyping, the embryos after *in situ* were dehydrated through two washes with methanol. Then methanol was replaced with 1ml of Murray's solution (mixture of 2:1 benzyl benzoate and benzyl alcohol) [Note: Murray's buffer is toxic so handle with care], and incubated O/N at 4°C. For imaging, the embryos were transferred to a glass dish and for each embryo the tail and yolk were removed using a needle and forceps. The remaining embryo was transferred to a glass slide and embedded in Murray's solution and covered with a cover slip. Each embryo was imaged at 10X magnification with the Axio Imager.A2 and processed with Zen blue. The embryos were positioned to get a clear view of the cardiac cells by rotating the cover slip. All the imaged embryos were transferred individually to a tube filled with methanol. The DNA was extracted as mentioned before.

Genotype for mutants using the KASP reaction.

Reagent	Volume
2X KASP master mix	5 µl

Gene Specific KASP assay mix	0.14 µl
DNA	1 µl
MQW	3.86 µl

The reactions were set up in a 96 well plate and include at least 3 WT controls and 3 no-template controls (NTCs) for each plate. qPCR was run and HEX and FAM were selected. WT and NTC were marked and data obtained was further analyzed.

2.9 Immunostaining

2.9.1 Embryo preparation

The embryos were treated with PTU and after removing their chorions were fixed at the right stage O/N in 2 ml 4% paraformaldehyde in 2ml tubes at 4°C. This process is same as mentioned before.

2.9.2 Day 1

3X 15 minutes PBS/0.1% Tween washes were given. The samples were permeabilized using proteinase K (10 µg/mL) in 0.1% PBST at room temperature for 40 minutes (96 hpf stage). They were washed in 1ml PBDT (PBS, 1% BSA, 1% DMSO, 0.5% Triton-X 100), and blocked with the B-buffer (for 5ml: 4.05 ml PBST, 500 µl sheep serum, 200 µl 20% Triton-X, 250 µl 20% BSA) for more than 1 hour. This was then incubated in primary antibody in 500 µl I-buffer (for 5ml: 4.5 ml PBST, 50 µl sheep serum, 200 µl 20% Triton-X, 250 µl 20% BSA) O/N at 4°C.

2.9.3 Day 2

4X 20 minutes washes were given in PBDT and the samples were incubated in secondary antibody in I-buffer O/N at 4°C or 2-3 hours at room temperature (gentle shaking). They were washed with DAPI (1:1000 in PBST) for 10 minutes and then 6X 15 minutes PBS/ Tween washes were given. These samples can either be stored in PBS/ Tween at 4°C or imaged on the same day.

Antibody	Dilutions Used	Host Organism
Primary Antibody		
HA	1:100	Rat
MF20	1:100	Mouse
Secondary Antibody		
Anti-Rat Alexa Fluor 488	1:400	Goat
Anti-Mouse IgG1 568	1:400	Goat

Table 6 Antibodies used for Immunostaining to check in 1XHA founders.

2.9.4 Imaging IF

Confocal microscope LSM 800 Examinar (Upright; Airyscan and Confocal) and LSM 700 (Upright Confocal) were used to take the images. The embryos were mounted in 1% low melting agarose. A z-stack of the heart was taken and images saved in the original format which were later analyzed.

2.10 Live Imaging

Confocal microscope LSM 800 Examinar (Upright; Airyscan and Confocal) was used for this purpose. The embryos were mounted in 1% low melting agarose (for 2 ml: 1ml 2% Agarose and 1ml 0.4% Tricaine) and after taking the image, each embryo was taken into a separate tube for genotyping.

2.11 Microinjection

All injections were performed on a single-cell stage embryo. We firstly make molds using egg water and 2% Agarose, then make needles using the capillary and the machine (Micropipette Puller) program 33. The injection mix was made and carefully inserted in the needle, after which using the injection assembly you inject 1nl into the cell of each embryo. To measure the volume injected the oil drop method was used. After injecting, put the embryos in egg water and place the plate in the incubator with 28.5°C temperature.

2.12 High Resolution Melting (HRM)

HRM was used for checking the efficiency of the primers and sgRNAs designed for CRISPR (Table 2, 3, 4 and 5). The software Eco was used to perform the PCR with an HRM curve. Start with injecting the embryos with the synthesized sgRNA and keep controls of un-injected embryos (at least 4 per primer set), then the DNA was extracted of each embryo using NaOH at 95°C for 20 minutes and then adding 1M Tris (pH 8.0) to stop the reaction. This genomic DNA was used to perform HRM. Each primer set was checked for both Yellow and Blue SYBR.

The HRM conditions were as follows:

50°C	2 minutes	
95°C	10 minutes	
95°C	10 seconds	X 40 cycles
60°C	30 seconds	
95°C	15 seconds	HRM Curve
55°C	15 seconds	
95°C	15 seconds	

2.13 Heart Isolation and qPCR

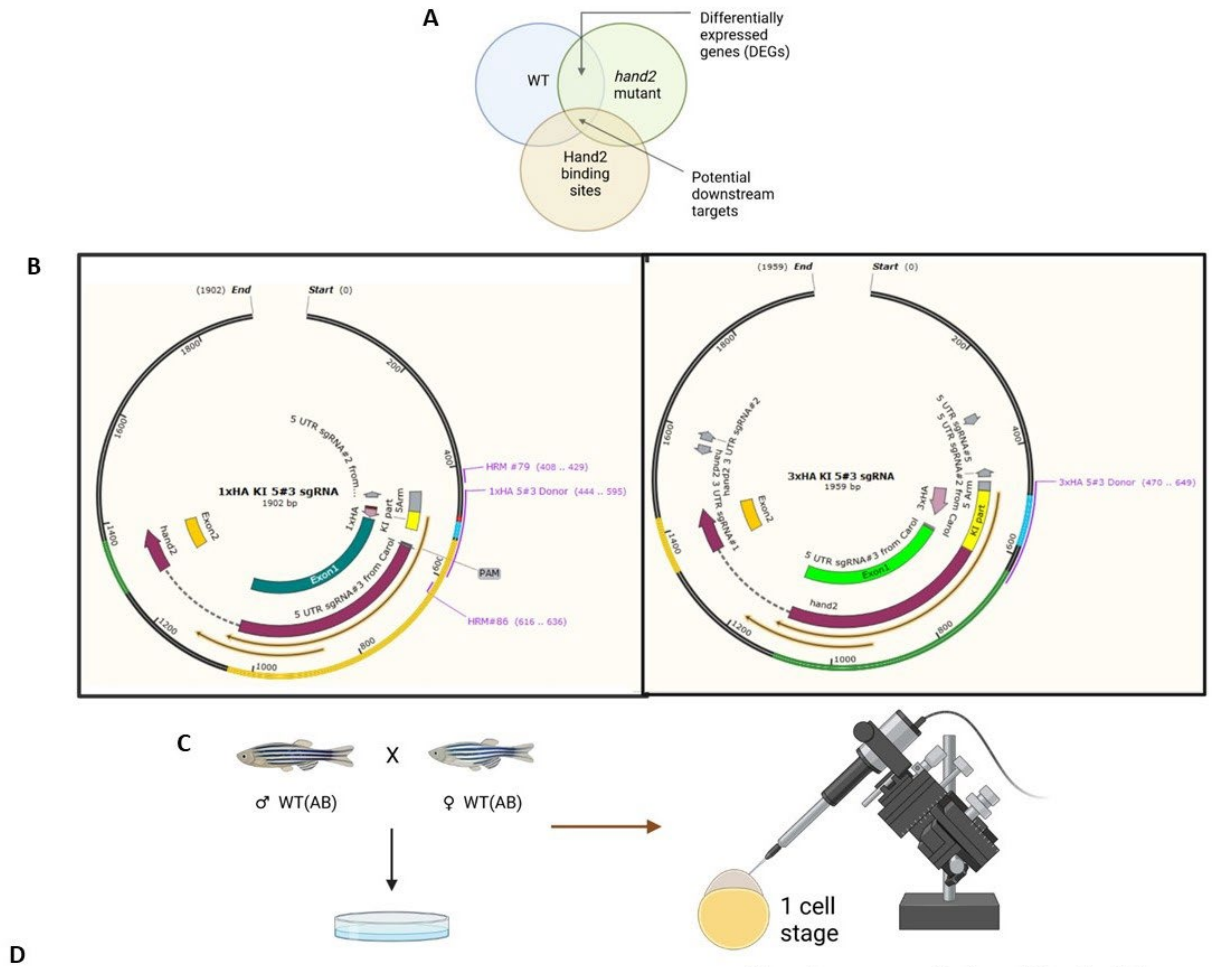
Tg(myl7:EGFP)^{twu26} was in-crossed to give a GFP background to the myocardium for the ease of heart dissections. The embryos were injected with the sgRNAs that are targeting the enhancer of *pdgfra* and hearts were dissected at 24hpf. A total of 60 injected as well as 60 un-injected hearts were isolated. These were put in TRIzol and frozen at -80°C before RNA extraction. RNA extraction was performed using the RNA extraction kit (Qiagen (miRNeasy Micro kit)) and was further converted to cDNA (cDNA was synthesized from RNA using SuperScript III cDNA kit.). The qPCR was performed (Bio-Rad) and each sample having technical triplicates and three biological replicates.

Chapter 3 Results

3.1 Generation of *hand2* 1XHA and 3XHA allele by oligonucleotide based knock-in (KI)

To look at the direct downstream targets of Hand2, the strategy followed was to first get the RNA-seq data of the wildtype and the *hand2* mutants (Figure 7(A)). This was obtained by first dissecting the hearts and sorting the cardiomyocytes. This analysis gave us a list of differentially expressed genes (DEGs). Now, the aim is to perform a Hand2 ChIP-seq, but in zebrafish there is no good antibody available for Hand2. For this reason, we aimed to generate a zebrafish line with the HA tag (Figure 7 (B)). The sequence was inserted into the zebrafish genome by the oligonucleotide method and using the CRISPR/Cas9 system (Figure 7(C)). Few months after putting the zebrafish in the system, they were used for crossing for founder screening. In total, we screened for 33 zebrafish for 3XHA out of which we got one potential founder (Figure 7(D)). The F1 were put in the system and screened for the good F1 after they reached adulthood. I had screened for 139 zebrafish for F1 but none of them showed the desired insertion (Figure 7(E)).

For 1XHA, 19 zebrafish were screened for the founder (Figure 7(F)). Out of these 2 of them have a good insertion, but one has a mutation in one amino acid while the other one has in two amino acids. To check the expression pattern we wanted to see if it is the same as Hand2 so Immunostaining was performed but due to some error in the experiment, I could not see any fluorescence in the embryos.


A
Differentially expressed genes (DEGs)
WT
hand2 mutant
Hand2 binding sites
Potential downstream targets
B
1xHA KI 5#3 sgRNA
1902 bp
HRM #79 (408 .. 429)
1xHA 5#3 Donor (444 .. 595)
HRM#86 (616 .. 636)
PAM
3xHA KI 5#3 sgRNA
1959 bp
3xHA 5#3 Donor (470 .. 649)
C
♂ WT(AB)
X
♀ WT(AB)
1 cell stage
D
(a)
Screening Founder Round 1
Screening Founder Round 2
DNA Ldder: 100 bp
3XHA 300bp; WT 247bp
(b)
Aligned sequence of selected F0 zebrafish

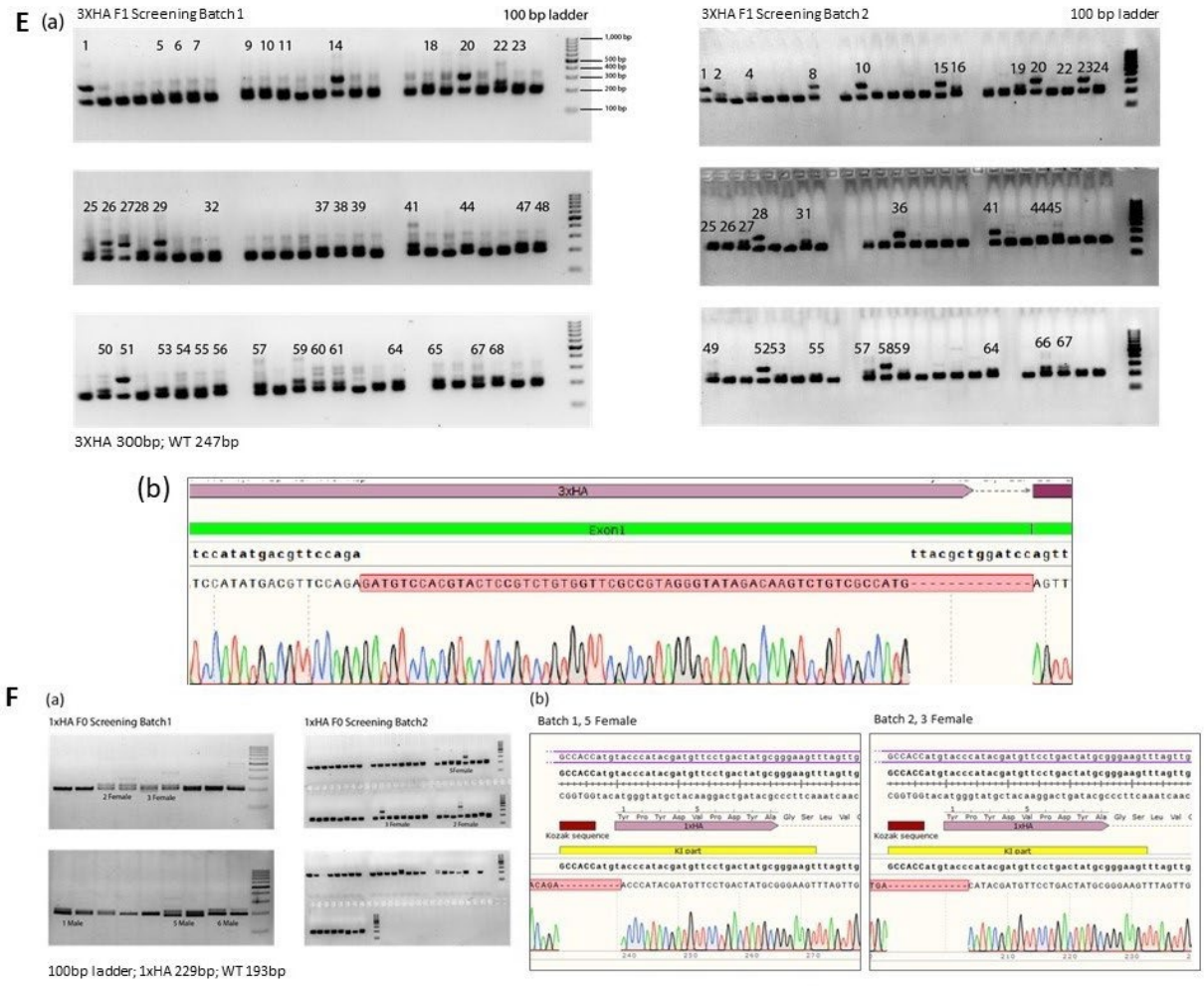


Figure 7 Founder and F1 screening of 1XHA and 3XHA. **A.** Representation of the strategy to define the direct downstream target of Hand2. **B.** The *hand2* locus with the HA insertion. Both 1XHA left and 3XHA right. **C.** Representation of the CRISPR/Cas9 injection into the zebrafish 1 cell stage embryo. **D.** (a) Gel images of the founder screening for 3XHA (b) Sequencing results of the founder for 3XHA. **E.** (a) Gel images of 3XHA F1 screening, the WT band observed at 247bp and 3XHA band at 300bp. (b) Sequencing results show a random insertion within the 3XHA insertion. **F.** (a) Gel images of the founder screening of 1XHA. (b) Sequencing results showing the aligned sequence of 1XHA founders.

3.2 Generation of *hand2* 1XHA allele by PCR product based KI.

This is similar to the previous line but this time we wanted to add the tag with fluorescence such that screening becomes easier. The construct included two loxP sites along with BFP and mCherry. Therefore, here in the absence of cre it should give out BFP fluorescence whereas in the presence of cre it should show mCherry.

All the parts were separately amplified and then fused together. This was then inserted into the T-vector and then checked for its sequence. After the correct sequence was obtained, it was injected with the sgRNA into 1 cell stage wildtype embryo. Because of no BFP positive embryos, we designed new primers with 5' AmC6 end-protections, which gave better results. The embryos still showed low BFP expression but around 30 BFP positive embryos are currently in the system, which will be tested for transmission to F1 after they grow to adults.

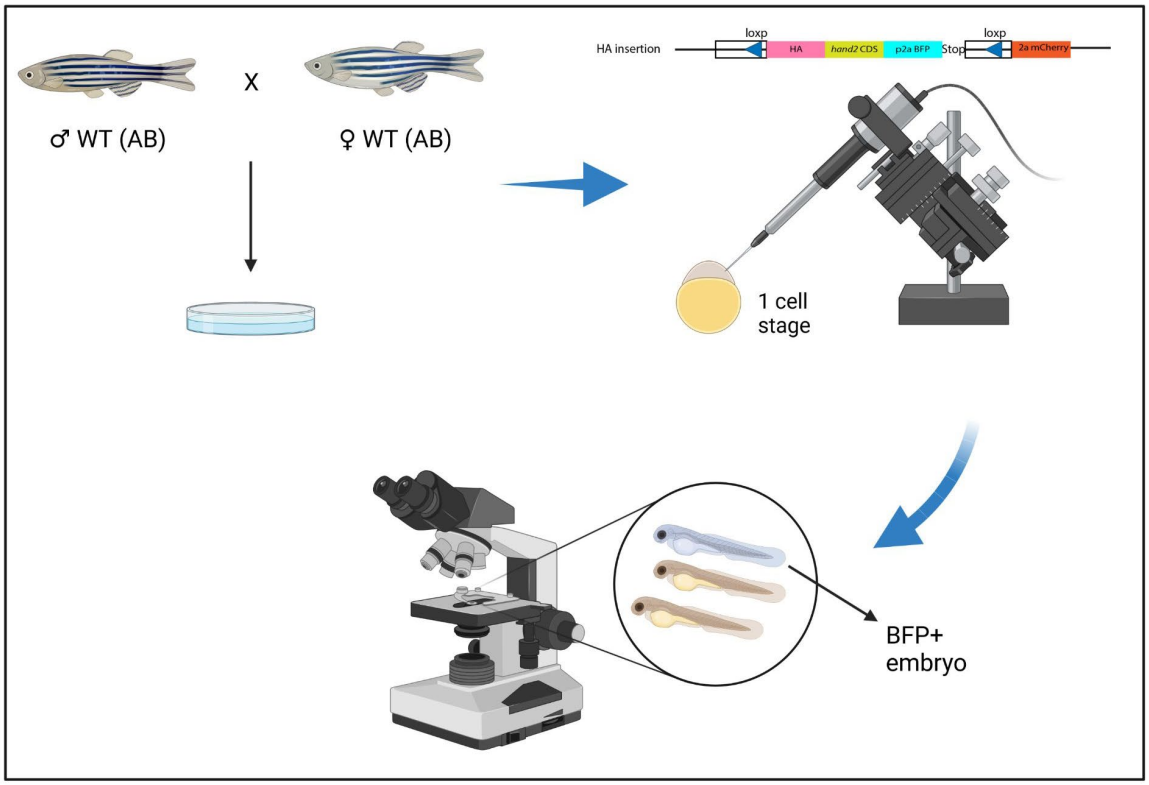


Figure 8 Representation of generation of line. You can see step by step how the two zebrafish of opposite genders are crossed which produces a few hundred embryos

which will then be injected with our injection mix containing CRISPR/Cas9, sgRNA and PCR product. This can be then checked at 48 hpf for BFP expression.

3.3 *pdgfra* overexpression (OE) in different cell types can partially rescue the phenotype.

The experiments were aimed at overexpressing platelet-derived growth factor receptor alpha using different promoters and checking if that can rescue the *hand2* mutant phenotype. In the *hand2* mutants we observe that the cardiac lineage cells fail to migrate. Now, in the RNA-seq analysis of the cadiomyocytes (CMs) of *hand2* mutants in comparison to wildtype reveal a list of differentially expressed genes (DEGs). This also has *pdgfra* as one of the DEGs that upon global KO leads to the same cardiac bifida phenotype. These data suggest that *pdgfra* might be a downstream target of Hand2.

Tg(myl7: pdgfrα-p2a-GFP) was crossed with *hand2 FLD* where *myl7* is a myocardium specific promoter so it will cause overexpression of *pdgfra* in the myocardium. *In situ* was performed and it revealed that infact there was a rescue in the phenotype but it was a partial rescue since the linear heart tube was not formed at 24 hpf (Figure 9 (a)). This was performed also in the endocardium by using the *fli1a* promoter that is specific for endothelium. *Tg(fli1a: pdgfrα, GFP)* was crossed with *hand2 FLD* which also gave the similar results as myocardium. This was then repeated by overexpressing in the myocardium and endocardium simultaneously, which also revealed only the partial rescue of the phenotype.

The next step was to understand how Hand2 regulates *pdgfra* and for that we combined the ATAC-seq data for the CMs at 24 hpf with the H3K27ac ChIP-seq data to find that Hand2 binds to the enhancer region of *pdgfra* (experiments and analysis done by Yanli Xu). To check if binding of Hand2 to *pdgfra* enhancer regulates its expression we did a KO of the enhancer region using the CRISPR/Cas9 method. The sgRNAs were checked using HRM (only 3 out of 12 were suitable) and then used for injection. Until now out of 18 zebrafish that I screened I do not have any founder.

To check the *pdgfra* mRNA expression level a separate experiment was done where *Tg(myl:EGFP^{twu26})* in-cross embryos were used for injections to provide the GFP background. The relative mRNA expression of *pdgfra* in the injected samples was significantly lower than the uninjected samples, which indicate that Hand2 can bind in the enhancer region of *pdgfra* to regulate the expression of *pdgfra* (Figure 9(F)).

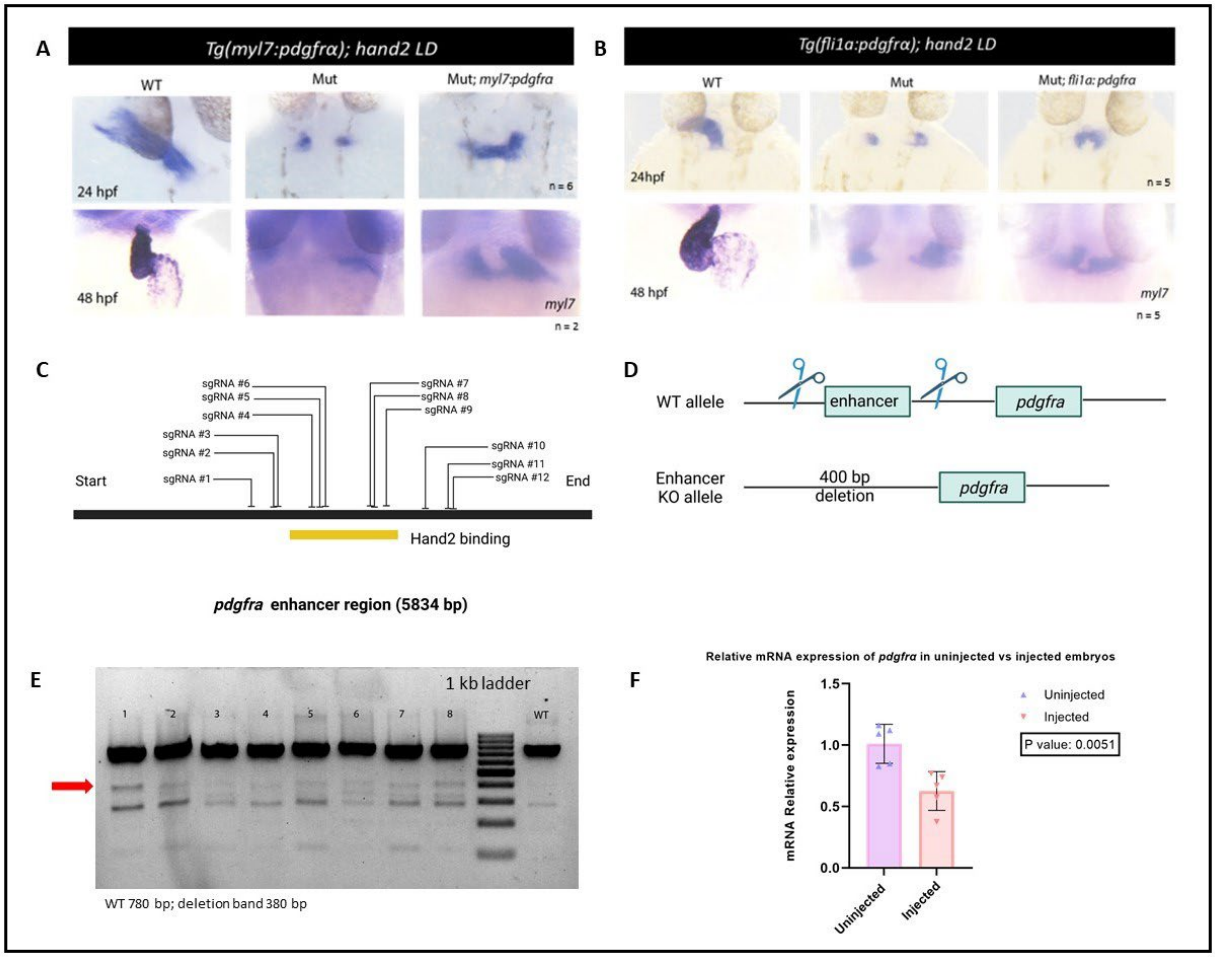


Figure 9 Hand2 regulates the expression of *pdgfra* by binding to its enhancer region. A. The cardiac phenotype observed in the WT, homozygous mutants where the cardiomyocytes fail to fuse at the midline and homozygous mutants with *pdgfra* OE in myocardium at 24hpf and 48 hpf**. B.** Comparison of phenotype of WT, homozygous mutants and mutants with *pdgfra* OE in endocardium. **C.** Representation of sgRNA sites on *pdgfra* enhancer region. **D.** Diagrammatic representation of cutting of the enhancer region after CRISPR. **E.** Gel images showing faint deletion bands after

injection marked by red arrow. **F.** The graph showing the relative mRNA expression of *pdgfra* reduced in the injected in comparison with the un-injected.

The *pdgfra* OE was performed using another promoter *eh22* and the results were compared with the *myl7* and the *fli1a* promoter. The data observed shows that there seems to be a better rescue in the cardiac bifida phenotype upon overexpressing *pdgfra* and *hand2* using the *eh22* promoter (Figure 10).

In another experiment *hand2* and *pdgfra* mRNA were injected into the 1 cell stage *hand2* mutant embryos. These samples were fixed at 20 hpf and *in situ* was performed which reveals that for *hand2* mRNA injection there was a good rescue in phenotype but for *pdgfra* mRNA injection no embryos were observed with a rescue in phenotype (Figure 10).

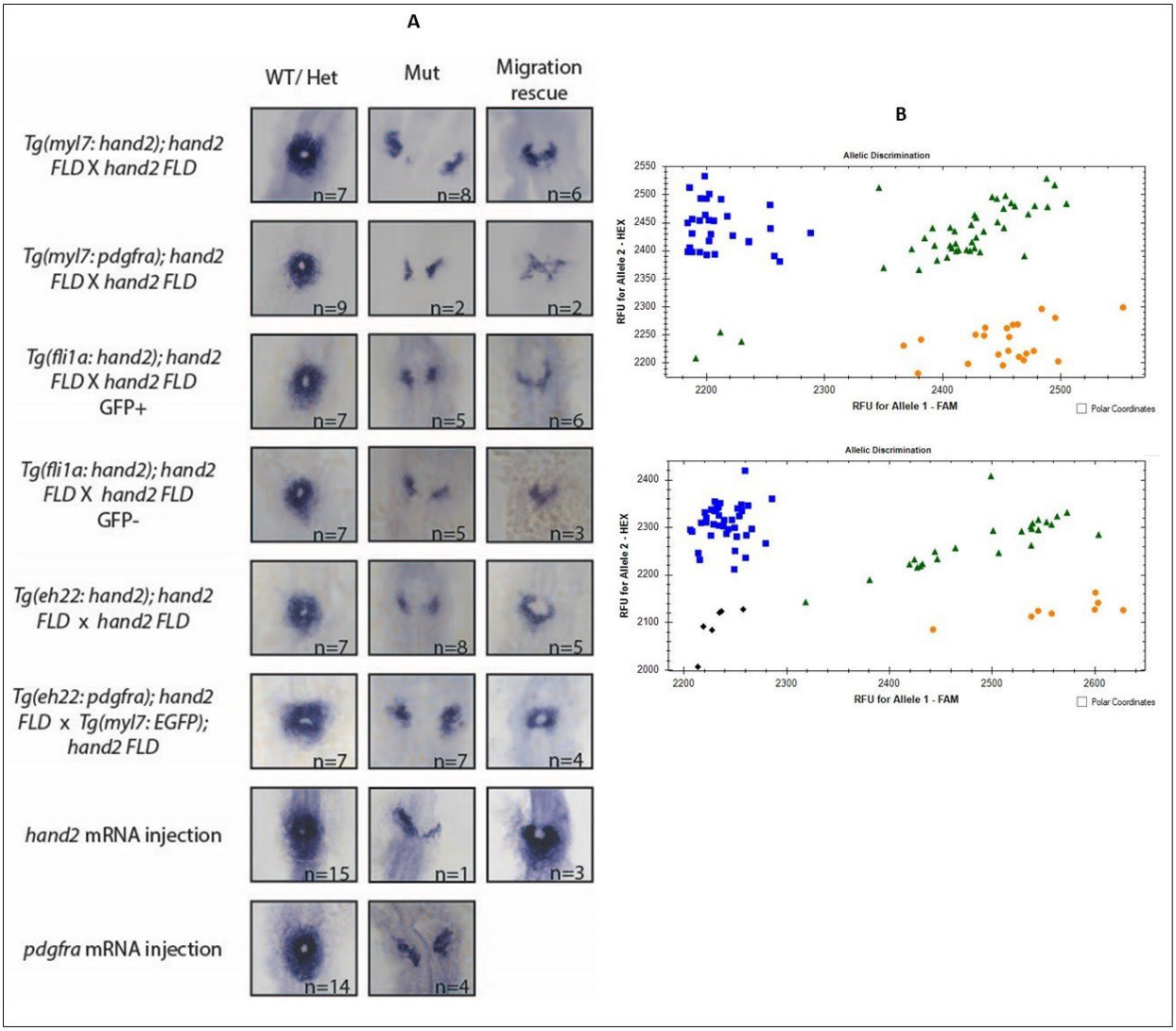


Figure 10 OE of *pdgfra* using the *eh22* promoter can cause a better rescue in the phenotype. **A**. The cardiac phenotype observed in the WT, heterozygous, homoyzgous and homozygous with OE observed at 20 hpf. **B.** The genotype of all individual *in situ* embryos using KASP based technique.

3.4 Mosaic KO of *timp2a* could partially rescue the valve defect *hand2* endothelial mutants.

In the DEGs *timp2a* was seen to be upregulated in the *hand2* endothelial mutants. Therefore, we performed a rescue experiment in which we checked if the KO of *timp2a* could lead to rescue in valve defects in the *hand2* endocardial mutants. We synthesized the sgRNAs and injected it into the endocardial mutants and did live imaging, which revealed that in fact we could observe migration of some cells to the valve interstitial cells (VICs). However, the VICs are still absent so this is also a partial rescue in the phenotype. To prove this further, we created a *timp2a* KO stable line. Firstly, we injected the wildtype embryos with the sgRNAs along with CRISPR/Cas9 and we observed that there was some deletion in the injected embryos. Then these embryos were put in the system and after a few months, they were screened for founder. The HRM analysis revealed two good founders, one female with 50 bp deletion and 2 bp insertion, and one male with 67 bp deletion and 9 bp insertion.

These founders were crossed with *Tg(kdrl:Cre); hand2 FLD* and *Tg(myl7: EGFP) Tg(kdrl:Cre-mCherry); hand2*$^{floxed/floxed}$ individually and put in the system to grow. When these zebrafish are ready for screening, they can be crossed together and checked for the rescue in the valve defect phenotype.

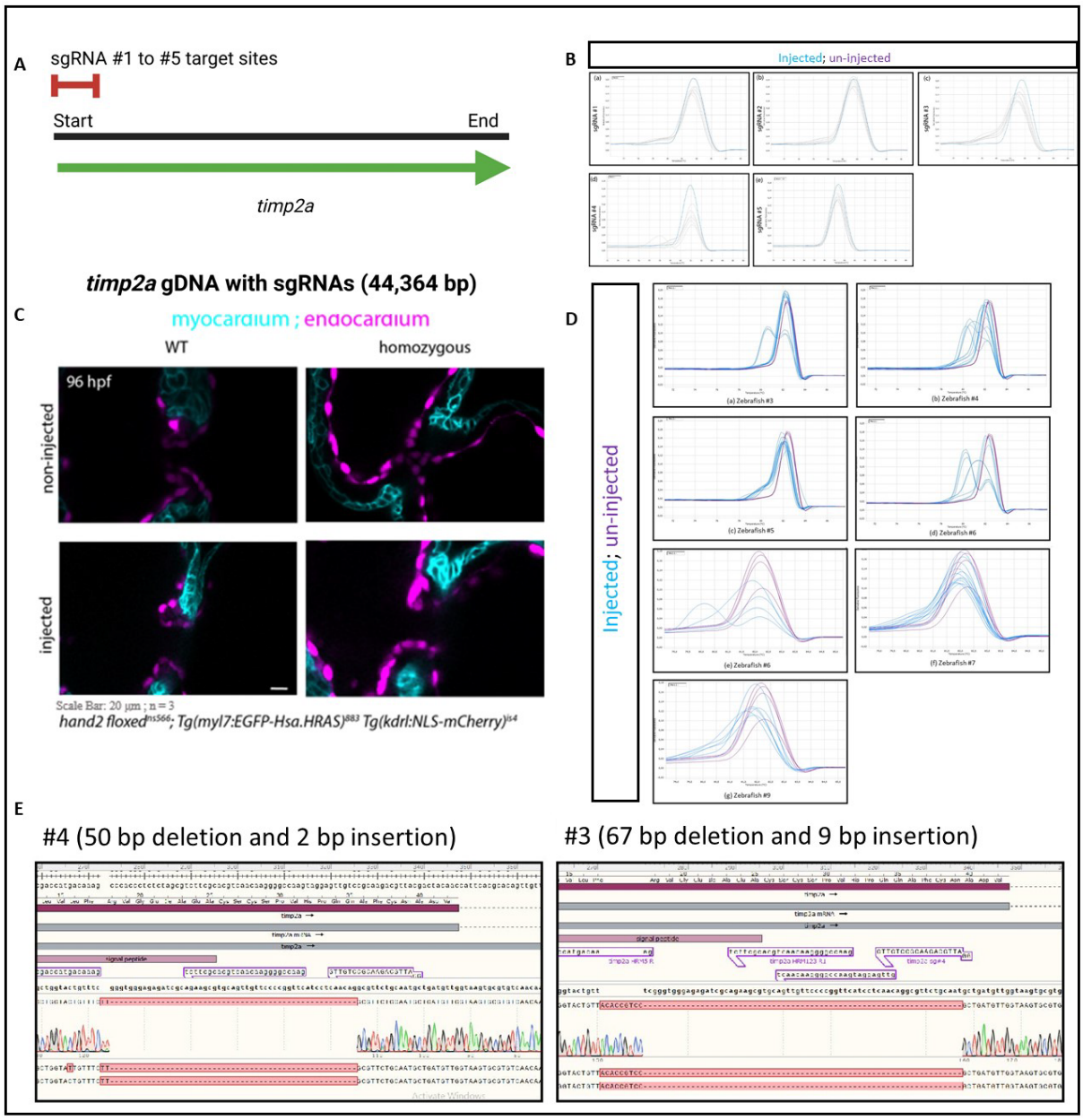


Figure 11 KO of *timp2a* could partially rescue the phenotype of valve defect. A. Representation of the sgRNA target sites on *timp2a* gDNA. **B.** HRM results of sgRNA show that 4 out of 5 sgRNA were good for injection. It is a melt curve with temperature on the x-axis and derivative fluorescence on the y-axis. **C.** Comparison of WT and *timp2a* KO mutant phenotype. Here, we look at the VICs and the gap between the endocardium and myocardium in the valve region. **D.** HRM results showing that fish

number 3, 4 and 6 show a good deviation from the WT and with a greater frequency. **E.** Sequencing results showing the desired deletion in the timp2a locus in fish 3 and 4.

Chapter 4 Discussion

4.1 Generation of lines with HA tag.

Since, there does not exist a good antibody for Hand2 in zebrafish we decided generate a line with HA tag. We thought of doing this by making use of the oligonucleotide-mediated integration of epitope-coding sequences into the zebrafish genome (Burg et al., 2016). While designing the insertion sequence we made sure to put the spacer, which increases the feasibility of the insertion (Burg et al., 2018).

In the founder screening for 3XHA we found only one good founder, which already says that the efficiency is quite low. Then upon screening 139 zebrafish for F1, we did not get any F1 with the proper insertion sequence. This tells that the germline transmission frequency is quite low. One reason behind this could be the fact that the size of insertion of the 3XHA sequence is 141 bp, which is quite big, which can be affecting its transmission to the next generation. Similarly, upon screening for 1XHA founder (KI size 42 bp), we found 2 founders out of 19, which is already more in comparison to 3XHA. Their F1 is in the system and growing so we can't say anything about the germline transmission but while screening for founders since our method involves screening through the F1 embryos it already gives us an idea that the germline transmission rate will be higher. However, the two founders obtained do not completely align with the designed sequence because they have small mutations. One can check if the expression pattern after immunostaining HA would be the same as that of the Hand2 expression pattern. If indeed it is similar, we can use the line to perform ChIP-se and find the downstream targets of Hand2, which is something unknown in the field. This would be very helpful in understanding the signaling pathways of cardiac development, which will take us one-step closer to understanding and helping people with CHD.

The simplified CRISPR/Cas9 knock-in approach has been found to be very efficient in the vertebrate model system where they observed the KI of large inserts with >40% efficiency and germline transmission rates of over 65% efficiency (Nath et al., 2022). The second type of zebrafish line that we tried to generate was the HA line with fluorescence tag attached to it. In this method, we made use of directly inserting the PCR product into the injection mix. Initially, even after changing the concentration of

the PCR product in the injection mix we were not able to achieve the presence of BFP positive embryos. We then modified our construct by making use of the newly found method where they used primers with 5' AmC6 end-protections, which increased the integration efficiency. In addition, the design using 2A peptides for linkage made it possible to integrate multiple cassettes (Mi & Andersson, 2023). The observation in the experiments performed by me suggests that we do have some BFP positive embryos but since BFP was not expressed in the complete embryo, the germline transmission of it will be low. But, if indeed it works fine we will get this line which will make screening so much easier. This will take away the step of checking the genotype of the organisms using a PCR based method which is a very tedious job, and will replace it with just observing the embryos under microscope for fluorescence.

4.2 Hand2 regulates *pdgfra* expression

Intrinsic and extrinsic factors are both found to be important in the myocardial movement towards the midline to form the primitive heart tube. The extrinsic factors include the factors secreted by the endoderm, which promote the bilateral cardiac lineage cell populations to merge. Very recently, it has been shown that among one of the many intrinsic factors *pdgfra* plays some role in cardiac fusion as one of the intrinsic mechanisms. This takes place through the interaction of *pdgfra* with phosphoinositide 3-kinase (PI3K) intracellular signaling pathway (Shrestha et al., 2023). This dependency of *pdgfra* for cardiac fusion is also something we saw in our experiments since the *pdgfra* mutants show cardiac bifida phenotype and the main aim was to find out if *pdgfra* is a downstream target of Hand2.

In the experiment where we OE *pdgfra* in myocardium and endocardium we observe a rescue in the cardiac bifida phenotype of the *hand2* mutants. Nevertheless, this rescue is a partial rescue, which can be because the promoters that we used i.e. *myl7* and *fli1a* might be functioning after the function of *hand2* starts. Therefore, Hand2 might be acting on the cardiac progenitor cells at very early stages. Hence, we think that using a promoter that starts acting earlier can be helpful in generating a better rescue. This is also something that we took further, and tried to use the *hand2* enhancer to OE *pdgfra* by generating the zebrafish line *Tg(eh22- hand2/pdgfrα-p2a-GFP).* Here, the *in situ*

data suggests that at 20 hpf a better rescue in the phenotype was observed in *eh22 promoter* as compared to *myl7* and *fli1a* promoters. We also tried to check the cardiac bifida phenotype rescue by injecting the *hand2* and *pdgfra* mRNAs into the 1-cell stage of *hand2* mutant embryos. In this experiment, we could not make any concrete conclusion as there were only 4 homologous embryos in the *pdgfra* mRNA injection embryos, and none of those show a good migration. This can be because the sample size was quite low.

In the other experiment, where we performed the KO of *pdgfra* enhancer, we saw in the RT-qPCR results that in the injected embryos there is lower mRNA expression of *pdgfra,* which is the result we expected since the ATAC-seq and ChIP-seq data suggests that Hand2 binds to the *pdgfra* enhancer region and since we KO enhancer the Hand2 can no longer bind and regulate the expression.

The functional study and qPCR analysis both suggest that somehow Hand2 is responsible for regulating the expression of *pdgfra,* making it a downstream target of Hand2.

4.3 Hand2 regulates *timp2a* expression

Timp2a was found to be upregulated in the *hand2* mutants so in the rescue experiments, we did the KO of *timp2a* in the *hand2* endocardium mutants and the live imaging showed us how the KO of *timp2a* could lead to partial rescue of the phenotype. In the homozygous condition, we saw that the gap between the endocardium and myocardium was much reduced. Therefore, in the stable line experiment we crossed the founders separately with *Tg(kdrl:Cre); hand2 FLD* and *Tg(myl7: EGFP) Tg(kdrl:Cre-mCherry); hand2*$^{floxed/floxed}$ because when together it will be lethal. Therefore, after these zebrafish are ready to cross they can be crossed and the phenotype can be studied. This experiment will confirm that *timp2a* is indeed a downstream target of Hand2.

Future directions

For the first project, we aim to screen for more 1XHA founders which has no mutation in the insert, and we can also repeat the immunostaining for 1XHA founder since we did not see any staining in the embryos. This will help us check if the founders can be used for ChIP sequencing. We have the F1 generation from these two founders growing in the system with EGFP background in myocardium, and these can be screened for F1 after they reach adulthood.

In the second project with the HA line with fluorescent tags, we will genotype the BFP positive fish that are currently in the system and check their sequence. Further, we will check its germline transmission to the F1 generation.

The third project will continue with screening for the *pdgfra* enhancer KO founder so that we can perform *in situ* to check the phenotype and make a conclusion if Hand2 is regulating the expression via binding to the enhancer region. We also plan to perform enhancer KO injections again to ensure that in the end we have a founder for *pdgfra* enhancer KO fish.

In the final project of *timp2a* KO fish, what remains is to cross the fish which are in the system after they reach adulthood and perform live imaging to check the valve defect phenotype and see if that is rescued. The fish will take about 2 months from now to be ready for crossing.

References

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