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**PARTIAL CRANIOFACIAL CARTILAGE RESCUE IN *ace/fgf8* MUTANTS
FROM COMPENSATORY SIGNALING PERMEATING FROM THE
VENTRICLE OF *Danio rerio***

A Thesis Presented

By

DOUGLAS ALEXANDER CALEDA II

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

SEPTEMBER 2017

Molecular and Cellular Biology

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**PARTIAL CRANIOFACIAL CARTILAGE RESCUE IN FGF8 MUTANTS FROM
COMPENSATORY SIGNALING PERMEATING FROM THE VENTRICLE OF
*DANIO RERIO***

A Thesis Presented

By

DOUGLAS ALEXANDER CALEDA II

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ABSTRACT

PARTIAL CRANIOFACIAL CARTILAGE RESCUE IN *ACE/FGF8* MUTANTS FROM COMPENSATORY SIGNALING PERMEATING FROM THE DEVELOPING VENTRICLE IN ZEBRAFISH

SEPTEMBER 2017

DOUGLAS A. CALEDA II, B.S., UNIVERSITY OF MASSACHUSETTS AMHERST
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Directed by: Professor Craig Albertson

Examples of asymmetric organs are found throughout the animal kingdom. Whether it is superficial like the fiddler crab's claw or within an organism like our visceral organs, asymmetries have repeatedly evolved in nature. However, the genetic and developmental origins for asymmetric organ development remain unclear, especially for superficially paired structures. Within zebrafish, a striking example of asymmetry occurs within the *ace/fgf8* mutant. The pharyngeal cartilages of these mutants develop asymmetrically 35% of the time, with more cartilages developing on the left or right side of the head, but the origins of this asymmetry are unknown. A significant proportion of mutants also exhibit *situs inversus*, whereby the visceral organs develop on the opposite side of the body. Here we seek to understand the temporal window most sensitive to giving rise to this asymmetry, and to understand if there is a correlation between the developing heart field and pharyngeal cartilage with respect to the direction of the asymmetry.

Wild type (WT) zebrafish were exposed to SU5402 during different periods of development, and heart position as well as cartilage development was observed within

the developing larvae. The direction of asymmetry (i.e., left or right biased) was also recorded in *ace/fgf8* mutant heart position and cartilage number to observe if there was a correlation between the two developing fields. SU5402 experiments revealed that the time window most sensitive to the development of cartilage asymmetries was during heart looping and pharyngeal arch segmentation. Furthermore, *ace/fgf8* mutants exhibited a robust correlation between ventricle position and the side of cartilage asymmetry, with more cartilages forming on the side where the ventricle is located. Given the close proximity of the heart and pharyngeal cartilage fields we suggest that the heart field is influencing the developing cartilage, with signaling permeating from the developing heart to the pharyngeal mesoderm to provide a buffer on the side of the developing ventricle.

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CHAPTER 1

INTRODUCTION

Understanding how and when signals are expressed to coordinate embryonic development remains an important question. The knowledge gained from such research has benefited the scientific community and society in many ways. For example, one obvious benefit is that we now have a much better understanding about the origins of many human diseases. Documenting patterns of normal gene signaling and understanding how genetic mutations lead to disease phenotypes allows scientists to predict human disease through genetic screening. Sometimes, treatments can even be provided for certain diseases caused by genetic mutations (Verma, 2013). In addition, this research can help scientists learn more about the basic principles of development, including how animals are organized along various axes of polarity.

Asymmetric development in vertebrate embryos

While the signals that coordinate development along anterior-posterior and dorsal-ventral axes are well studied, less is known about development along the left-right (LR) axis. Vertebrates superficially appear symmetric; however, this observation might only be skin deep. Looking inside the body cavity, it is clear that vertebrate organs are not positioned symmetrically. For instance, the liver and spleen do not line up perfectly in the middle, but rather are asymmetrically situated. The stomach is positioned to the left of the mid-line while the gallbladder develops on the right. The heart too is not only positioned asymmetrically in the body, but it is an asymmetric organ itself. The lungs are also asymmetric, with the right side having more lobes than the left. In short, our left and right sides are not perfect mirror images of one another.

The molecular and developmental processes that lead to asymmetric organ development have been well studied in several laboratory model organisms (Levin, 2005). In zebrafish (Long et. al 2003), investigators found that left-right (LR) asymmetric development of the viscera and diencephalon requires the gene *southpaw*. *Southpaw* is part of the *nodal* subfamily. When *southpaw* is disrupted, the diencephalon and visceral organs do not exhibit the wild type pattern of asymmetry, but rather development of these structures is “randomized” relative to the midline. Cardiac jogging and looping, which are normally asymmetric, also depend on *southpaw*, and when it is disrupted, both are severely affected. Long and colleagues (2003) came up with a model for the development of LR asymmetry in the diencephalon and visceral organs in zebrafish (Fig 1). The model depicts how signaling is initiated at the ciliated node and relayed to the left side of the embryo, which in turn leads to the asymmetric positioning of the visceral organs and diencephalon. This is the symmetry “breaking” event in zebrafish.

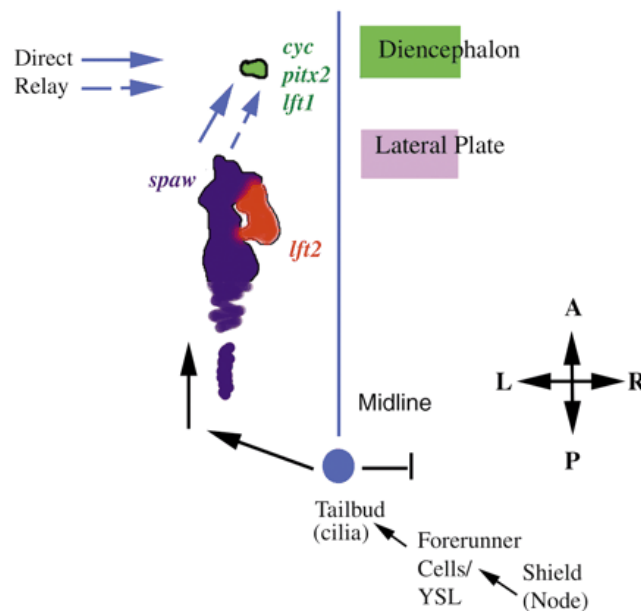


Figure 1 Model of LR asymmetry: In this model (Long et. al, 2003), the dorsal forerunner cells form the ciliated node (i.e., Kupfer’s vesicle), which upregulates *southpaw*, on the left side of the embryo. *Southpaw* in turn leads to asymmetric expression of other genes including *cyclops*, *lft2*, *piti2*, and *lft1*, which all play a role in normal LR asymmetric development. Green represents diencephalon, purple is lateral plate mesoderm, and red is heart.

Here, I explore the question of whether there is more to asymmetric organ development than previously thought. Beyond those organs that are obviously asymmetric, could there be a wider influence of asymmetric signaling during embryonic development? In particular my Master's Thesis addresses two outstanding questions in the field: 1) Do superficially paired structures know their left from right? 2) If so, does this latent laterality arise due to interactions between symmetric and asymmetric developmental fields?

My PI Craig Albertson investigated the first question while working with *ace/fgf8* mutants when he was a postdoc (e.g., Albertson and Yelick, 2005). *Fgf8* is expressed throughout the developing embryo, in structures such as the brain, heart, pharyngeal arches, and limbs (Howe et al., 2013). He noted that *ace/fgf8* mutants had aberrant LR orientation for the brain and visceral organs and showed this was likely due to *fgf8*'s role in dorsal forerunner cell development. This cell population gives rise to the ciliated node (called Kupfer's vesicle in zebrafish), and in a proportion of *ace/fgf8* mutants this structure does not form. Correspondingly, *southpaw* was shown to be reversed or bilaterally expressed in *ace/fgf8* mutants, which explains the ultimate defects seen in the brain, viscera and heart. He also noted that *fgf8* is necessary for proper symmetric development of the pharyngeal skeleton. Specifically, a subset of mutants showed an asymmetry in cartilage and bone development, where mutants would develop more skeletal elements on one side of the face (Fig 2). While *fgf8* was known to play a role in cartilage development (Walshe and Mason, 2003), this asymmetric phenotype was surprising.

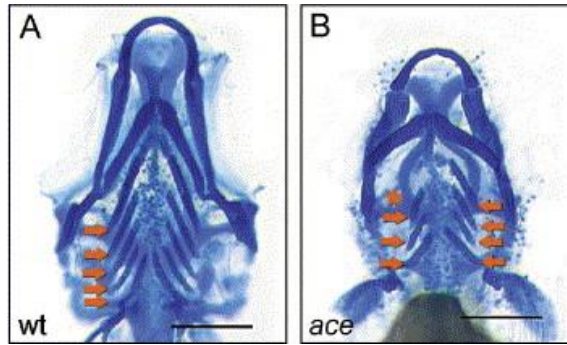


Figure 2 Cartilage of WT and *ace/fgf8* mutant zebrafish (Albertson & Yelick, 2005): Comparison of WT cartilage and *ace/fgf8* mutant cartilage. Mutant cartilage is 1) truncated compared to the WT zebrafish and 2) missing cartilage directionally on one side.

Moreover, the asymmetry was not randomized, but rather directional, with significantly more animals missing elements from the right side of the head. This suggests that there could be a compensatory signal that does not overtly affect craniofacial cartilage development in WT zebrafish, but affects the development in *ace/fgf8* mutants. Another developmental field in the *ace/fgf8* mutants must be interacting with the cartilage field.

Interactions between developmental fields as part of normal development

Interactions between developmental fields are a common event in development. One such example comes from liver and lung specification (Serls et al, 2006). The signals involved in the specification of the liver and lung are *Fgfs*, which activate target genes that instruct organ specification. Serls and colleagues (2006) did multiple *in vitro* experiments, one showing that the quantity and proximity of cardiac mesoderm influences the lung and liver specification via outpocketing of the foregut endoderm.

They also performed dose dependent experiments to see how the levels of *Fgf1* and *Fgf2* influence specification, and found that they influence *albumin* and *Nkx2.1* levels and instruct liver and lung specification. Specifically, with low levels of *Fgf's*, *albumin* is

present and the liver is specified. At high levels of *Fgf*'s, *Nkx2.1* is present and lung specification occurs.

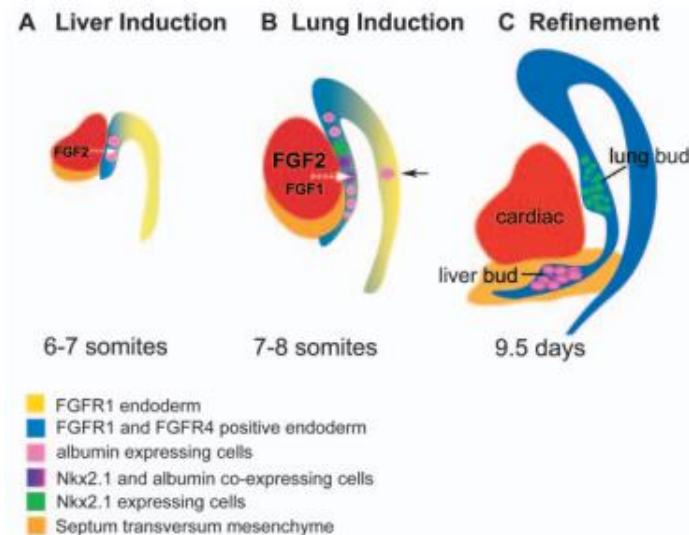


Figure 3 Lung & Liver model (Serls et al., 2006): A) During 6-7 somites stage, Fgf2 is expressed in the cardiac mesoderm before albumin is activated in foregut endoderm. The levels of Fgf are not high enough to lead to lung specification. B) From 7-8 somites stage, the dose of fgf increases as Fgf1 is also expressed in the cardiac mesoderm, and with higher levels of Fgf and Nkx2.1 activation, lung is induced in the foregut endoderm. C) Refinement of cell commitment occurs through positive and negative feedback throughout the germ layers.

Based on their findings, they came to the conclusion that temporal manipulation and concentrations of *fgf*'s emanating from the cardiac mesoderm are necessary for proper foregut endoderm patterning (Fig 3). These findings provide a clear example of how signals emanating from the cardiac mesoderm can influence nearby developing tissue. Specifically, how development of lung and liver depend on signals emanating from the heart field.

Another notable study comes from Bell and colleagues (2006) who examined the origins for asymmetry in pelvic spine reduction in three-spine sticklebacks. Specifically, they wanted to understand why the vestige on the left was typically larger than the one on the right. The gene responsible for pelvic spine reduction is *pitx1* (Shapiro et. al., 2004), which is typically expressed in the developing hindlimb buds of all vertebrates, but

expression is lost in stickleback without pelvic spines. Notably in mice lacking *pitx1*, hind limbs are also asymmetrically reduced with the left side being slightly larger (Fig 4 A)(Shapiro et. al., 2004). Bell et al 2006 postulated that the left pelvic vestige in stickleback (and left hindlimb in *pitx1* ^{-/-} mice) is larger because of compensatory signaling from *pitx2*. *Pitx2* is a homolog of *pitx1*, is a target of *nodal* signaling, and is normally expressed on the left side of the embryonic midline during organ development (Fig 4B). Since organ and limb development occur at the same time and within the lateral plate mesoderm, the authors suggest that expression of *pitx2* leads to longer spine vestiges on the left side (Bell et. al, 2006).

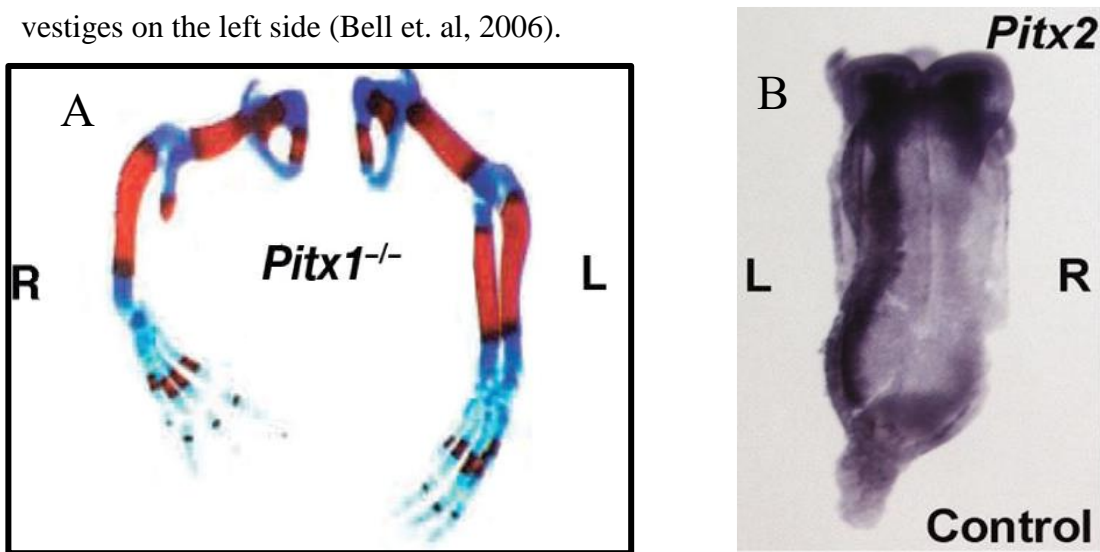


Figure 4 Asymmetric pelvic reduction in mice: A) Reduction of hind limbs in the *Pitx1* mutant mice, where the left side is longer than the right in most cases (Marcil et al., 2003) B) Expression of *Pitx2* in the lateral plate mesoderm of mice, which is only on the left side (Murray & Gridley, 2006).

Craniofacial and heart development in the zebrafish

In order to gain a full understanding about what could underlie the craniofacial asymmetry observed in zebrafish *ace/fgf8* mutants; we must understand the fate map of cardiac and cartilage cells and the stages of craniofacial development (Fig 5, 6). Cardiac precursors are located laterally on the zebrafish embryo, and they migrate medially and

meet in the middle at 20 hpf (Kessler et al., 2012). Craniofacial cartilage development is a multi-step process, and around 20 hpf the precursor cells are close to the cardiac cells.

As stated above, there are multiple steps that lead to cartilage development. The first step involves cranial neural crest cell (CNC) migration (Klymkowsky et al., 2010). First the CNC are specified at the neural-non-neural border of the dorsal ectoderm during neurulation. Next, these cells undergo epithelial-mesenchymal transition and migrate ventrally and populate the pharyngeal arches. In zebrafish, they begin to migrate at 12 hpf, and by 15-16 hpf they begin to move into the pharyngeal arches. These arches begin as a uniform strip of pharyngeal endoderm, and must first undergo segmentation in order to receive the CNC (Choe et al, 2014). The first two arches start segmenting around 20 hpf, with segmentation of arch 3-7 occurring every four hours. After the arches containing CNCs undergo segmentation, cells must condense in order for the cartilage to form and grow. Condensation can be due to enhanced mitotic activity, and/or cells aggregating to the center of a condensation (Hall and Miyake, 2000). Once condensation is initiated, boundaries are established within the pouches. From there, the cells adhere, proliferate, and begin to grow in a coordinated manner. Once the cells have reached a critical size, they begin to differentiate into cartilage. The condensation and growth occurs from 36 hpf to 8 dpf. Pharyngeal arch one gives rise to meckel's cartilage and the palatoquadrate, while arch two gives rise to the ceratohyal, basihyal and hyosymplectic. Pharyngeal arches 3-7 give rise to the five ceratobranchials cartilages.

While pharyngeal cartilage development is taking place, the heart begins to form as well. As noted with the fate map, heart cells begin to migrate at 15 hpf and meet in the middle around 20 hpf. From 24-36 hpf, the heart undergoes jogging and looping. The

jogging seen within WT zebrafish goes to the left of the zebrafish, and as the heart loops, the ventricle is positioned up to the right, with the atrium down and to the left.

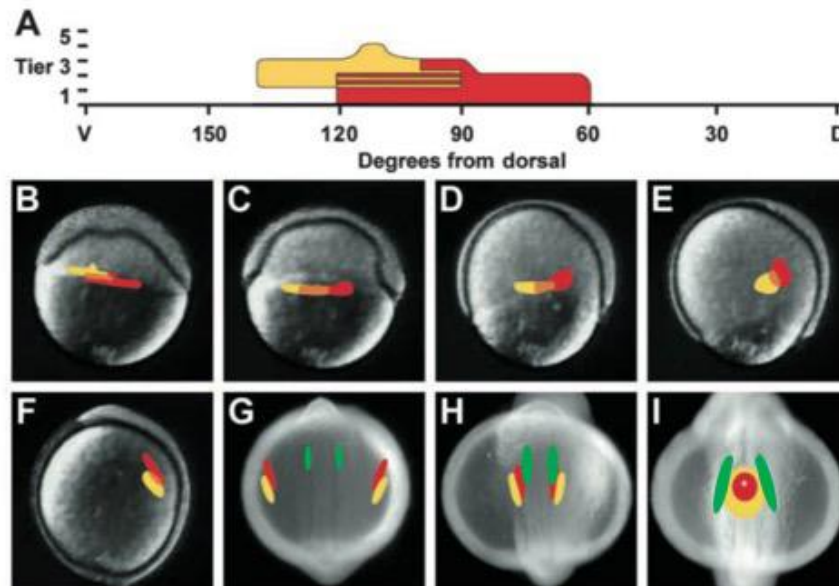


Figure 5 Fate map of heart and cartilage cells (Modified from Stainier et al., 1993; Keegan et al., 2004; Wada et al., 2005): A) red represents ventricular myocardial precursors, yellow represents atrial myocardial precursors. Representative of where along the embryo the cells are located. (B-F) The background images were taken from Karlstrom and Kane with the precursor cells locations. (G-I) The same background images (Karlstrom & Kane, 1996) But this time with not only the cardiac fate map, but with the cartilage as well (green).

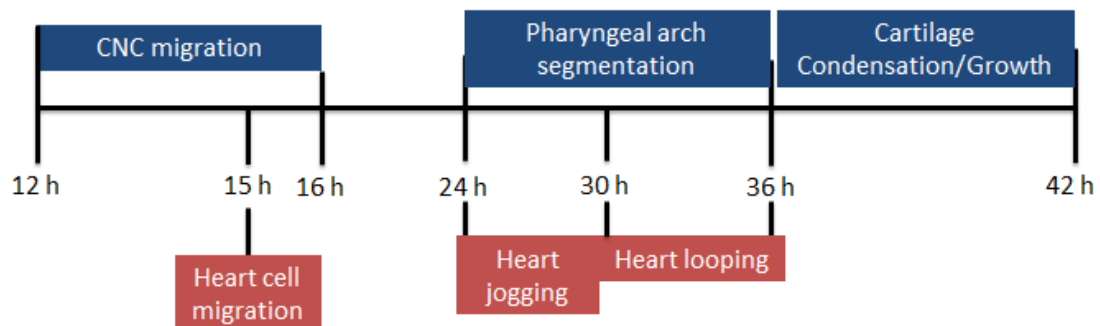


Figure 6 Timeline of cartilage and heart development: The timeline below indicates the developmental stages crucial for normal development in a zebrafish. As seen, multiple stages are involved.

Origins for craniofacial asymmetries in the zebrafish *ace/fgf8* mutant

Based on the body of literature presented above, we hypothesize that asymmetric cartilage development in *ace/fgf8* mutants is due to asymmetric signaling from the developing ventricle. Our reasoning builds on published studies that indicate that (1) that *ace/fgf8* mutants exhibit craniofacial asymmetries with a directional bias and (2) interactions between symmetric and asymmetric development fields can lead to asymmetric phenotypes. Further, we believe the heart is the source of the asymmetric signal based on recent work that demonstrates the intimate association between ventricle and pharyngeal arch development. (Choudrey & Trede, 2013, Choe et al, 2014).

Specific aims of the thesis

Our main hypothesis will be tested through three specific aims:

AIM #1: Determine if CNC migration asymmetries lead to craniofacial asymmetries

Hypothesis: Craniofacial asymmetries do not arise during CNC migration stages.

My first aim was to assess the likelihood that asymmetries arise during (CNC) migration stages. CNC's migrate at 12 hpf and move into the pharyngeal arches by 15-16 hpf (Klymkowsky et al, 2010). Since *fgf8* plays a role in CNC specification and migration (Creuzet, 2009; Klymkowsky et al, 2010), there is potential for asymmetric migration of CNC's, which could lead to cartilage asymmetries. To test this, we carried out two experiments. The first one involved using the small molecule inhibitor SU5402 to disrupt Fgf signaling in WT zebrafish between 10-16 hpf. Next, we crossed the *ace/fgf8* mutation into the *sox10:gfp* reporter line in order to observe and track CNC migration in mutants.

AIM #2 Determine if Pharyngeal arch segmentation stages lead to craniofacial asymmetry

Hypothesis: Craniofacial cartilage asymmetries do arise during pharyngeal arch segmentation stages and follow the direction of heart looping

My second aim was to determine if cartilage asymmetries arise during pharyngeal arch segmentation and heart looping stages. Pharyngeal pouches begin to form at 20 hpf, with a new one grown every four hours (Choe and Crump, 2014). Concurrently, ventricle cells differentiate between 16-22 hpf, and the heart jogs to the left at 24 hpf and loops to the right soon thereafter (Chen et al, 1995; Choudhry and Trede, 2013). Not only is the development of the heart and pouches coordinated temporally, but both fields are in close proximity of one another. To test if cartilage asymmetries arise during pharyngeal arch segmentation stages, we used SU5402 again, during 24-36 hpf. To test if the direction of the asymmetry is influenced by the heart field, we also observed a large sample of mutants and tracked ventricle location (i.e., left versus right) and the direction of pharyngeal cartilage asymmetry.

AIM #3 Determine if Pharyngeal cartilage condensation stages when craniofacial asymmetries arise

Hypothesis: Craniofacial cartilage asymmetries do not arise during pharyngeal cartilage condensation stages.

Our next aim was to determine if asymmetric cell death in the pharyngeal pouches causes cartilage asymmetries. Programmed cell death is a normal part of development, and there is potential for this to occur asymmetrically within *ace/fgf8* mutants. Fgf's play a role in cell survival, and so it is possible that this process contributes to asymmetric

cartilage development. (Trumpp et al, 1999). To test this, we used SU5402 at post-segmentation stages, 36- 42 hpf. To supplement this, we also used an apoptosis kit to measure cell death within the *ace/fgf8* mutant zebrafish at 40 hpf.

CHAPTER 2

MATERIALS AND METHODS

Zebrafish husbandry and embryo collection

Zebrafish were maintained in the Albertson fish facility in Morrill II at about 28.5°C in a 14 hr light, 10 hr dark light cycle. We used the wild type EW and AB zebrafish lines to obtain embryos for SU5402 experiments. We used the *sox10:gfp^{ace/+}* line of zebrafish to obtain mutants. We identified heterozygous males and females, and bred them to get embryos that were 25% homozygous mutants. We used this line for CNC migration imaging, cartilage tracking, and apoptosis assays. We crossed three females and two males within each line using false-bottom breeding cages. Embryos were maintained using techniques previously described (Westerfield, 1995).

***ace/fgf8* mutant tracking**

Ace/fgf8 mutant embryos were identified by a lack of cerebellum by 24 hpf (Figure 7), as previously noted (Reifers, 1998). At 48 hpf, heart orientation in mutants was also noted, and mutants were separated from each other based on ventricle and atrium orientation. Zebrafish with ventricles on the right and atriums on the left, the wild type (WT) phenotype, were kept in one dish (Fig 8, from French et al., 2012). Zebrafish with left ventricles and right atriums, the reversed heart phenotype, were kept in a dish separated from the normal heart zebrafish to distinguish between the two.



Figure 7 WT vs. *ace/fgf8* mutant: On the left is the WT phenotype, where the cerebellum is located in between the two regions of the brain, it is indicated with the red arrow. On the right is the *ace/fgf8* phenotype, where there is no cerebellum, as indicated by the black arrow. Black bar represents 500 μm .

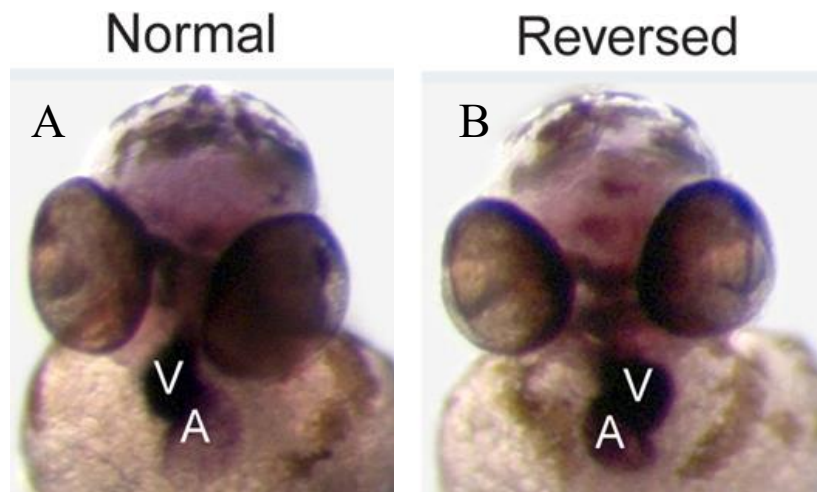


Figure 8 Images of heart orientation: (modified from French et al., 2012): (Ventral view) A) The main phenotype that was observed was the ventricle to the right and atrium to the left, as this is the WT phenotype. B) The second phenotype seen was the reversed heart, where the ventricle was up to the left and atrium down to the right.

SU5402 experiments

SU5402 powder (EMD Millipore, cat#572630) was brought up to a 5 mM SU5402 stock solution in DMSO, aliquoted and stored at -20°C . SU5402 inhibits all Fgf receptors. For Fgf receptor inhibition, SU5402 has an IC_{50} of 0.03 μM . However, previous reports indicate this dosage is ineffective to elicit a phenotypic response *in vivo*.

Following previous work (Walshe & Mason, 2003; Nechiporuk & Raible, 2008; Nicoli et al, 2009; Poss et al, 2000; Molina et al, 2007; Griffin & Kimelman, 2003) we dosed with a range from 1-50 μM and found 10 μM reliably recapitulated the asymmetry mutant phenotype without also producing catastrophic developmental defects. For treatments, we took zebrafish at the start of the time window (up to 12 hrs) and use 3x4 tissue culture plates with two mesh basket inserts, one for experimental animals and one for control. Control zebrafish were treated with an equal volume of DMSO. During the treatment window, zebrafish were kept at 28.5°C. After the treatment, we washed the embryos with fresh embryo water several times and then placed them in larvae dishes. Heart scoring was done in the same manner described above.

The *sox10:gfp:ace/fgf8* line

We observed craniofacial development in *sox10:gfp:ace/fgf8* animals. Specifically, we used a Leica MF15 dissecting microscope in the Albertson lab to observe fluorescent *sox10:gfp:ace/fgf8* embryos at 16 hpf. We crossed parents that were heterozygous to get a yield of 25% homozygous mutants. Fluorescent embryos were set aside from non-fluorescent embryos, and CNC migration was observed. At 24 hpf, the embryos were separated again based on whether they were mutant or not as described above, and at 26 hpf, they were imaged once again. At 48 hpf, they were separated based on heart orientation the same way as above. At 72 hpf and 6 dpf, they were observed for cartilage development.

Cartilage Staining

Zebrafish set for staining were fixed in 4% paraformaldehyde at 6 dpf for two hours. For cartilage staining, we followed a previous study with slight modifications

(Walker & Kimmel, 2006). For the bleaching step, we stopped the reaction after 10 minutes. For clearing solution #1, we kept zebrafish in the solution for 2 hours. For clearing solution #2, we kept zebrafish in the solution overnight. Zebrafish were stored in 80% glycerol.

Apoptosis Assay

Zebrafish were fixed at 40 hpf in 4% paraformaldehyde. They stayed in fix at room temperature for three days, and then the ApopTag Plus Peroxidase *In situ* apoptosis detection kit was used to observe cell death. This assay works by labeling DNA strand breaks through the indirect TUNEL method. We scored apoptosis by the presence of orange dots within the embryo. Minor adjustments were made to the manufacturer's instructions for preparing the embryos (EMD Millipore). Instead of using the slides provided by EMD Millipore, we used 1.5 mL tubes. For the protocol itself, we used PBSt instead of PBS, skipped the xylene and methyl green steps, and stored the specimens in 80% glycerol. We also did several washes of PBSt after the embryos were fixed in 4% paraformaldehyde.

Imaging

All bright field and fluorescent imaging was done with a Leica DFC450 camera mounted to a Leica MF15 stereomicroscope in the Albertson lab.

CHAPTER 3

RESULTS

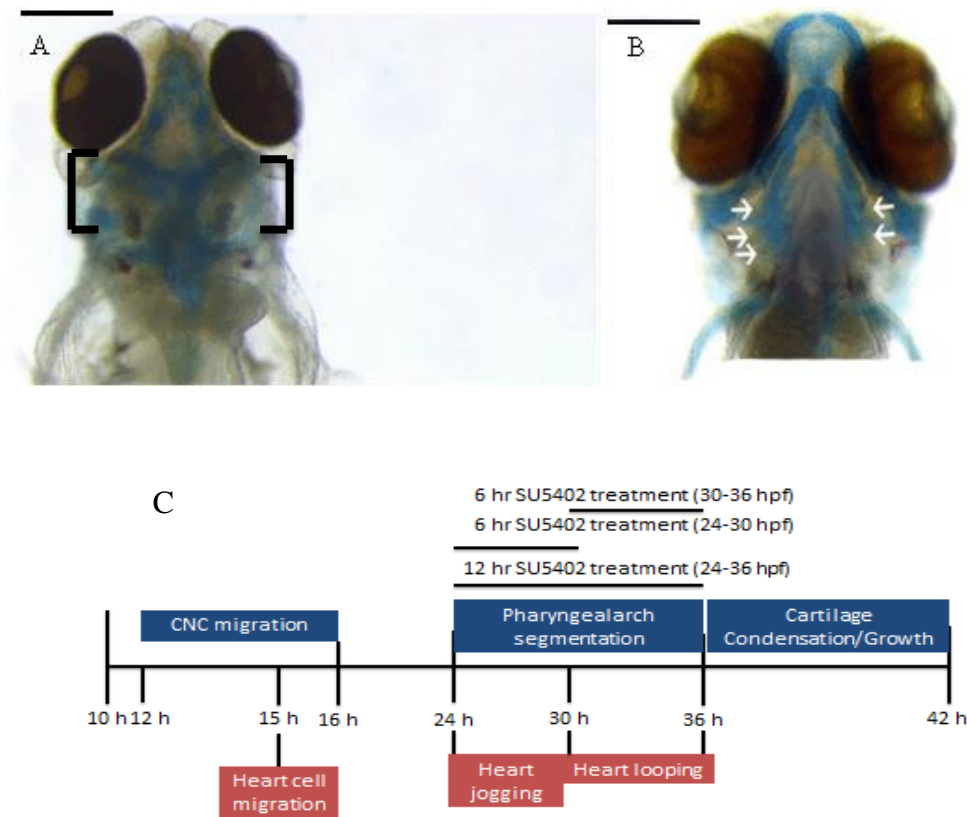
SU5402 treatment recapitulates the *ace/fgf8* mutant phenotype

Fgf8 is known to be required for cartilage formation, however it was not known what stage *fgf8* loss leads to cartilage asymmetry (Walsh & Mason, 2003; Albertson & Yelick, 2005). SU5402 is a potent inhibitor of Fgf receptors. When zebrafish are exposed to 25 μM SU5402 for 24 hours at an early stage, all of the cartilage elements are lost (Walsh & Mason, 2003). To properly recapitulate the *ace/fgf8* mutant phenotype, we explored previous research that used SU5402 (Walshe & Mason, 2003; Nechiporuk & Raible, 2008; Nicoli et al, 2009; Poss et al, 2000; Molina et al, 2007; Griffin & Kimelman, 2003) as well as various experiments done throughout the year. We tested concentrations ranging from 1 μM to 25 μM to determine what value most similarly replicated the mutant cartilage phenotype (Figure 2A, B). While we were experimenting with the concentration value, we also did different time windows of SU5402 exposure. In the beginning, we did not focus on specific time windows, as we were trying to determine if it was possible to replicate the *ace/fgf8* mutant cartilage phenotype in SU5402 treated zebrafish. Once we found it was possible, we then had to determine the length of time that worked best for exposure. We tried both 6 and 12 hour treatments.

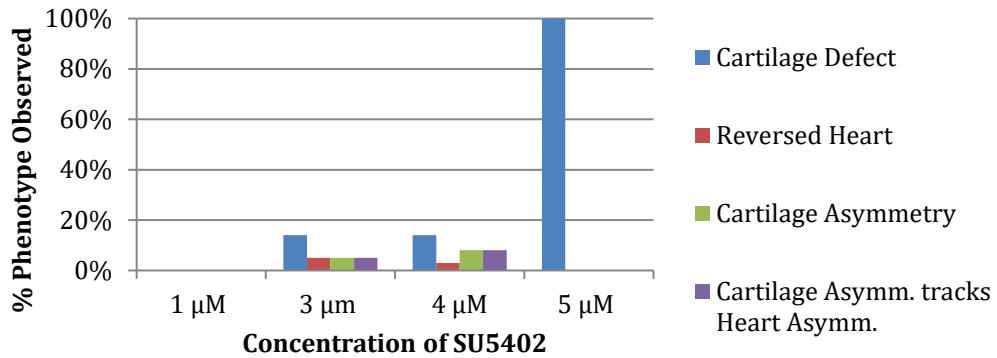
We found that the 12 hour window of treatment could not replicate the mutant cartilage phenotype from 24-36 hpf. At 4 μM , the frequency of asymmetries was much lower than what was seen in the *ace/fgf8* mutant (Figure 2C). At 5 μM during a 12 hour window from 24-36 hpf, 100% of the zebrafish exhibited a loss of ceratobranchials, with

truncation of the ceratohyal and meckel's cartilage (Figure 2C). We then decided to try shorter windows with a higher dose of inhibitor.

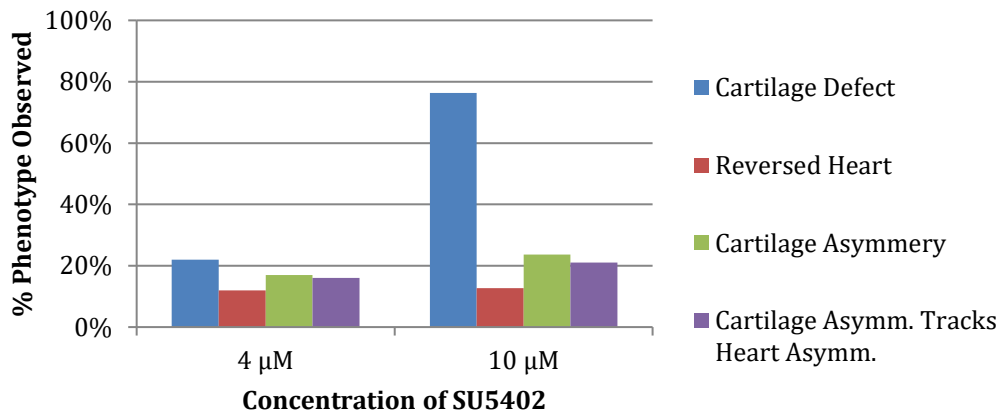
For the 6 hour window, 4 μM once again disrupted cartilage development, but again at a lower frequency than in *ace/fgf8* mutants. At 10 μM however, this concentration effectively disrupted cartilage development in WT zebrafish including a higher frequency of cartilage asymmetries (Figure 2B). When we tried 25 μM , this concentration created the same issues as 5 μM during the 12 hour window (Figure 2A). Based on these results, we found that we could best approximate the *ace/fgf8* mutant cartilage asymmetry at a dose of 10 μM for 6 hours.



9D) 12 hr SU5402 Treatment, 24-36 hpf



9E) 6 hr SU5402 Treatment, 24-30 hpf



9F) 6 hr SU5402 Treatment, 30-36 hpf

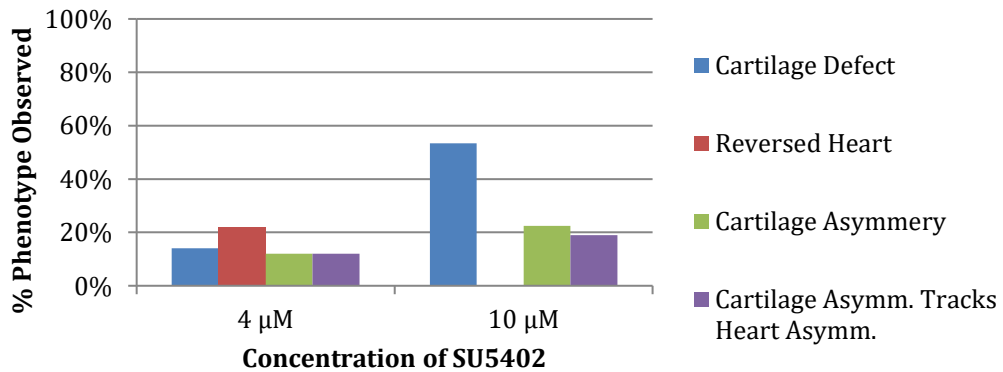


Figure 9 Recapitulating the *ace/fgf8* mutant asymmetry phenotype: A) 25 μM of SU5402 exposure during a 6 hour treatment window. Ceratobranchials 1-5 are missing, and meckel's cartilage and ceratohyal are truncated. The black scale bar represents 200 μm . B) 10 μM of SU5402 during a 6 hour window treatment. Ceratobranchials are missing from the left and right, with more on the right. The black scale bar represents 200 μm . C) Timeline of initial SU5402 treatments, with the 12 hour treatment from 24-36 hpf and the six hour treatments from 24-30 and 30-36 hpf. D) A percentage graph for the treatments at 12 hours with 1 μM , 3 μM , 4 μM , and 5 μM of SU5402. A 12 hour treatment does not recapitulate the *ace/fgf8* asymmetry. E) 6 hour treatment from 24-30 hpf for 4 μM and 10 μM . 4 μM causes asymmetries at a 17% rate, while 10 μM does so at a 24% rate. F) 6 hour treatment from 30-36 hpf. Again, 4 μM causes asymmetries at a 12% rate, while 10 μM causes asymmetries at a 22% rate.

Asymmetries do not arise at CNC migration stages

The next test was to see if CNC migration is asymmetric in *ace/fgf8* mutants. We looked during and at the end of migration to see if CNC were equal on each side of the developing embryo (Figure 10). 59 zebrafish embryos were observed at this time frame, derived from an incross between *ace/+* carriers. At 16 hpf, none had obvious asymmetric migration. At 26 hpf, homozygous mutants could be reliably identified, but when CNC were imaged again, no asymmetries were noted. However, by 72 hpf, asymmetries within the developing cartilages were noted in about 25% of mutants, as expected. At 6 dpf, the cartilage asymmetries were still present.

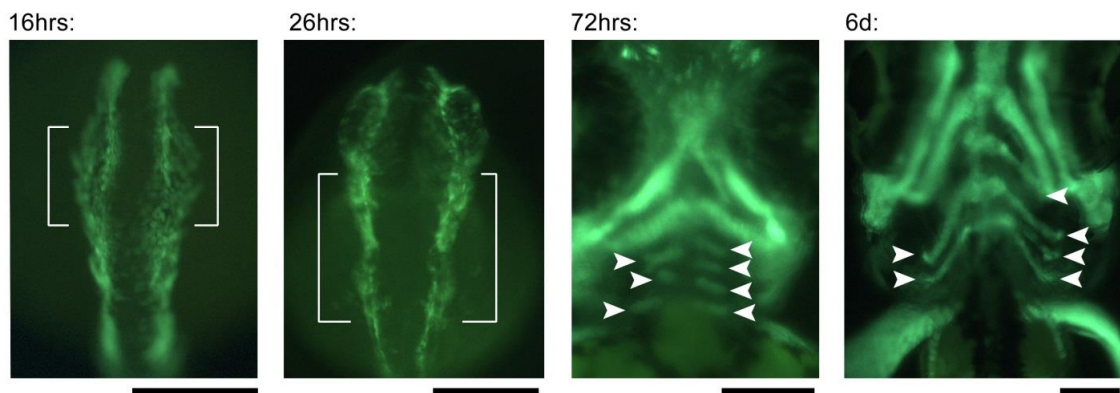


Figure 10 CNC migration: Equal CNC migration at 16 hpf. Zebrafish were then tracked as they grew to 26 hpf. They were imaged at 72 hpf and 6 dpf, where cartilage asymmetry was observed despite no obvious CNC migration asymmetry. Black bars represent 200 μm .

Next we treated wild-type embryos with 10 μ M SU5402 at CNC induction/migration stages, 10-16 hpf. We used 85 zebrafish for SU5402 treatment and 20 for DMSO carrier controls. Only 16 out of 85 had cartilage defects, and only 7 had a reversed heart (Figure 4). None of the SU5402 exposed zebrafish exhibited any cartilage asymmetry. For the DMSO carrier control zebrafish, 0 of them had cartilage defects or heart inversion.

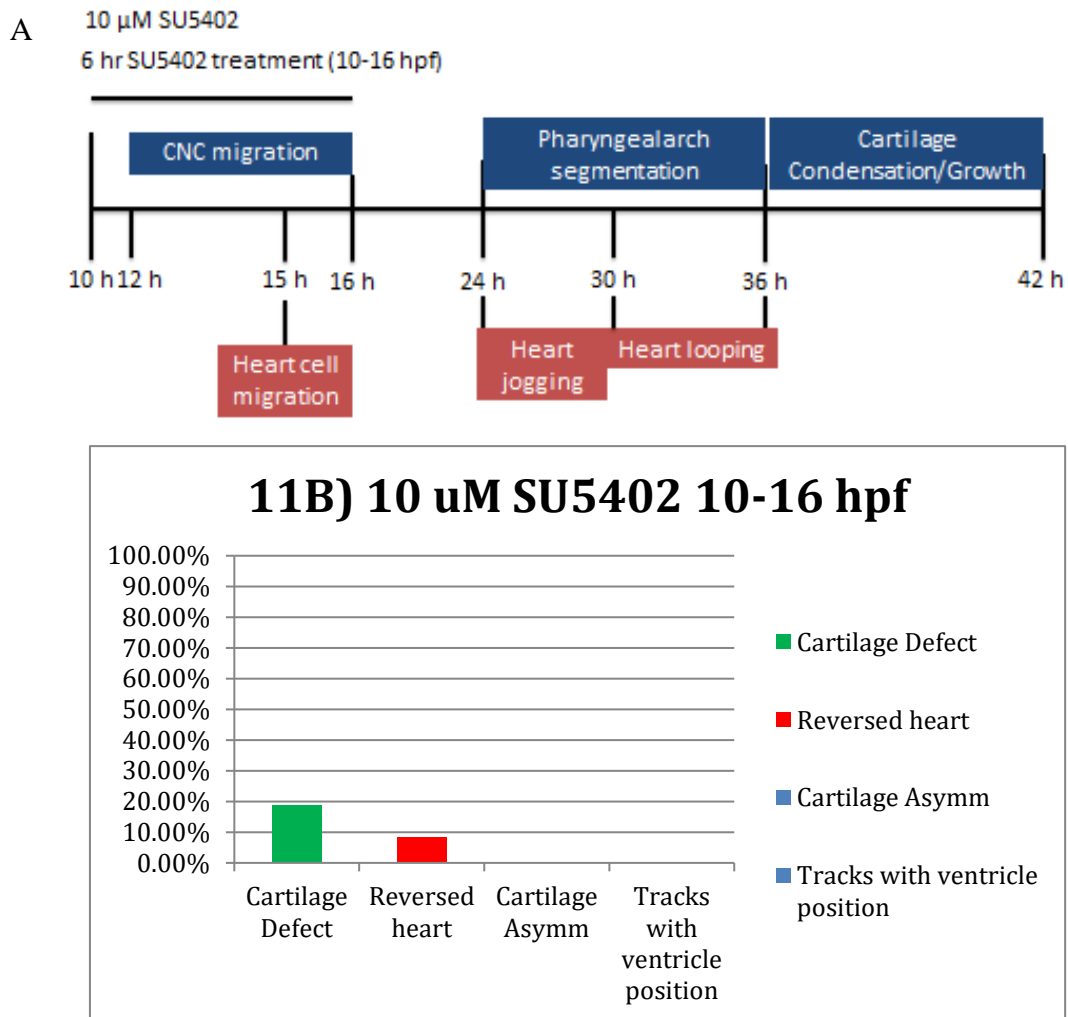


Figure 11 SU5402 treatment from 10-16 hpf: Timeline of SU5402 treatment from 10-16 hpf. B) Graph of SU5402 from 10-16 hpf: On the y axis is % of phenotype observed for the 85 total zebrafish. Only 18.8% experience cartilage defects such as missing ceratobranchials on either side, while only 8.2% had a reversed heart. None of them had cartilage asymmetry. All 20 controls had WT phenotype for both cartilage and heart.

**Asymmetries arise at the pharyngeal arch segmentation stage and are correlated
with ventricular positions**

We tracked development of 304 *ace/fgf8* mutant zebrafish to observe if heart looping and cartilage development had a correlation, as they share the same developmental window and occur adjacent to one another (Choudrey & Trede, 2013, Choe et al 2014). Of these fish, 254 had the normal phenotype for heart orientation, and 50 of them had reversed hearts, with the ventricle developing on the left and atrium on the right. In mutants with normal heart situs, cartilage asymmetries were observed in 33% of the animals. In animals with reversed heart situs, cartilage asymmetries were observed in 40% of the animals, (Figures 13A-C). Regardless of the direction of the ventricle, cartilage asymmetries tracked with the position of this structure. Specifically, 84 animals with ventricles on the right also exhibited cartilage asymmetries, and of these 69 (82%) exhibited more cartilages on the right-side of the head. For the reversed heart, 20 exhibited cartilage asymmetries, and of these 17 (85%) had more cartilages on the left.

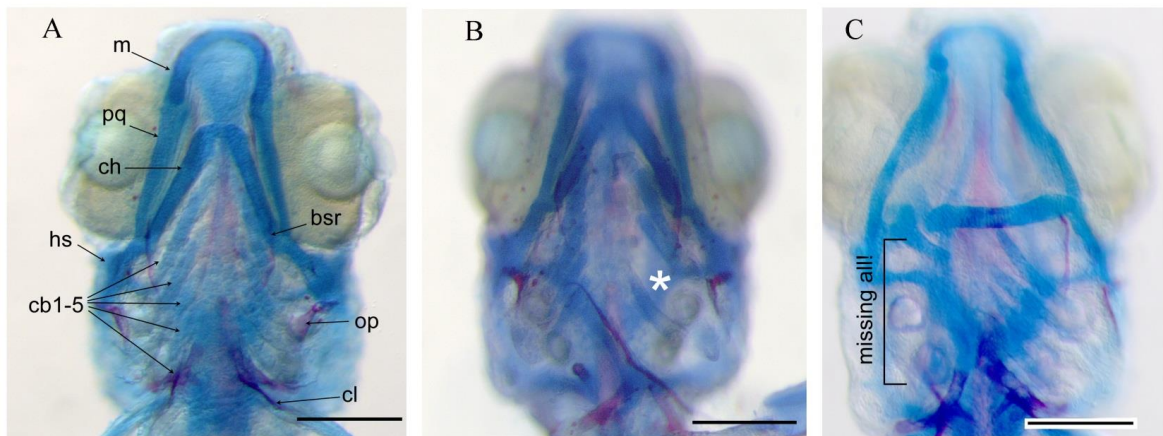


Figure 12 Zebrafish cartilage: A) Image of a 6 dpf WT zebrafish, with labelled cartilage and bone. Abbreviations stand for: m, meckel's cartilage; pq, palatoquadrate; ch, ceratohyal, bsr, branchiostegal ray; hs, hyosymplectic; op, opercle; cl, cleithrum; cb, ceratobranchials. Black scale bar represents 200 μ m. B) Example of an *ace/fgf8* mutant with the normal heart phenotype, where the ventricle was located on the right. On the right, it has all 5 ceratobranchials while on the left one is missing. Black scale bar represents 200 μ m. C) Example of a reversed heart *ace/fgf8* mutant, where not only the ceratobranchials are missing on the right but the ceratohyal is truncated. Black scale bar represents 200 μ m.

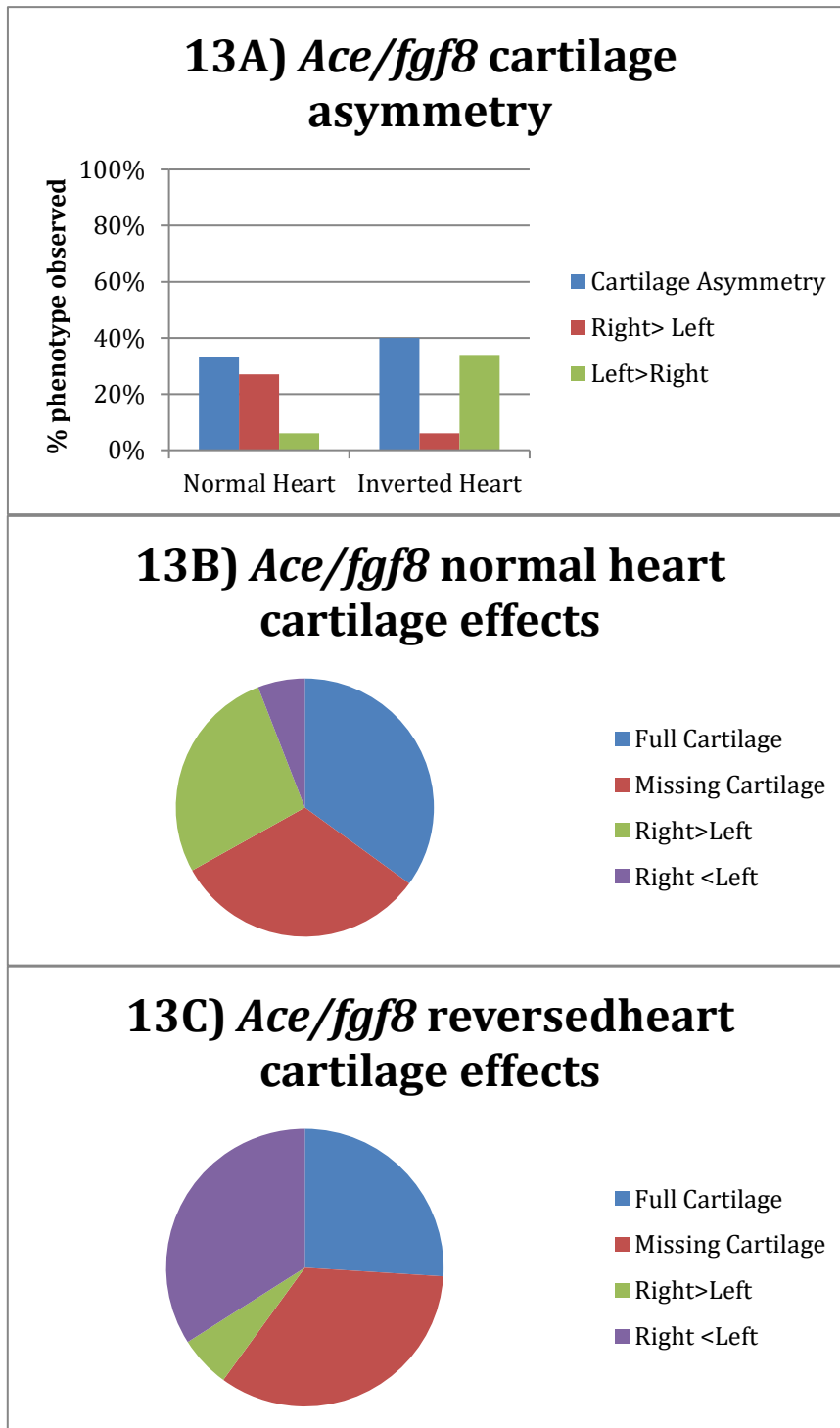
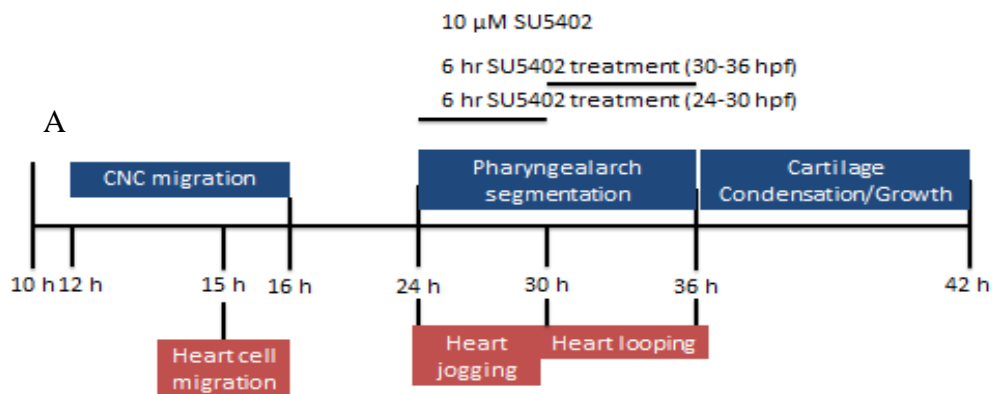


Figure 13 Asymmetry within *ace/fgf8* mutants A) The y axis is % phenotype observed of the mutant zebrafish. The sample size for normal heart mutants is 254, while for the reversed heart it is 50. 33% of the WT heart zebrafish experience asymmetry, with 27% of the asymmetries having more of the cartilage on the right than left, and 6% has more cartilage on the left than right. For the reversed heart, 40% of the mutants experienced cartilage asymmetry, with 34% having more on the left than right and 6% having more on the right than left B) Pie chart of all cartilage phenotypes observed in normal heart *ace/fgf8* mutants C) Pie chart of all cartilage phenotypes observed in reversed heart *ace/fgf8* mutants.

SU5402 exposure from 24-30 and 30-36 hpf leads to cartilage asymmetries

In zebrafish segmentation of the pharyngeal arches occurs from 20-36 hpf (Choe et al, 2014). Heart jogging and looping occur between 24-36 hpf (Choudrey & Trede, 2013). To most effectively cover these time points, we performed two six hour experiments from 24-30 hpf and 30-36 hpf. The total number of experimental zebrafish for these time points was 68 and 70, respectively (Figure 14A, B). During these time points, cartilage defects happened at a higher rate than the treatments at CNC migration stages. Not only are cartilage defects higher, but cartilage asymmetries occurring at a rate of ~ 23.6% during 24-30 hpf and ~22.4% during 30-36 hpf. Within the zebrafish that developed cartilage asymmetries the direction of the asymmetry tracked with the position of the ventricle 93% of the time when treated between 24-30 hpf and 87% of the time when treated between 30-36 hpf. These percentages are close to the percentages seen in *ace/fgf8* mutants. It is possible that there were no reversed hearts in the 30-36 hpf treatment because the heart is finishing up looping during this time point (Choudrey & Trede, 2013).



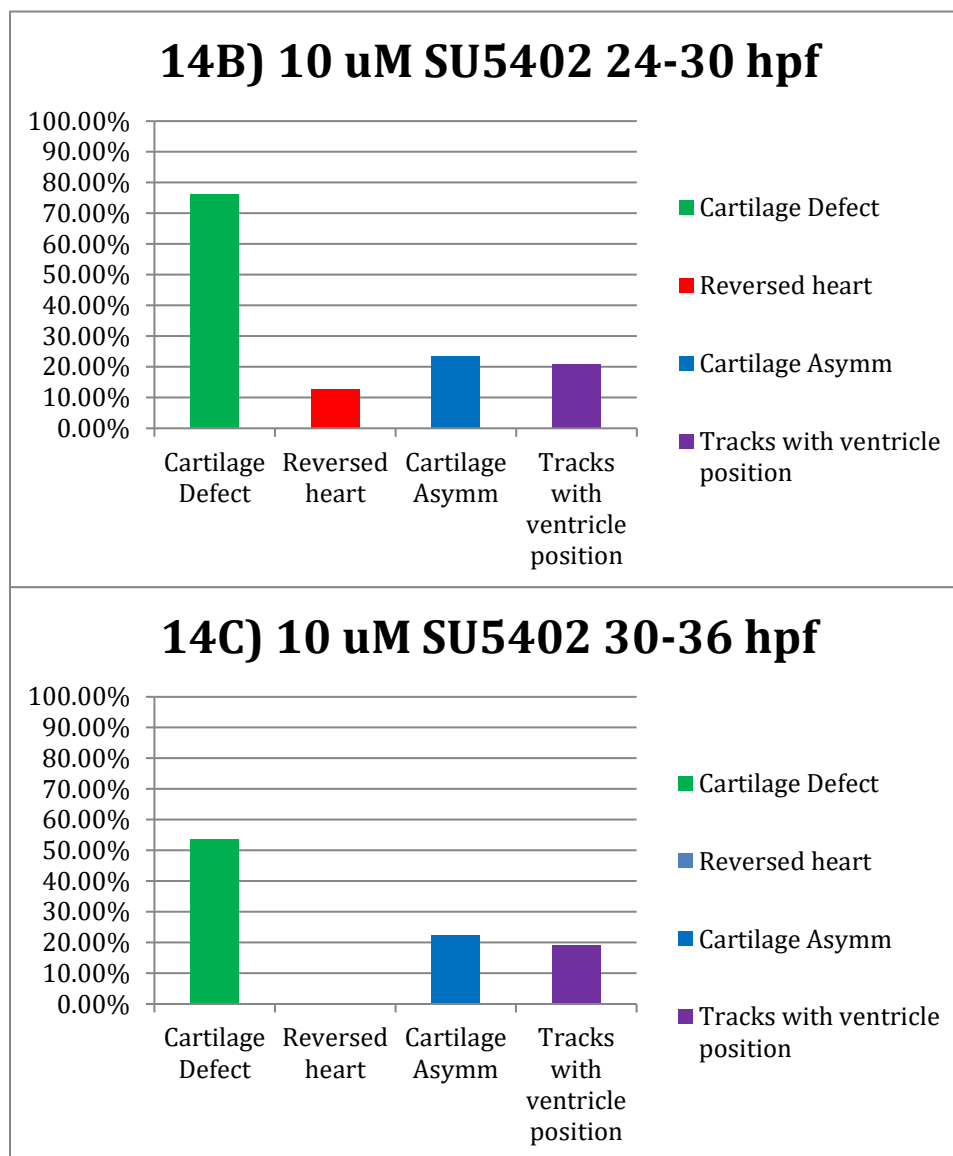


Figure 14 SU5402 treatments from 24-30 hpf & 30-36 hpf: A) Timeline of SU5402 treatments from 24-30 hpf and 30-36 hpf. B) Graph of SU5402 effects from 24-30 hpf: Cartilage asymmetry is seen in 23.6% of the zebrafish exposed to SU5402. Of these, most track the direction of the ventricle. 12.7% experience a reversed heart, and the total that experienced cartilage defects was 76.3%. DMSO control zebrafish experienced no cartilage defects and no inversion of the heart. C) Graph of SU5402 effects from 30-36 hpf: Cartilage asymmetry and directional asymmetry here are seen at 22.4% and 19%, respectively. There is no heart inversion occurring from treatment at this time, and cartilage defect occurs at 53.4%. n= 70. DMSO control zebrafish experienced no cartilage defects and no inversion of the heart

Asymmetries do not arise during post segmentation/ cartilage condensation stages

To assess cell death at post-segmentation stages, an apoptosis assay was done with *ace/fgf8* mutants and WT siblings at 40 hpf. Within the WT zebrafish (n=25), there was little to no apoptosis noted at this stage (Figure 15). In the *ace/fgf8* mutants (n=24), however, apoptosis occurred in several spots. Apoptosis was found in the tail, lens, heart, and head for all 24 zebrafish. These data are consistent with known roles for Fgfs in cell survival, however no cell death was observed within the developing pharyngeal arches. While it cannot be ruled out that cell death may contribute to jaw asymmetries in *ace/fgf8* mutants at other stages, these data suggest that asymmetries do not arise due to lateralized cell death at condensation stages.

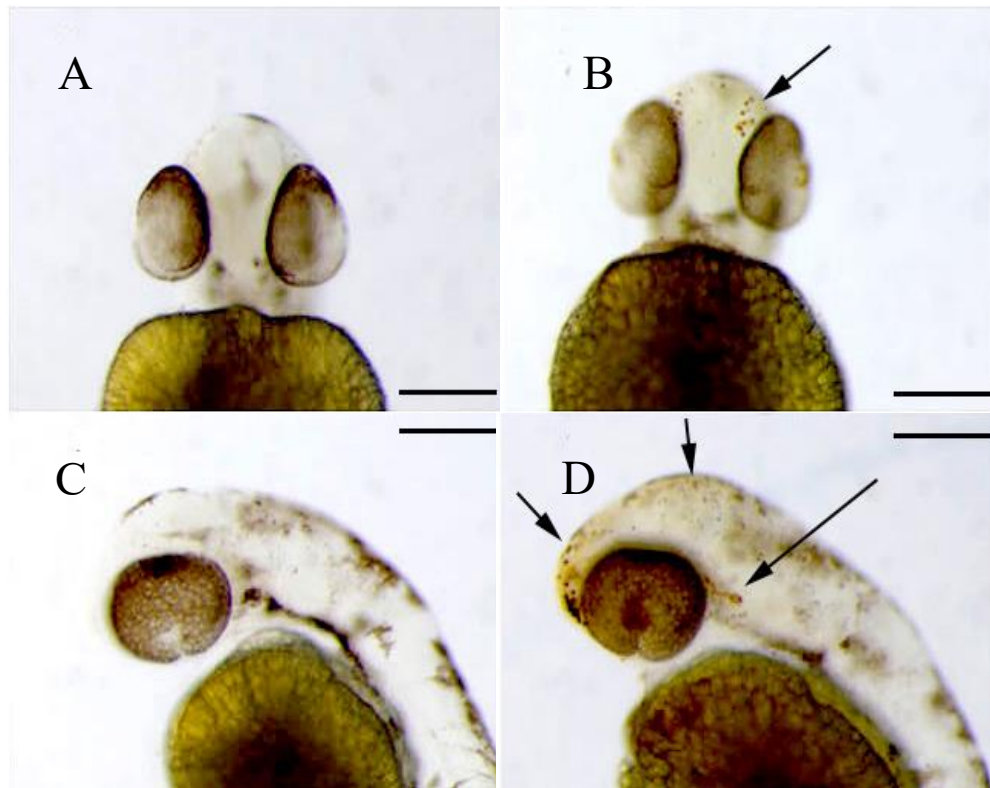


Figure 15 Apoptosis assay of WT and *ace/fgf8* mutant zebrafish: Orange dots represent apoptosis within the specimens (A and B are ventral view. C and D are lateral view). A) WT zebrafish at 40 hpf with no apoptosis. B) *ace/fgf8* mutant at 40 hpf, with visible apoptosis in the front of the head, the eye, and the heart. C) Lateral view of the WT zebrafish at 40 hpf, with no apoptosis present. D) Lateral view of the *ace/fgf8* mutant at 40 hpf, with apoptosis behind the eye and at the top of the head. Black bars represent 200 μm in all panels.

To complement the apoptosis assays, we treated wild type animals with 10 μM SU5402 at post-segmentation stages, 36-42 hpf. For this experiment, 70 zebrafish were used for the experimental treatment and 36 were used for DMSO carrier control treatment. Of the 70, 33 had cartilage defects, and 4 had reversed hearts, but none exhibited any cartilage asymmetry (Figure 16B). For the DMSO control zebrafish, 0 exhibited cartilage defects or heart reversal (not shown). These data complement those presented above and suggest that cartilage asymmetries do not arise at this stage.

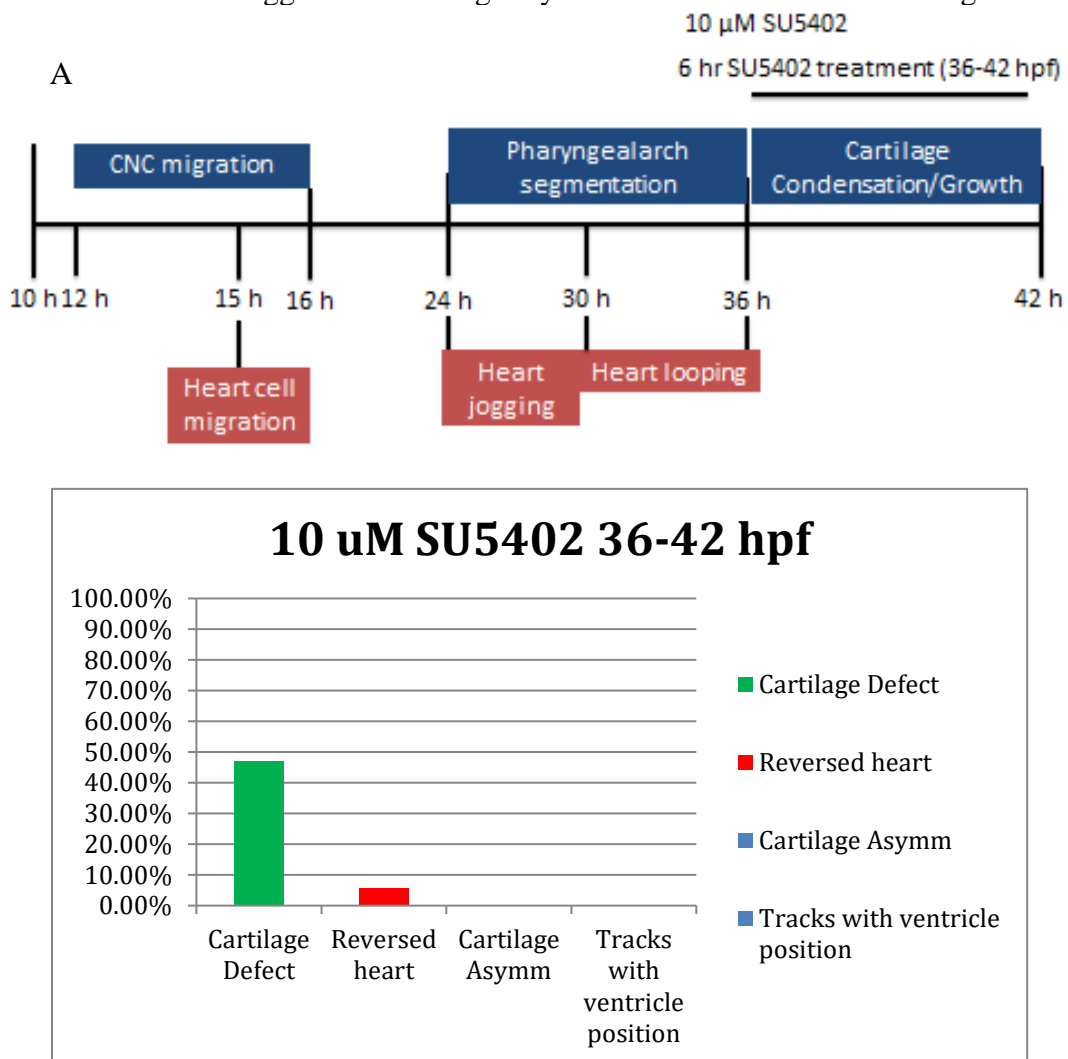


Figure 16 SU5402 treatment from 36-42 hpf: A) Timeline of treatment from 36-42 hpf during condensation stage, with 10 μM SU502 B) Graph of SU5402 exposure effects from 36-42 hpf: The y axis is % of phenotype the observed, n=70. From 36-42 hpf, no cartilage asymmetry occurs, and cartilage defects occur at a 47.1% rate.

CHAPTER 4

DISCUSSION

Pharyngeal arch segmentation/ heart looping is the sensitive time for cartilage asymmetry

Consistent with our hypothesis, there were no cartilage asymmetries did not arise during CNC migration stages (10-16 hpf) or the pharyngeal arch growth stages (36-42 hpf) when embryos were treated with SU5402. During pharyngeal arch segmentation (24-36 hpf), we do see craniofacial asymmetries, which is also consistent with our hypothesis. Not only are craniofacial asymmetries observed but they were also directional in the sense that whichever side the ventricle was on, there was more cartilage on that side than the opposite side. This strongly supports our overarching hypothesis.

Cartilage asymmetries still arise without asymmetric CNC migration in *ace/fgf8* mutants

We bred the *ace* mutation into the *sox10:gfp* reporter line. Surprisingly, CNC migration appeared relatively unaffected in *ace/fgf8* mutants. In addition, no obvious asymmetries were observed in CNCs. We examined the migration carefully, but for future work it will be helpful to use a more powerful scope to meticulously examine the CNC migration, and to test if this trend holds.

No obvious apoptosis within *ace/fgf8* mutants

Similar to the point above, surveying mutants for apoptosis provided basic insight into what is going on within the *ace/fgf8* mutants. We were able to look carefully at apoptosis in many parts of the embryo, including the cartilage, but further assays will help. Using a stronger microscope, and dissecting out the yolk and looking at the

cartilage will offer a better understanding on apoptosis within these mutants. Sectioning and co-staining after TUNEL staining will be important next steps.

***Ace/fgf8* mutant and SU5402 treated zebrafish cartilage asymmetry correlates with ventricle location**

Within the *ace/fgf8* mutants, whenever there was a cartilage asymmetry, 82% and 85% of the time it tracked with the side of the ventricle. In the SU5402 treated zebrafish this percentage was slightly higher, at 93% and 87% of the time depending on when the treatment occurred (see above). This is consistent with our hypothesis, that heart looping likely plays a role in the development of cartilage asymmetries in *ace/fgf8* mutants. Collectively these data strongly implicate the developing heart field, especially the ventricle, in providing a compensatory signal that partially rescues the pharyngeal arch, and hence the cartilage, phenotype in *ace/fgf8* mutants on one side of the larvae. Testing this hypothesis as well as identifying the specific signal(s) should be the focus of future investigations.

Direct or indirect molecular cue influencing cartilage asymmetry

One possibility that we did not explore fully with this work is that a molecular cue working independent of heart or pharyngeal arch development influences the cartilage asymmetry within the *ace/fgf8* mutant zebrafish. While we ruled out CNC migration as a causative stage, it is possible that asymmetries are due to an earlier stage, such as the development of the ciliated node. It is also possible that they arise from another structure such as the forebrain. However, we find this to be unlikely and assert that the heart remains a prime candidate for the asymmetry in cartilage. Our work shows a correlation between

heart and cartilage asymmetries, and other work shows that both the developing ventricle and cartilage share important signals during development, such as *wnt11r*.

Wnt11r as the potential compensatory signal

Due to the cartilage asymmetry arising during pharyngeal arch segmentation and heart looping, future investigation should target signal(s) behind the compensation in cartilage development. Within the heart, Choudrey and Trede (2013) noted that *tbx1* expression is stronger in the ventricle than the atrium, and that it is required for ventricle differentiation, and necessary for heart looping. They also found that *wnt11r* is activated by *tbx1* in the ventricle. *Tbx1* and *wnt11r* also play important roles, together with *fgf8* during pharyngeal arch segmentation (Choe et al, 2014). Specifically, these investigators found that *tbx1* controls pharyngeal pouch formation by controlling mesodermal expression of *wnt11r* and *fgf8*. All three factors are necessary for pouch formation. *Wnt11r* causes the initial outpocketing of the pouches, while *fgf8* promotes the outgrowth of the pouches. Notably, they also found that when *wnt11r* was restored in *tbx1* mutants it was more effective at rescuing pouches than was *fgf8* restoration. Thus, ventricle and pouch formation both require mesodermal expression of *wnt11r*.

Building on this evidence and based on the results from my thesis, we have created a model to indicate what is possibly going on in *ace/fgf8* mutants (Figure 17).

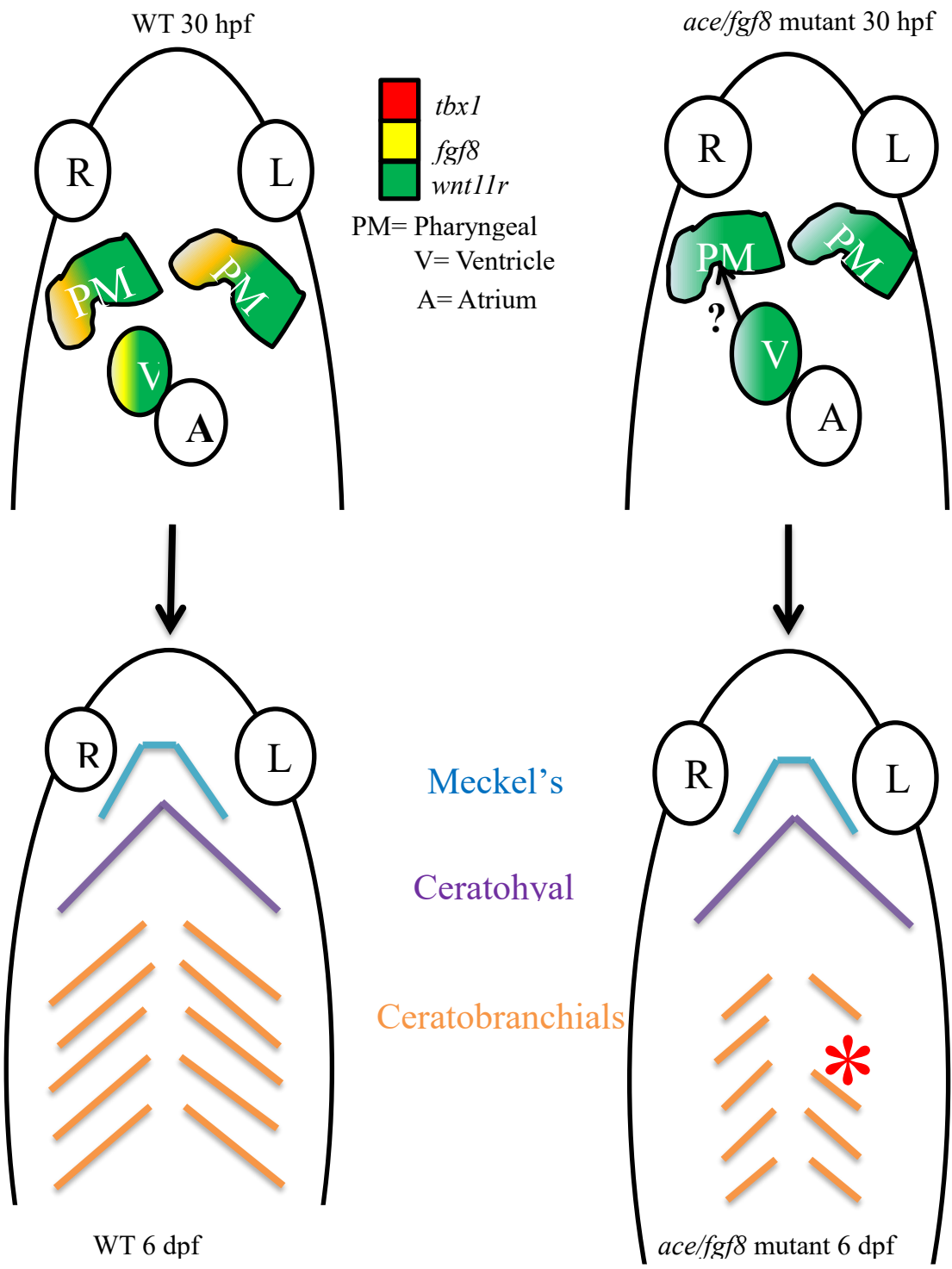


Figure 17 Proposed model for the molecular origin of cartilage asymmetries: this model accounts for signaling from the ventricular mesoderm as compensating for loss of *fgf8* in the pharyngeal mesoderm. . Via *Tbx1*, *wnt11r* is upregulated on the ventricle side but not the opposite side, leading to the asymmetry.

In the WT embryo above, *fgf8*, *wnt11r*, and *tbx1* are expressed in the pharyngeal mesoderm and the ventricle at 30 hpf. By 6 dpf, all of the craniofacial cartilage is present within the zebrafish. . As stated previously, all three of these signals are involved in proper pouch formation. In *wnt11r* mutants the pouch formation is destabilized, but *fgf8a* expression is sufficient to maintain some pouch outgrowth (e.g., *wnt11r* mutants possess reduced cartilage numbers Choe et al, 2014). *Ace/fgf8* mutants also exhibit reduced cartilage numbers, but to a lesser extent than *wnt11r* mutants, which is consistent with the idea that *wnt11r* is a more potent signal in maintaining pharyngeal arch segmentation (Choe et al, 2014). In *ace/fgf8* mutants, *fgf8* is not present in the pharyngeal mesoderm, so it cannot promote the outgrowth of pouches. Craniofacial cartilage still forms within the mutants. We propose the asymmetry arises from *wnt11r* permeating from the ventricle to the pharyngeal mesoderm on the same side, and this upregulation goes on to compensate for loss of *fgf8* expression. This leads to more cartilage forming on the ventricle side.

Bead/tissue implantation

One way in which gene/protein levels may be manipulated in a time/location specific manner is through bead/tissue implants. Bead implants could be done not only with SU5402 in WT zebrafish to knock down Fgf signaling in a targeted fashion, but also Wnt11r protein in *ace/fgf8* mutants to try to rescue cartilage defects. They also open up the possibility of taking cardiac mesoderm from one fish and implanting it into the left or right side of *ace/fgf8* mutants. These studies could go on to show that targeted knockdown on one side with SU5402 is more effective than soaking embryos in SU5402

embryo water, and provide spatial resolution for the phenotype. By using beads coated in Wnt11r protein in *ace/fgf8* mutants on the side opposite of the ventricle, we would expect to see less cartilage asymmetry.

CHAPTER 5

CONCLUSIONS

The results of this study have provided insight to the time of development that is most sensitive for cartilage asymmetry in response to loss of Fgf signaling. It has also provided insight into the dosage of SU5402 that most efficiently recreates the asymmetry phenotype of *ace/fgf8* zebrafish. 10 μ M during a 6 hour window (between 24-30 or 30-36 hpf) was found to be the best treatment, as 12 hours was too strong. The frequency of asymmetries was still lower in the SU5402 treated zebrafish, and never reached the 33-40% frequency observed within the mutants. There is the possibility that this frequency is difficult to fully recapitulate, as development is robust within the wild type zebrafish, so even though there is a knockdown during the time frame they can recuperate. Within the *ace/fgf8* mutants, *fgf8* expression is absent throughout development, whereas the SU5402 treatment inhibits Fgf receptors while the zebrafish are exposed to it. In contrast to the frequency of asymmetries in general, we were able to increase the frequency in which the direction of cartilage asymmetry matched that of the ventricle using SU5402. Whereas the frequency of cartilage tracking the heart was 82% and 85% in mutant, it was up to 93% when treating animals at segmentation stages.

Through further investigations using the *ace/fgf8* mutant, we can potentially gain further insights into the influence of asymmetric signaling in other organ systems. So, does the body know it's left from right? My thesis suggests that for the jaw, the answer is yes, and that this occurs as the developing heart field, which is asymmetric, interacts with the developing pharyngeal arch field, which is symmetric. Normally this laterality is hidden by a robust developmental program (e.g., both *fgf8a* and *wnt11r* working to drive

PA segmentation), however when development is destabilized the asymmetry reveals itself.

Not only does this work represent another example of interactions between developmental fields, but it provides insight into the origins of this interesting asymmetric defect. We are one step closer to knowing the specific signal and tissue origins, as we have narrowed down not only the time frame but have shown it tracks with an organ that is close by, similar to the example of lung and liver specification (Serls et. al, 2006). It's a wonder how more asymmetries do not arise, as there's widespread asymmetric signaling early in development. We have seen how in sticklebacks and mice a mutation in one gene can lead to an asymmetry within paired structures (Shapiro et. al, 2004; Bell et. al, 2006). These studies combined with my own, reveal how tenuous bilateral symmetry can be. In both examples, the absence of one gene leads to normal asymmetric signals "bleeding" into another developmental field, giving rise to abnormal asymmetries.

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