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A Molecular Basis for Craniofacial Laterality in Fishes

A Capstone Project Submitted in Partial Fulfillment of the
Requirements of the Renée Crown University Honors Program at
Syracuse University

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and Renée Crown University Honors

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Abstract

Laterality, the preference for development on one side of the body, is essential to the vertebrate body plan. While the vertebrate skeleton usually develops symmetrically, the processes underlying craniofacial laterality are not well understood. Using zebrafish and cichlids as model organisms, this study focuses on the molecular basis of symmetric craniofacial development. *Fgf8*, Retinoic Acid, and *wnt11* were examined to analyze their involvement in regulating craniofacial laterality. *Fgf8* is known to play a role in proper jaw development. Because *fgf8* and Retinoic Acid interact to achieve laterality of the somites during somitogenesis, the role of Retinoic Acid in regulating craniofacial laterality was also investigated. *Wnt11* was analyzed because it was recently mapped to a chromosomal region in cichlids that is associated with the regulation of asymmetric jaw development. Wnt signaling is also known to be involved in regulating asymmetries in the developing heart field. By comparing the jaws of wild type and Retinoic Acid deficient zebrafish, we show that Retinoic Acid function promotes symmetric craniofacial development. Using whole-mount in situ hybridization (WISH), we provide evidence that *Fgf8* and Retinoic Acid interact to achieve laterality of the pharyngeal cartilages. Additionally, WISH was used to show the asymmetric expression of *wnt11* in developing wild type cichlids. By treating cichlid embryos with LiCl, we show that the over expression of Wnt signaling can

induce craniofacial asymmetries. We provide potential models to explain the roles of *fgf8*, Retinoic Acid, and *wnt11* in regulating craniofacial laterality.

Table of Contents

Acknowledgements	i
Introduction	1
Methods	6
Results	9
Discussion	16
References	21
Capstone Summary	23

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Introduction

In establishing the vertebrate body plan, laterality, the preference for development on one side of the body, is of fundamental importance. While visceral organs such as the brain, heart, and gut exhibit asymmetries along the left-right (LR) axis, the vertebrate skeleton usually develops symmetrically. Although much is known about the genetic basis of visceral organ asymmetries, the processes underlying craniofacial laterality are not as well understood. Up to 70% of birth defects are characterized by craniofacial malformations (Hall, 1999). Two of the most common birth defects, Hemifacial Microsomia and Treacher-Collins Syndrome, involve asymmetric jaw development (Albertson and Yelick, 2005). Identifying the molecular basis of craniofacial laterality is crucial for understanding these and related birth defects.

The zebrafish (*Danio rerio*) is one of the only model organisms that has been used to study the molecular basis of craniofacial laterality (Albertson and Yelick, 2005; 2007). Several features of zebrafish make them an ideal candidate for vertebrate genetic and developmental studies. They are small, freshwater fish that are maintained relatively easily and inexpensively in a laboratory setting. Zebrafish develop rapidly and exhibit high fecundity, which allows for mutagenesis screens. Their eggs are fertilized externally, and their embryos are transparent making it fairly easy to observe

developmental events. Also, the zebrafish genome has been completely sequenced, which allows for the easy identification of specific genes. While zebrafish have fewer cells and simpler spatial patterns than higher vertebrates, they possess the same muscle and skeletal types. Finally, many craniofacial mutations have been identified in zebrafish (Yelick and Schilling, 2002). These many advantages of zebrafish make them an ideal model for our experiments.

Cichlids were also used as a model organism for this study. Like zebrafish, cichlids are small, tropical fish that exhibit high fecundity, rapid development, and are easily maintained in a laboratory setting. They also have multiple genomic resources available to researchers, which simplifies genetic experiments. Additionally, cichlids exhibit remarkable levels of anatomical diversity. One cichlid species that feeds on scales has naturally occurring asymmetric jaws, a characteristic that was particularly useful for this study.

A number of different molecules were examined over the course of this study to analyze their involvement in regulating craniofacial laterality. One such molecule, *Fgf8*, is known to play a role in proper jaw development. *Fgf8* is one of several fibroblast growth factors (FGFs), which are intercellular signaling molecules that play various roles in development. It has been shown that *fgf8* is required for both the asymmetric development of the visceral organs and the symmetric development of the jaw (Albertson and Yelick, 2005). *Fgf8* was found to

be associated with the presence of Kupffer's Vesicle (KV), a ciliated organ that forms early in development. After KV develops, the cilia covering its surface vibrate and create a fluid flow. This initiates a series of events that ultimately lead to the asymmetric development of the visceral organs and brain. When KV is present, the organs develop asymmetrically as expected. When *fgf8* was absent, one-third of the zebrafish mutants lacked KV altogether. This suggests that *fgf8* plays a role in the morphogenesis of KV. It was also shown that without *fgf8*, the pharyngeal cartilages of the jaw develop asymmetrically (Figure 1). Therefore, *fgf8* plays at least two distinct roles in the establishment of vertebrate laterality. It leads to the formation of KV, which results in asymmetric visceral organ development, and it also plays a role in the symmetric development of the craniofacial skeleton (Albertson and Yelick, 2005).

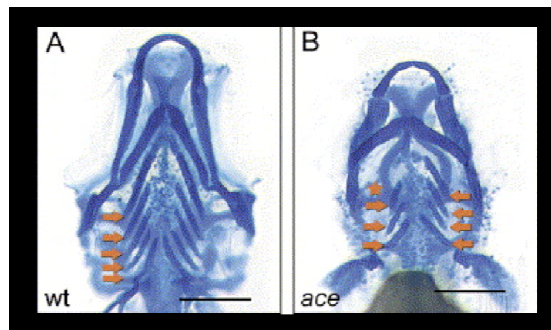


Figure 1 –
Asymmetric
development of
the pharyngeal
cartilages in *fgf8*
(*ace*) mutants
(Albertson and
Yelick, 2005).

Retinoic Acid, a Vitamin A derivative and intercellular signaling molecule, is also suspected to play a role in craniofacial laterality regulation. Retinoic Acid is known to be important in establishing the symmetry of other systems in the vertebrate body. For example, during somitogenesis, the somites, or precursors to muscle cells, develop

symmetrically along the LR axis. It has been shown that both Retinoic Acid and *fgf8* are necessary for the proper symmetric development of the somites (Kawakami et al., 2005). When either Retinoic Acid or *fgf8* is absent, the somites develop asymmetrically along the LR axis (Figure 2). It was also found that without *fgf8*, Retinoic Acid is over expressed. This suggests that Fgf8 acts as an inhibitor of Retinoic Acid production. Just as Fgf8 and Retinoic Acid regulate each other during somitogenesis, we suspected that a similar process may be occurring in the developing jaw, and that Retinoic Acid and Fgf8 regulate each other as antagonistic pairs to achieve craniofacial symmetry.

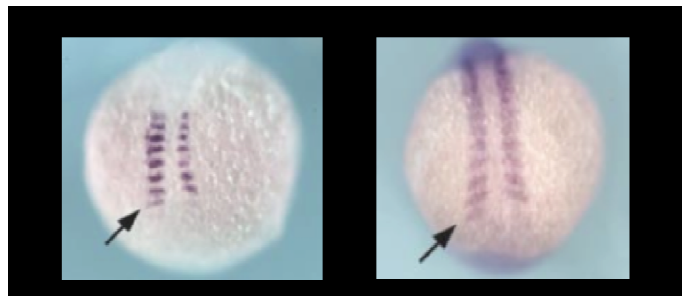
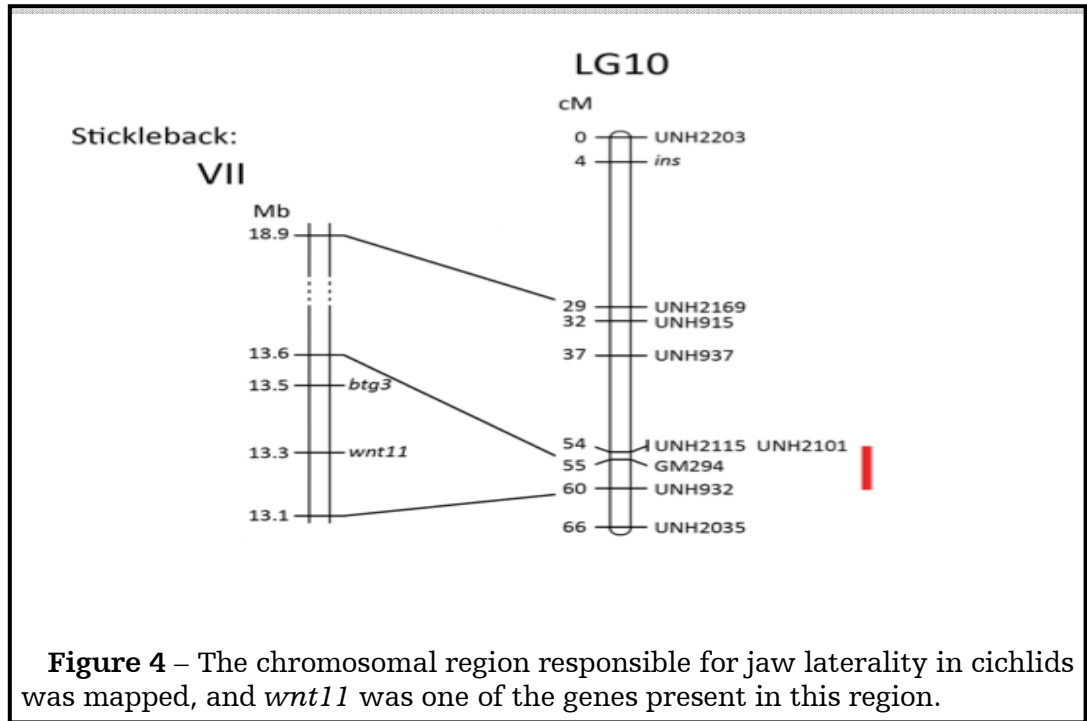
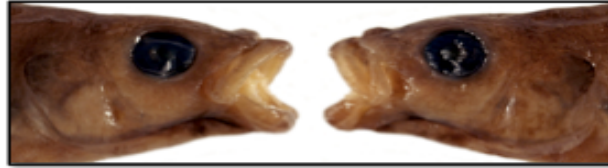


Figure 2 – Asymmetric somite development in Retinoic Acid mutants (Kawakami, 2005).

The role of *wnt11* in the regulation of craniofacial symmetries was also investigated during the course of this study. This focus arose due to a recent study on *Perissodus microlepis*, a scale-eating cichlid species (Figure 3). These fish have evolved naturally occurring asymmetric jaws, which they use to feed on the scales of other fish by attacking them at an angle from behind (Stewart and Albertson, 2010). In analyzing the genetic profile of jaw asymmetry in cichlids, a small region on chromosome 10 was found to be involved in the regulation of

Figure 3 – Scale-eating cichlids have naturally occurring asymmetric jaws.

their jaw asymmetry. In this chromosomal region, *wnt11* was one of the genes that was present (Figure 4).



In addition to the suspicion that *wnt11* plays a role in regulating laterality in the jaws of scale-eating cichlids, *wnt11* was chosen for this study because it is known that Wnts play a role in establishing cardiac asymmetry. Like Fgf8 signaling, Wnt signaling early in development leads to the formation of KV in zebrafish. An asymmetric signal is then sent from KV to the lateral plate mesoderm (LPM), and this ultimately results in the asymmetric development of the heart field (Lin and Xu, 2009). Given the roles of Wnt signaling in cardiac asymmetries, we

suspected that, similarly to *fgf8*, Wnts (more specifically, *wnt11*) might also play a role in the regulation of craniofacial symmetries.

Methods

Whole-Mount In Situ Hybridization (WISH)

To analyze gene expression during development, WISH analyses were used. Methods generally follow Albertson and Yelick (2005). Specifically, embryos were collected and fixed in 4% paraformaldehyde (PFA) overnight at 4°C. They were then dehydrated using an ascending methanol series, left overnight at 4°C, and rehydrated back into Phosphate-buffered solution with 0.1% Tween-20 (PBSt). Older embryos were bleached using a 10% solution of H₂O₂/PBSt to reduce pigmentation. Tissues were digested in a 1:5000 dilution of Proteinase K:PBSt and refixed in 4% PFA for 30 minutes. Embryos were then put in a prehybridization solution (PHS) (50% formamide, 5x SSC, 0.1% Tween-20, 4.6 mM citric acid, DEPC-H₂O) for 2-3 hours at 70°C. The PHS solution was removed, and the embryos were left to incubate at 70°C overnight in hybridization solution, which contained PHS as well as 50 µg/ml heparin, 50 µg/ml tRNA, and the selected genetic probe. The probes used were generated from cDNA of *fgf8*, *wnt11*,

raldh2, and *dlx2*. To synthesize the probes, plasmids were transformed using selected cDNA and the Invitrogen One Shot TOP10 chemically competent procedure. After being cultured in a solution containing 50mg/mL Ampicillin, the transformed plasmids were prepped according to the Eppendorf Fast Plasmid Mini procedure. They were then cut to form linear plasmids using plasmid specific restriction enzymes. An extraction was performed in phenol chloroform, and then the cleaned linear plasmid was precipitated. The linear plasmid was transcribed using a specific polymerase. After being precipitated, the newly generated RNA probe was fractionated. The hydrolyzed probe was then precipitated before being used in WISH experiments. The nucleic acid probes contain dig-tagged nucleotides, which makes it possible to observe the localization of the genes of interest. The following morning, embryos were washed in graded prehybridization solutions at 70°C into 2x SSC followed by 0.2x SSC. A pre-block solution containing Boehringer blocking reagent, maleic acid buffer (MAB: 100 mM Maleic Acid, 150 mM NaCl, 7.5 g/L NaOH, 0.1% Tween-20, pH 7.5), and lamb serum was added. After at least 3 hours, a block solution was added containing α -dig antibody, and the embryos incubated overnight at 4°C. The following day, the embryos were washed in MAB as well as AP buffer (60 mM Tris-HCl, 60 mM NaCl, 30 mM MgCl₂, 0.1% Tween-20). They were then put in a staining solution containing BCIP (5-bromo-4-chloro-3-indolyl phosphate in 100% dimethylformamide), NBT (nitro

blue tetrazolium in 70% dimethylformamide), and AP Buffer and left until sufficiently stained. The reaction was stopped with PBSt washes, and embryos were refixed in 4% PFA. To remove background staining, embryos were dehydrated in 100% methanol and left overnight. They were then rehydrated back into 100% PBSt, and, finally, stepped into an 80% glycerol solution for storage.

WISH was used to observe *fgf8*, *raldh2*, and *wnt11* gene expression. Using WISH, we stained *ace* mutants, which lack *fgf8*, for the expression of *raldh2*, which encodes a protein necessary for Retinoic Acid synthesis. The protocol was also used to observe *fgf8* expression in *nls* mutants, which lack *raldh2*. WISH was also used to observe the expression of *dlx2* in *nls* mutants. *Dlx2* is a gene expressed in migrating neural crest cells, which are the cells that form the pharyngeal cartilages. Finally, wild type cichlids were also stained for *wnt11* expression at various stages in development to look for asymmetric expression.

LiCl Treatments

To identify the roles of Wnt signaling during jaw development, cichlid embryos were treated with LiCl at different stages for various intervals of time. LiCl is a GSK3 inhibitor. The inhibition of GSK3 leads to the over expression of Wnt signaling, likely including *wnt11* signaling. Cichlids at 2-10 days post fertilization (dpf) were treated

with varying amounts of LiCl (5 μ M – 30 μ M per liter of H₂O) for different time intervals, which are specified in the results.

Clearing and Staining

To better visualize the jaw cartilages of the zebrafish and cichlids, a protocol for clearing and staining as described by Walker and Kimmel (2007) was used. Embryos were collected at appropriate stages and dehydrated in ethanol. Cartilages of the jaw were stained in a solution containing Alcian blue (1 mL of a solution containing 0.02% Alcian blue, 70% ethanol, and 60 μ M MgCl₂; 10 μ L of solution containing 0.5% Alzarine Red) and left overnight. The next day, embryos were rinsed in H₂O before being bleached in a 1:1 solution of 2% KOH and 3% H₂O₂ for 10 minutes. After another wash in H₂O, embryos were enzymatically cleared in a solution of 20% glycerol and 0.25% KOH for 1-2 hours, followed by another clearing solution made up of 50% glycerol and 0.25% KOH. After being left overnight in the second clearing solution, the embryos were put into 80% glycerol for storage.

Results

Role of Retinoic Acid in craniofacial development

To confirm the importance of Retinoic Acid in the regulation of craniofacial asymmetries, the jaws of wild type zebrafish and the jaws of zebrafish that lacked Retinoic Acid (*nls* mutants) were compared.

After the fish were cleared and stained, we observed asymmetric pharyngeal cartilages in *nls* mutants (Figure 5). We also saw that these mutants had underdeveloped jaws, which lacked several pharyngeal cartilages. Of the 25 *nls* mutants that were analyzed, only 7 developed any posterior cartilages. Of those 7 zebrafish, 5 had asymmetric pharyngeal cartilages. This observation leads to the conclusion that Retinoic Acid is involved in the symmetric development of the pharyngeal cartilages.

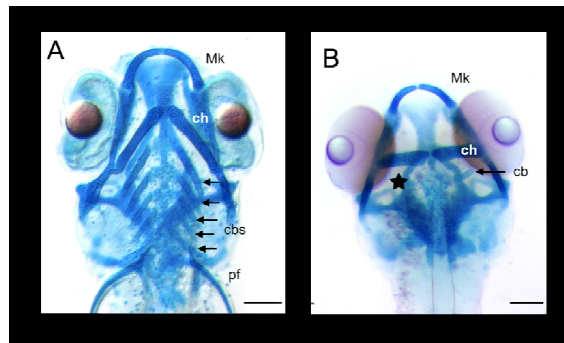


Figure 5 - Asymmetric pharyngeal cartilages in *nls* mutants. (A) Wild type (wt) zebrafish with symmetric pharyngeal cartilages. (B) *Nls* mutant with asymmetric pharyngeal cartilages. Star denotes missing cartilage. Abbreviations: cbs, ceratobranchial cartilages; ch, ceratohyal cartilage; Mk, Meckel's cartilage; pf, pectoral fin. Scale bars equal 200µm.

Retinoic Acid and *fgf8* expression during craniofacial development

Because it was known that *fgf8* was also necessary for the development of symmetric pharyngeal cartilages (Figure 1), we observed the expression of *fgf8* in *nls/raldh2* mutants as well as *raldh2* expression in *ace/fgf8* mutants using WISH. *Raldh2* expression was observed because the gene encodes an enzyme necessary for Retinoic Acid synthesis. In mutants lacking *fgf8*, we saw that *raldh2* was over

expressed as compared to wild type zebrafish (Figure 6A). This observation is consistent with *Fgf8*'s role as an inhibitor of Retinoic Acid production. Notably, 25% of *fgf8* mutants also exhibited asymmetric *raldh2* expression in the lateral mesoderm (Figure 6B-C). *Fgf8* expression in 33% of *nls* mutants was also asymmetric in either the lateral mesoderm (possibly the heart field) or in the pharyngeal endoderm (Figure 6D-E). While the percentages of asymmetries were rather small, the sample sizes for this experiment were also small (*nls* mutants: n=8; *fgf8* mutants: n=12). This experiment should be repeated in future studies with larger sample sizes to provide more accurate statistics on the frequency of asymmetric *fgf8* and *raldh2* expression.

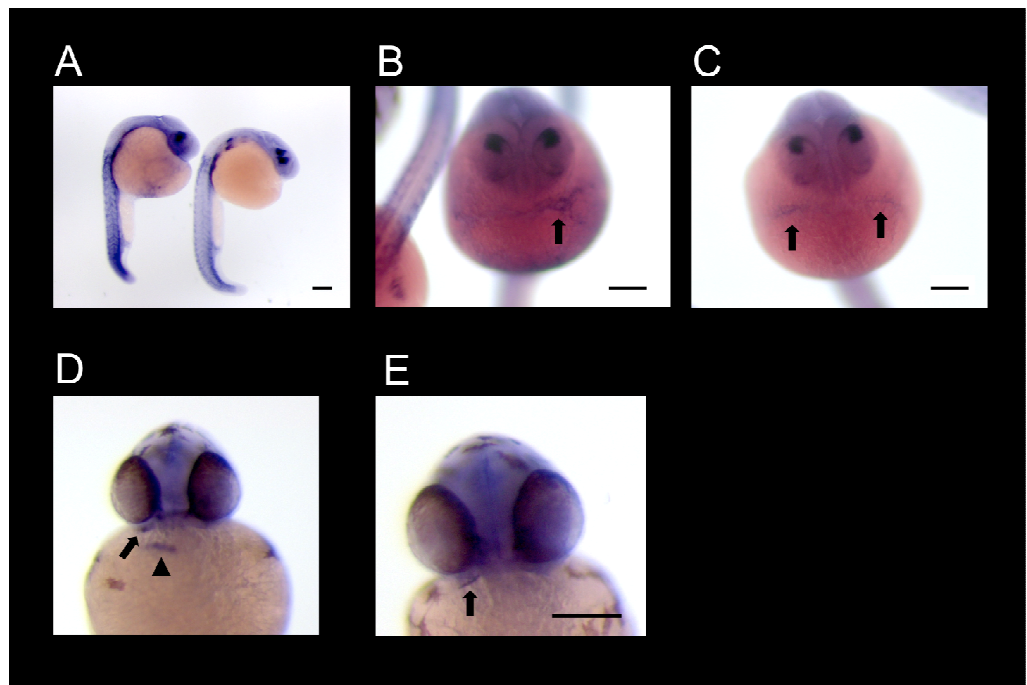


Figure 6 - Asymmetric *raldh2* and *fgf8* expression. (A) Comparison of a *fgf8* mutant (left) and a wt zebrafish (right). Note that *raldh2* expression is upregulated in zebrafish lacking *fgf8*. (B) *Fgf8* mutant exhibiting asymmetric *raldh2* expression in lateral mesoderm (arrow). (C) Wt embryos showing symmetric *raldh2* expression in the lateral mesoderm (arrows) (seen in 25% of mutants, n=8). (D-E) *Nls* mutants show asymmetric expression of *fgf8* in the pharyngeal endoderm (arrows) and lateral mesoderm (arrowhead) (seen in 33% of mutants, n=12). Scale bars

Neural Crest Cell migration in Retinoic Acid deficient mutants

In zebrafish lacking Retinoic Acid, the pharyngeal arches are underdeveloped and asymmetric along the LR axis. Using WISH, we observed that neural crest cells, which will eventually form the pharyngeal cartilages, migrate asymmetrically to the pharyngeal endoderm (Figure 7).

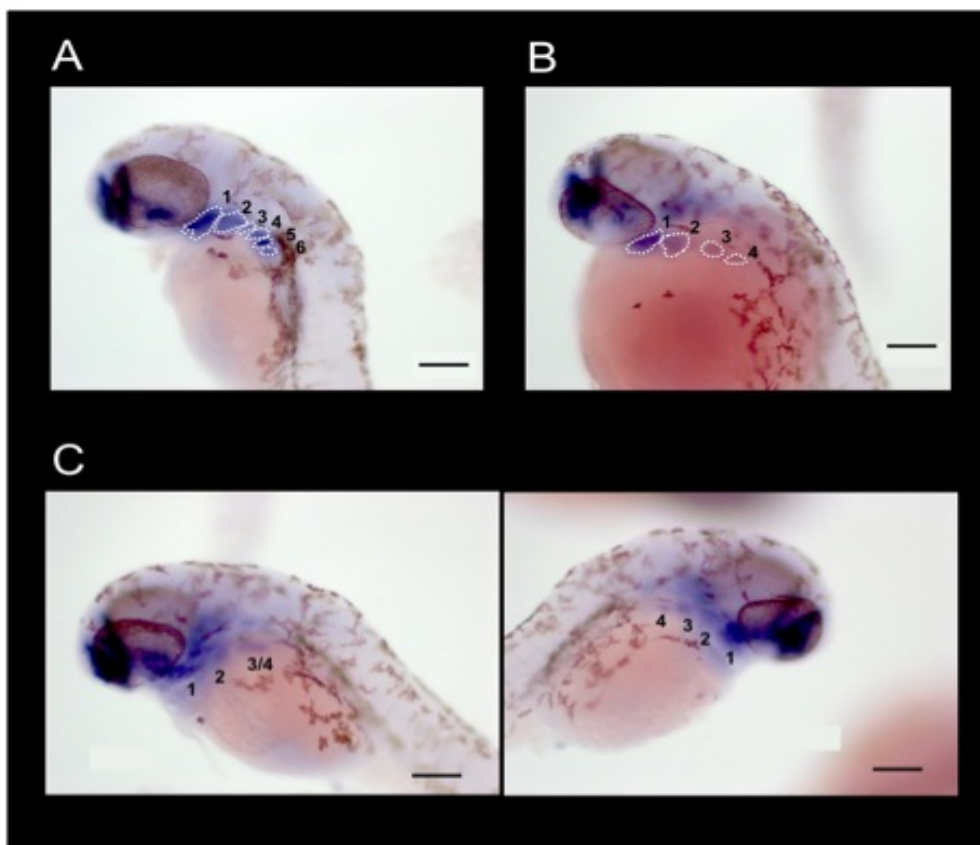


Figure 7 - Asymmetric pharyngeal arch development in *nls* mutants. (A) At 32 hours post-fertilization, wt zebrafish possess 6 pharyngeal arches, which will give rise to pharyngeal cartilages. (B) *Nls* mutants lack posterior pharyngeal arches. (C) Left and Right view of *nls* mutant lacking pharyngeal arches. These mutants show asymmetries in the number of arches present on the left and right sides at 32 hours post-fertilization, consistent with asymmetric defects in pharyngeal cartilages (53% showed abnormal arch formation; n = 58). Scale bars equal 100 μ m.

Roles of *wnt11* in craniofacial development

To study the role of *wnt11* in regulating craniofacial laterality, we used WISH to observe *wnt11* expression in wild type cichlids. Because little is known about the activity of *wnt11* during early cichlid development, we stained embryos from 5-8 days post fertilization (dpf) to find when *wnt11* is most active. Surprisingly, we found *wnt11* expression in cichlid embryos was asymmetric around the developing pharyngeal cartilages at 6 and 8 dpf (Figure 8). The number of cichlids with asymmetric *wnt11* expression was quantified, and we found that 75% of cichlids showed asymmetric expression at 8 dpf (Table 1). Asymmetric *wnt11* expression was also seen in 25% of 6 dpf cichlid embryos. We also saw that 75% of those with asymmetric *wnt11* expression showed the asymmetry on the left side of the jaw (Table 2).

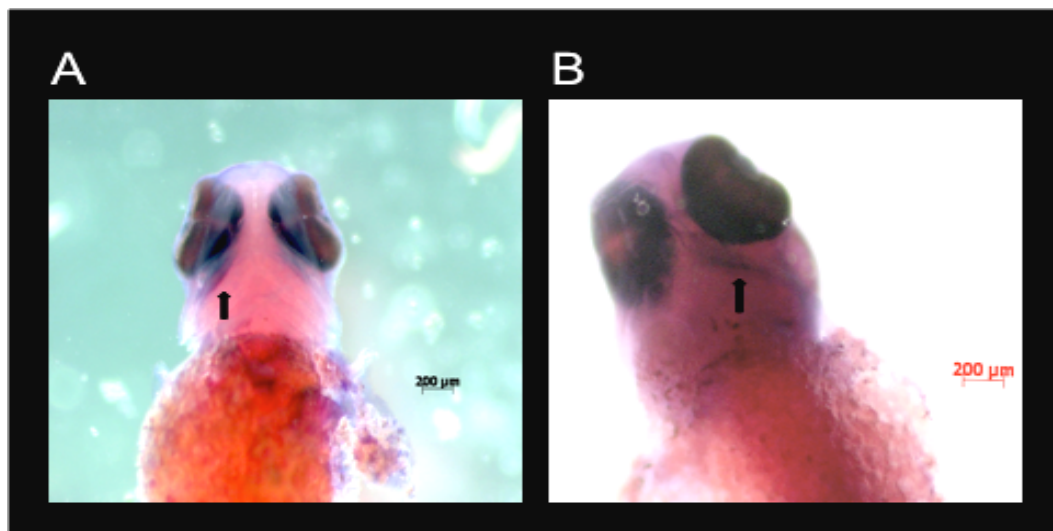


Figure 8 – Asymmetric *wnt11* expression in wild type cichlids in the pharyngeal endoderm (arrows) at (A) 6 days post fertilization (dpf) and (B) 8 dpf. Asymmetries on both the left and right sides were seen.

Asymmetric <i>wnt11</i> expression		
dpf	# cichlids	% asymmetric
6	4	25%
7	4	0%
8	4	75%

Table 1 – At 8 dpf, the majority of wild type cichlids (75%) had asymmetric *wnt11* expression in the pharyngeal endoderm.

Asymmetric <i>wnt11</i> expression (n=4)	
Right	25%
Left	75%

Table 2 – Of the 4 cichlids that exhibited asymmetric *wnt11* expression, 75% showed the asymmetry on the left side.

In addition to looking at *wnt11* activity in wild type cichlid development, we over expressed the canonical Wnt pathway to find what effects this may have on developing embryos. We found that when Wnt is over expressed, the jaw does not properly form. When

wild type cichlids were treated with 30 μM LiCl for 24 hours at 2 dpf, grown to 8 dpf, and cleared and stained, the jaw was underdeveloped and malformed. There were also asymmetries seen in the front of the jaw of some of the treated embryos (Figure 9B-C). Using the same protocol but a lower dosage of LiCl (10 μM), the pharyngeal cartilages were more fully developed, but the length and width of the jaw varied (Figure 9D-E). This experiment was repeated with 10 μM of LiCl for 24 hours, but we used cichlids that were 3 dpf, and we saw no significant cartilage or bone formation in the embryos after they were grown to 7 dpf (Figure 9F). In summary, the results of this experiment indicate that 30 μM LiCl treatments have more of an effect on inducing asymmetries than 10 μM treatments, and over expressing Wnt signaling at 3 dpf is more deleterious to the formation of jaw cartilages than over expressing Wnt signaling at 2 dpf. Because the neural crest cells are migrating to the jaw at 2 dpf and then differentiating into cartilage cells at 3 dpf, these results suggest that Wnt signaling is crucial for the differentiation of jaw cartilages, but not necessarily for the migration of precursor cells.

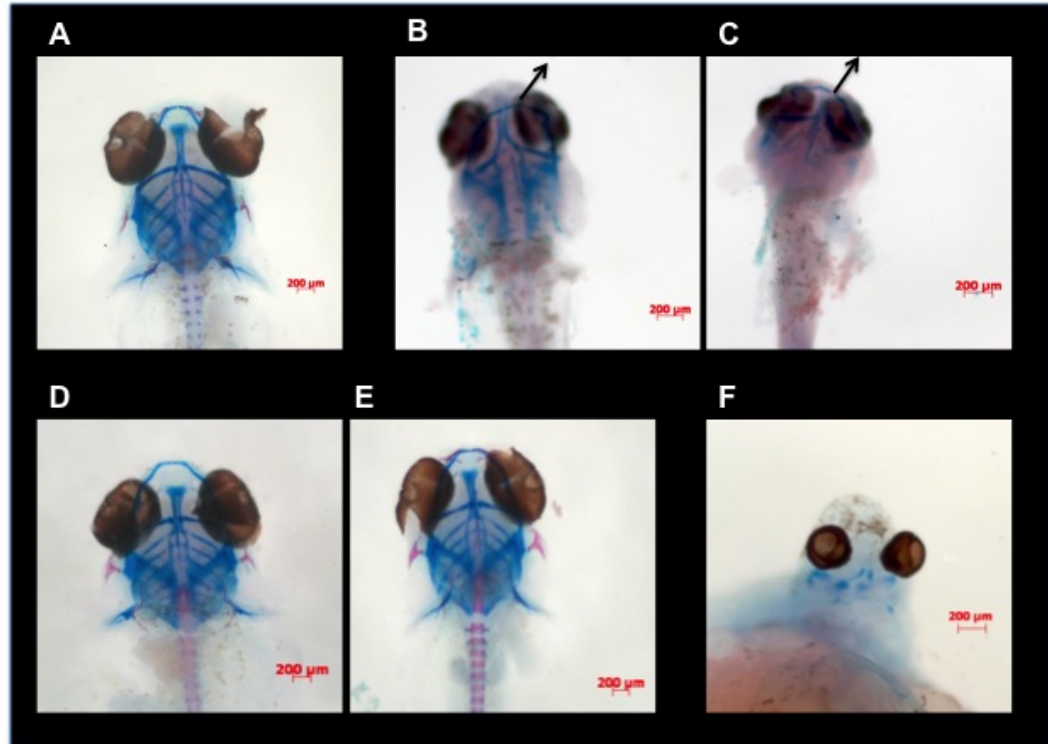


Figure 9 – *Wnt11* is necessary for proper jaw formation. (A) Wild type, untreated cichlid with proper jaw formation. (B-C) Over expression of Wnt signaling at 2 dpf with 30 μ M of LiCl for 24 hours treatment results in underdeveloped jaw cartilages. Asymmetries were also seen (arrows) (n=15). (D-E) The length and width of the jaw varies when Wnt signaling is over expressed at 2 dpf with a treatment of 10 μ M LiCl for 24 hours (n=16). (F) When treated with 10 μ M LiCl for 24 hours at 3 dpf, there is little development of the pharyngeal cartilages (n=10).

To further explore the effects of over expressing Wnt signaling over more extended periods of craniofacial development, additional experiments with LiCl were performed. Using older embryos (5-10 dpf), a lower dosage of LiCl (10 μ M), and shorter treatments (12 hours), we saw that the over expression of Wnt signaling induced jaw asymmetries (Figure 10). Most of the asymmetries were seen in cichlids treated at 6 dpf, but some asymmetries were also seen at 7 dpf (Table 3). Of the cichlids with asymmetric jaws, there was no significant preference for the right or left sides of the jaw.

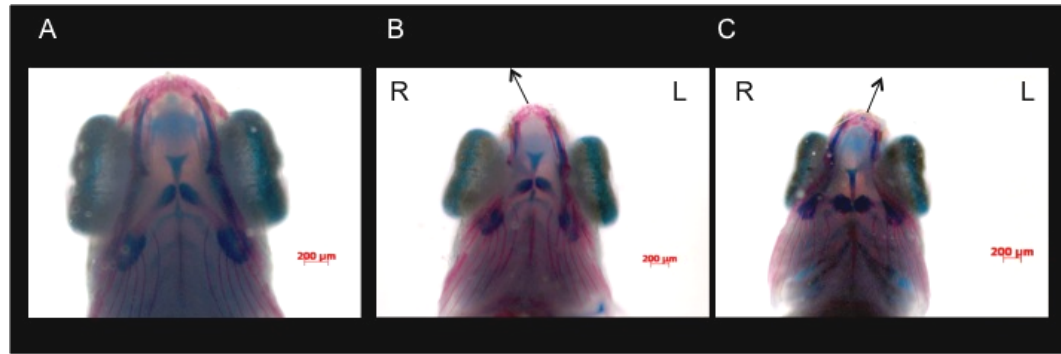


Figure 10 – Over expression of Wnt signaling at 6 dpf induced asymmetries in the jaws of wild type cichlids. (A) Untreated cichlid with a symmetric jaw. (B-C) Asymmetric jaws in cichlid treated with 10 μ M LiCl for 12 hours at 6 dpf.

Asymmetric Jaw Development in Cichlids Over Expressing Wnt Signaling		
Dpf	# Treated Cichlids	% Asymmetric
5	17	0%
6	15	66.7%
7	15	13%
8	23	0%
9	22	0%
10	2	0%

Table 3 – When *wnt11* signaling is over expressed at 6 and 7 dpf, asymmetries are observed in the cichlid jaw.

Discussion

Based on our results, we have concluded that *fgf8*, Retinoic Acid, and *wnt11* are active during craniofacial development and help establish and/or maintain symmetry of the craniofacial skeleton. When either *fgf8* or Retinoic Acid is absent, the pharyngeal cartilages develop asymmetrically along the LR axis (Figure 1 & 5). *Fgf8* mutants show an over expression of *raldh2*, which provides evidence of Retinoic Acid's role as an inhibitor of *fgf8* expression (Figure 6A). Furthermore, *fgf8*

expression was shown to be asymmetric in Retinoic Acid mutants, and *raldh2* expression was asymmetric in *fgf8* mutants (Figure 6B-E). These results suggest that Retinoic Acid and *fgf8* are acting as antagonistic molecules during the development of the craniofacial skeleton. Specifically, Fgf8 and Retinoic Acid appear to regulate the expression of each other to achieve laterality of the pharyngeal cartilages much like they do during somitogenesis.

During fish development, both Wnt and *fgf8* signaling lead to the proper development of KV. Signals are then sent from KV to the lateral mesoderm, where Fgf8 signaling and Wnt signaling both play a role in establishing cardiac laterality. One hypothesis is that asymmetric gene expression in the lateral mesoderm (Figure 6B-D), which is adjacent to the developing pharynx, might then lead to asymmetric development of the pharyngeal endoderm (Figure 6D-E). This, in turn, could lead to aberrant and asymmetric segmentation of the pharyngeal endoderm (Figure 7), resulting in the formation of asymmetric numbers of pharyngeal cartilages (Figure 5).

Fgf8 and Retinoic Acid balance the expression of each other during craniofacial development, and we suspect that *wnt11* may also play a role in this interaction. Fgfs and Wnts have been shown to interact in many other systems during development. For example, in mice, during the induction of the otic placode in inner ear development, FGF signaling regulates *wnt8a* (Urness et al., 2010). *Fgf8*

expression is positively regulated by Wnt signaling in digit and interdigit formation in mice (Villacorte et al., 2009). It has also been suggested that FGFs might control the activation of Wnt signaling during frontal and parietal bone formation (Quarto et al., 2009). Finally, it has been shown that *Fgf8* expression suppresses Wnt activity during the patterning of the cerebral cortex in mice (Shimogori et al, 2004). As these examples demonstrate, it is not uncommon for Wnts and Fgfs to interact during vertebrate development. While the results of this study did not specifically demonstrate the interaction of Wnts and Fgfs during craniofacial development, it is possible that one might be involved in regulating the other, and this would be an interesting question to address in future studies.

Although the nature of the interaction between *wnt11* and both Retinoic Acid and *fgf8* is still unknown, the results of this study led to speculations about the importance of *wnt11* and the role it might be playing in regulating craniofacial laterality. During cardiac development, numerous asymmetric signals lead to normal asymmetric heart formation. Because the heart field is in close proximity to the developing jaw, we speculate that some of these signals may spill over into the region of the developing craniofacial skeleton. This would result in asymmetric signals being sent from the underlying lateral mesoderm to the jaw endoderm. It is certainly possible that *wnt11* may be one of the signaling molecules involved in compensating for this

signaling asymmetry, which would explain why we observed asymmetric *wnt11* expression in the pharyngeal endoderm of wild type cichlids (Figure 8&9). In addition, if *wnt11* signaling is compensating in the pharyngeal endoderm for signals from the lateral mesoderm, it would be expected that an over expression of *wnt11* signaling would lead to asymmetric jaw development, which is consistent with our results (Figure 10).

Future Work

While we have preliminary evidence, further experimentation is necessary to increase statistical support for the conclusions of this study. For example, how often are asymmetries observed in Retinoic Acid deficient zebrafish? Experiments should be repeated to increase the number of mutants available for such analyses. For the *wnt11* studies, larger sample sizes would also give us a more accurate idea of the frequency of asymmetric gene expression and the time period that most of those asymmetries occur.

Furthermore, while we observed *raldh2* expression in *fgf8* mutants and *fgf8* expression in *nls* mutants, it would be interesting to incorporate *wnt11* into these experiments and observe the pattern of its expression in Retinoic Acid and *fgf8* mutants (and vice versa). Lastly, to further understand the roles of *wnt11* in regulating craniofacial laterality, it would be interesting to study the relation of cardiac development to

the formation of the pharyngeal cartilages. That is, when Wnts are over expressed, laterality is affected in jaw development, but is laterality of heart looping also affected? Is there a correlation between abnormal laterality of the heart and of the jaw? It might also be useful to observe the expression of *wnt11* in the developing heart field of LiCl treated fish and to extend this experiment by observing *fgf8* and *raldh2* expression in the region as well.

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Capstone Summary

Laterality, the preference for development on one side of the body, is essential to the vertebrate body plan. While organs such as the brain, heart, and gut exhibit asymmetries along the left-right (LR) axis, the vertebrate skeleton usually develops symmetrically. Although much is known about the genetic basis of visceral organ asymmetries, the processes underlying craniofacial laterality are not as well understood. Up to 70% of birth defects are characterized by craniofacial malformations (Hall, 1999). Two of the most common birth defects, Hemifacial Microsomia and Treacher-Collins Syndrome, involve asymmetric jaw development (Albertson and Yelick, 2005). Identifying the molecular basis of craniofacial laterality is crucial for understanding these and related birth defects.

Zebrafish (*Danio rerio*) and cichlids are two model organisms that have been used to study the molecular basis of craniofacial laterality. Several features of these fish make them ideal candidates for vertebrate genetic and developmental studies. They are small, freshwater fish that are maintained relatively easily and inexpensively in a laboratory setting. Zebrafish and cichlids develop rapidly and exhibit high fecundity. Also, their genomes have been completely sequenced, which allows for the easy identification of specific genes. While both species of fish have fewer cells and simpler spatial patterns

than higher vertebrates, they possess the same muscle and skeletal types (Albertson and Yelick, 2005; 2007).

A number of different molecules were examined over the course of this study to analyze their involvement in regulating craniofacial laterality. One such molecule, Fgf8, is known to play a role in proper jaw development. Fgf8 is one of several fibroblast growth factors (FGFs), which are intercellular signaling molecules that play various roles in development. It has been shown that *fgf8* is required for the asymmetric development of the jaw (Albertson and Yelick, 2005). That is, when *fgf8* is absent during development, the pharyngeal cartilages, or the cartilages of the jaw, develop asymmetrically along the LR axis (Figure 1, pg 3).

Retinoic Acid, a Vitamin A derivative and intercellular signaling molecule, is also suspected to play a role in craniofacial laterality regulation. Retinoic Acid is known to be important in establishing the symmetry of other systems in the vertebrate body. For example, during somitogenesis, the somites, or precursors to muscle cells, develop symmetrically along the LR axis (Figure 2, pg 4). It has been shown that both Retinoic Acid and *fgf8* are necessary for the proper symmetric development of the somites (Kawakami et al., 2005). When either Retinoic Acid or *fgf8* is absent, the somites develop asymmetrically along the LR axis. Just as Fgf8 and Retinoic Acid regulate each other during somitogenesis, we suspected that a similar process may be

occurring in the developing jaw, and that Retinoic Acid and Fgf8 regulate each other's expression to achieve craniofacial symmetry.

The role of *wnt11* in the regulation of craniofacial symmetries was also investigated during the course of this study. This focus arose due to a recent study on a scale-eating cichlid species (Figure 3, pg 4). These fish have evolved naturally occurring asymmetric jaws, which they use to feed on the scales of other fish by attacking them at an angle from behind (Stewart and Albertson, 2010). In analyzing the genetic profile of jaw asymmetry in cichlids, a small region on chromosome 10 was found to be involved in the regulation of their jaw asymmetry. In this chromosomal region, *wnt11* was one of the genes that was present (Figure 4, pg 5).

In addition to the suspicion that *wnt11* plays a role in regulating laterality in the jaws of scale-eating cichlids, *wnt11* was chosen for this study because it is known that Wnts play a role in establishing cardiac asymmetry (Lin and Xu, 2009). Because it is known that *fgf8* plays a role in regulating both cardiac asymmetries and craniofacial symmetries (Albertson and Yelick, 2005), we suspected that Wnts (more specifically, *wnt11*) might also play roles in both the regulation of cardiac asymmetries and craniofacial symmetries.

Methods

To analyze the roles of *fgf8*, Retinoic Acid, and *wnt11* in regulating craniofacial laterality, several methods were used. Whole-mount in situ hybridization (WISH) is a protocol used to stain embryos and observe the expression of selected genes at specific stages in development. Therefore, using WISH, we were able to observe where *fgf8*, *raldh2* (a gene necessary for Retinoic Acid synthesis), and *wnt11* genes were expressed early on in development.

To confirm the importance of Wnt and Retinoic Acid signaling during craniofacial development, the jaws of zebrafish and cichlids were cleared and stained. Using this protocol gave us the ability to visualize the formation of the jaw and analyze the deformities caused by the absence of Retinoic Acid and the over expression of Wnt signaling.

To identify the roles of *wnt11* signaling during jaw development, cichlid embryos were treated with LiCl at different stages for various intervals of time. Treatment with LiCl causes an over expression of Wnt signaling, including *wnt11* signaling.

Results and Discussion

To confirm the importance of Retinoic Acid in the regulation of craniofacial asymmetries, we compared the jaws of wild type zebrafish with the jaws of zebrafish that lacked Retinoic Acid (*nls* mutants). After clearing and staining the fish, we observed asymmetric

pharyngeal cartilages in Retinoic Acid mutants (Figure 5, pg 10). This observation leads to the conclusion that Retinoic Acid, like Fgf8, is necessary for symmetric development of the pharyngeal cartilages.

To analyze the interaction of Fgf8 and Retinoic Acid during craniofacial development, we used WISH analyses to observe the *raldh2* expression in *fgf8* mutants and *fgf8* expression in *raldh2* mutants. We found asymmetric expression in both cases: *raldh2* expression is asymmetric when Fgf8 signaling is absent, and *fgf8* expression is asymmetric when Retinoic Acid signaling is absent (Figure 6B-E, pg 11). These results suggest that Retinoic Acid and Fgf8 are acting as antagonistic molecules during the development of the craniofacial skeleton much like they do during somitogenesis.

To investigate the role of *wnt11* during craniofacial development, WISH analyses and LiCl treatments were performed. First, we observed the expression of *wnt11* in wild type cichlids using WISH. Surprisingly, we observed asymmetric *wnt11* expression in the forming cartilages of the jaw (Figure 8, pg 13). We then used LiCl treatments to investigate the effects of over expressing Wnt signaling on jaw formation. We found that when Wnt signaling is over expressed during specific points early in development, asymmetries can be induced in the cichlid jaw (Figure 10, pg 16). To explain this, we proposed a model for Wnt signaling during craniofacial development. As the jaw is forming, multiple other developmental processes are

occurring in the vertebrate body. One of these processes is asymmetric cardiac development, which occurs in close proximity to the developing jaw. We speculate that some of the signals from the heart field may spill over into the region of the developing craniofacial skeleton. This would result in asymmetric signals being sent from the heart field to the jaw. It is certainly possible that *wnt11* may be one of the signaling molecules involved in compensating for this signaling asymmetry, which would explain why we observed asymmetric *wnt11* expression in the developing jaw of wild type cichlids. In addition, if *wnt11* signaling is compensating in the jaw for signals from the heart field, it would be expected that an over expression of *wnt11* signaling would lead to asymmetric jaw development, which is consistent with our results.

While we have preliminary evidence, further experimentation is necessary to increase support for the conclusions of this study. Still, though, the results of this study have provided some insight into the mechanisms and molecular basis of craniofacial laterality. We now have a better understanding of the importance and function of *fgf8*, Retinoic Acid, and *wnt11* during craniofacial development.