THE ROLE OF THE PCP EFFECTOR PROTEIN FRITZ IN CONVERGENT EXTENSION, CILIOGENESIS AND HEDGEHOG SIGNALING

Presented by Srimoyee Ghosh

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____ ________________________
John Wallingford Date
Supervising Professor

____ ________________________
Ruth Buskirk Date
Honor's Advisor in Biology

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Abstract:

Fritz is one of the downstream players in the Planar Cell Polarity (PCP) pathway. PCP signaling was first identified in *Drosophila,* and many vertebrate homologues of PCP genes have been shown to be central to neural tube development and closure in vertebrate animals, including humans. Recently, it has also been shown that some PCP genes are involved in the formation of cilia. A critical function for cilia during embryonic development involves transducing Hedgehog signaling, which is essential for neural tube closure. However, the role of Fritz in vertebrate neural tube formation has not yet been studied.

The expression patterns of Fritz during the early development of *Xenopus laevis* embryos have been determined. Also, phenotypic changes in *Xenopus laevis* embryos have been observed when the Fritz protein is knocked down by antisense morpholino oligonucleotide injection— embryos failed to close their neural tube properly, indicating that Fritz is involved in neural tube closure as well as convergent extension. GFP fusion to Fritz and immunostaining reveal that Fritz protein is localized at the base of cilia. Morpholino injection resulted in defective cilia inside the neural tube and in the epidermis of these morphant embryos. These results indicated that Fritz is an essential gene for cilia formation. Furthermore, the expression level of Hedgehog target genes was greatly reduced within Fritz morphants. Fritz morphants also exhibit characteristic craniofacial defects associated with defective Hedgehog signaling. Though much work is left to determine the exact role of Fritz, these experiments illustrate that the Fritz gene is

involved in cilia formation, and consequently Hedgehog signaling, which is critical for neural tube closure and development in vertebrate animals.

Neural tube closure and Hedgehog signaling are two processes that are essential for proper vertebrate development. The neural tube later gives rise to the brain and spinal cord, while Hedgehog signaling is necessary for brain development, limb development, and establishment of the midline. Considering that Fritz plays a role in both of these processes, a better understanding of Fritz can provide vital information about early vertebrate development. More importantly, because a lack of Fritz disrupts these processes, one can gain insight into why certain developmental defects occur. These include Holoprosencephaly, which results in severe defects in brain formation, and Spina Bifida, a spinal cord formation defect that is one of the most common birth defects worldwide. It is my hope that my work on Fritz has contributed to the body of knowledge regarding these types of defects, and that my work may be someday used to help prevent or treat these defects, and save the lives of children worldwide.

Introduction:

The neural tube is an embryonic organ that gives rise to the brain and spinal cord in developing vertebrates. It begins as part of a cell layer known as ectoderm, which is a germ layer that can differentiate into either the epidermis or the central nervous system. In order for differential gene expression inducing the formation of the brain and spinal cord to occur in ectodermal cells, certain ectodermal cells must become a structure known as the neural tube.

Cells must undergo a number of movements in order for neural tube formation and closure to occur. The main movements that must occur involve an invagination of

tissue, followed by elevation of tissue upward, and finally a fusion of the two edges elongating towards one another. The actual mechanism of neural tube formation begins with tissue at the dorsal portion on an embryo invaginating to form a pocket, or neural plate, at the top of the embryo, and tops of the two sides of the pocket, the neural folds, elongating and coming together to form a tube[1].

Convergent extension involves the interdigitation of cells on one axis, which results in the elongation of tissue on the perpendicular axis[2]. During neural tube convergent extension, the two sides of the tube elongate increasingly and later curve towards one another. The top portions of the two edges of the tube then come together and fuse with one another, forming a complete tube. It is thought that a specific signaling pathway known as the Planar Cell Polarity (PCP) signaling pathway is responsible for convergent extension because knockdown (or eliminating the function) of genes in the PCP pathway has resulted in the disruption of this elongation of tissue [2]. Defects in the PCP signaling pathway result in a type of neural tube defect known as spina bifida, which is one of the most common neural tube defects in the humans.

PCP signaling is mainly known for effecting cell movements within the plane of the epithelium[3].Originally labeled “tissue polarity”, PCP signaling was first studied within the retina of *Oncopeltus fasciatus* [4]. However, major work on the PCP signaling pathway was done through a series of screens in *Drosophila melanogaster* [3]. PCP was originally studied in the fly wing, eye, abdomen and notum, and these initial studied were later followed by genetic screens that led to the discovery of key proteins in the PCP pathway [3, 5-7]. PCP signaling was then studied in vertebrates, and shown to play a role in convergent extension. Analysis in Xenopus and zebrafish convergent extension during

gastrulation and neurulation showed that orthologues of core PCP factors, including Dishevelled, Flamingo and Strabismus, have shown to control convergent extension specifically during neural tube closure [3, 8, 9].

The Frizzled/Flamingo PCP group of signaling proteins is conserved among many species and contributes to regulating cell polarity [3]. In this mechanism, reviewed by Seifert et al (2007), PCP signaling allows for an asymmetrical distribution of two different groups of proteins [10]. One of these groups involved Frizzled, Dishevelled and Diego. In this case Frizzled recruits Dishevelled to the cell membrane. This group of proteins allows for activation of Dishevelled effectors. The other group of proteins involves Strabismus and Prickle. Strabismus (whose vertebrate homolog is Vangl2) recruits Prickle to the cell membrane, which leads to a redistribution of Strabismus, causing there to be clusters of Strabismus and Prickle throughout the membrane. This group of proteins allows for activation of Prickle effectors. Prickle can interact with Dishevelled and cause a reduction in Dishevelled membrane localization. Diego (whose vertebrate homologues are Inversin and Diversin) competes with Prickled for Dishevelled, in order to reduce the effects of the negative feedback loop created by Prickle and Dishevelled. These interactions result in Dishevelled, Diego and Frizzled being localized to one side of the cell and Prickle and Strabismus being localized to the other side of the cell. Flamingo, another core PCP protein is localized at both sides of the cell.

In vertebrates, Wnt11 and Wnt 5a are ligands that transduce a signal through the Frizzled receptor [11]. This signal is then sent through Dishevelled to Rho and Rac GTPases. The PDZ domain of Dishevelled binds to DAAM1 (Dishevelled associated

activator of morphogenesis 1), which activates he Rho GTPase. Rho GTPase then activates the RHO-associated coiled coiled forming kinase, which then modulates the actin cytoskeleton [11]. Furthermore, the DEP domain of Dishevelled activates Rac GTPases, which then activates a Jun kinase, which also modulates the actin cytoskeleton. This specific part of the PCP signaling pathway is important to know because it may have implication in cell polarity and consequently convergent extension, the morphogenetic movement that is necessary for neural tube closure.

Within vertebrates, PCP signaling has been recently associated with an organelle known as the cilium. The cilium is a hair like structure that has structural and regulatory functions within eukaryotic cells [12]. The link between cilia and PCP signalling was first found in a study of the protein Inversin [3, 13]. Inversin, a ciliary protein that has sequence and structural similarities to PCP protein Diego, was found to directly interact with Dishevelled and to regulate Dishevelled stability. Inversin downregulates cytoplasmic, but not membrane bound Dishevelled. Considering that in the PCP pathway Dishevlled is tightly bound to the plasma membrane and in canonical Wnt signaling, Dishvelled shuttles between cytoplasm and different subcellular compartments, it seems that Inversin positively regulates PCP signaling and negatively regulates canonicical Wnt Signaling [3, 14, 15]. This showed evidence that PCP regulation was influenced by the cilium. Furthermore, links have also been shown between the PCP protein Strabismus/Vangl2 and the ciliary protein BBS4. This is also of particular significance because this link studies the relationship between BBS4 and PCP genes by observing convergent extension defects caused by each [16]. Knockdown of BBS4 resulted in phenotypes that were similar to knockdown of PCP genes. BBS4, which is a protein that

controls the microtubule organization of cilia, and therefore the structure of the cilia, is shown to have links to Strabismus/Vangl2 because knockdown of BBS4 exacerbates convergent extension defects in embryos lacking Strabismus/Vangl2. Considering that Strabismus/Vangl2 already play a role in convergent extension defects, this increase of convergent extension defects because of BBS4 indicates that there is some sort of relationship between these two proteins. Vangl2 also localizes to the basal body (which is a structure found at the base of cilia) and the ciliary axoneme (which is at the inner core of cilia), which was of interest because BBS4 proteins also localize to that area.

This connection between PCP signaling and cilia is important because cilia can regulate important signaling pathways in development, including Wnt signaling, PDGF signaling and Hedgehog (Shh) Signaling [12]. Hedgehog signaling is a signaling pathway necessary for formation of the midline, limb development, brain development, gastrointestinal tract formation, and lung formation, and neural tube closure [17]. Sonic Hedgehog signaling works through a series of protein protein interactions. First the sonic hedgehog ligand binds to Patched (a transmembrane protein). Patched then blocks Smoothened (another tramsmembrane protein), When Smoothened is blocked, transcription of an activated form of Gli transcription factors occurs. These factors include Gli 1(an activator), Gli 2(another activator), and Gli 3 (an activator or repressor.) It has been shown that cilia affect the Gli transcription factors, as well Patched and Smoothened [18]. This is done through a set of proteins known as IFT proteins. Cilia are assembled and maintained by these proteins, which are known as the Intraflagellar Transport System. Mutations in the IFT proteins have shown to cause mutations downstream of Patched. Mouse embryos that lack Patched and have mutations in IFT

proteins show the same phenotypes as embryos that have defective Shh signaling. Considering that a lack of Patched usually turns on Hedgehog signaling (since the Shh ligand inhibits Patched), these results show that IFT proteins affect a step in the signaling pathway downstream of Patched. IFT proteins have also shown to play a role in the processing of Gli activators and repressors [18]. In the absence of IFT proteins, the targets of Gli2 activators are not activated in the ventral neural tube. IFT proteins are also necessary for proteolytic processing of Gli3 to its repressor form. IFT mutants also have shown to lack Gli2 and Gli3.

Work done by Park et al. 2006 has shown that there is a link between PCP signaling and Hedgehog signaling through cilia [19]. Park studied the PCP effector genes Fuzzy and Inturned. Originally studied in *Drosophila*, Fuzzy and Inturned are needed by the core PCP proteins to organize actin cytoskeleton polarity [20, 21]. Knockdown of two PCP effector proteins, Fuzzy and Inturned, have resulted in both a loss of cilia as well as defective Hedgehog signaling. Specifically, knockdown of these PCP effector genes resulted in microtubules not being organized into apically projecting cilia, but rather into webs below the surface. This was similar to the loss of cilia due to a lack of the BBS4 protein, since nucleation (or formation) of microtubules was not affected, but rather because of problems in microtubules organization. Also, the apical actin cytoskeleton was less dense in the Fuzzy and Inturned morphants, which suggested that Inturned and Fuzzy controlled ciliogenesis by controlling the organization of the actin cytoskeleton. The proper organization of the actin cytoskeleton is necessary for the proper organization of microtubules. Cilia are made of microtubules, and thus by affecting actin cytoskeleton organization, Fuzzy and Inturned can affect cilia formation. This was of

importance because, knockdown of Fuzzy and Inturned also resulted in reduced expression levels of Hedgehog target genes, and resulted in phenotypes that were characteristic of defective Hedgehog signaling.

Apart from showing the link between Hedgehog signaling and cilia, Fuzzy and Inturned also affected convergent extension, As effector proteins (or modulator) of the PCP pathway, it seemed appropriate that Fuzzy and Inturned morphants also exhibited defects in convergent extension, since ties between convergent extension and the PCP pathway have been shown [8]. In vertebrates, knockdown of Fuzzy and Inturned resulted in through failures of neural tube closure as well as dorsal curvature, which are two phenotypes characteristic of defective convergent extension [1, 22].

This is of particular importance, because though work has been done on the PCP effector proteins Fuzzy and Inturned , no work has been in vertebrates done on the PCP effector protein Fritz. For this reason, I wished to study Fritz role in vertebrate development.

Fritz is an effector protein in the PCP signaling pathway. It was first studied in *Drosophila* by Collier et al. (2005) and was shown to control polarity of wing hair cells [20]. The Fritz protein was first discovered by Collier when he saw that Fritz mutants were showing that same phenotypes as Fuzzy and Inturned. He saw a disruption in the polarity of hair cells, which also occurred in Fuzzy and Inturned morphants. Wing hair cells in Fritz mutants also showed a high degree of cell autonomy, which meant that cells surrounding hair cells affected by Fritz did not produce external hair. This type of cell autonomy was also seen in other PCP effector mutants [23-25]. Fritz was also expressed during embryogenesis and Fritz mutants had disruption in the alignments of denticles,

which are scale like outgrowth that cover the skin of insects. Fuzzy and Inturned, the two other PCP effector proteins, also were expressed during embryonic stages in *Drosophila* and mutants in these two genes resulted in similar denticle phenotypes. Collier also showed that Fritz is downstream of core PCP components. Collier first directly expressed different core PCP proteins in a Fritz mutant. Upon doing this, he saw that polarity of hair cells was reversed in wild type cells when spiny-legs(Prickle) was expressed throughout the wings using an actin-Gal4 driver, however these phenotypic changes were not observed in the mutant Fritz wings, indicating that Fritz was acting downstream of a core PCP factor like Prickle. Also, when a clone of wing cells lack Fuzzy function, in order for hair growth to be induced in neighboring cells, both core PCP and PCP effector proteins are needed. To see if Fritz was also needed by these cells to induce hair growth, clone of Fuzzy lacking hair cells were induced in Fritz mutants, however their neighboring cells were unable to produce hairs, indicating that Fritz was necessary. Also, mutations in PCP effector proteins also do not block the asymmetrical localization of members of core PCP components. To see if this was also the case for Fritz, Collier looked at the localization of Frizzled, Dishevelled and Strabismus in Fritz mutants, and saw that their localization also was not affected, which was another line of evidence showing that Fritz was a PCP effector protein that was not upstream of the core proteins. All of these experiments indicated that Fritz played a role in cell polarity and could be characterized as a PCP effector protein that was downstream of core PCP proteins.

Because of its similarities to Fuzzy and Inturned, I hypothesized in my experiments that Fritz would act in a similar manner to Fuzzy and Inturned. I

hypothesized that knockdown of Fuzzy and Inturned would affect neural tube closure, cilia formation, and Hedgehog signaling. Through my work, I showed that Fritz is expressed in the neural tube and also other ciliated tissues, including ear, kidney and ciliated epidermal cell population, indicating that Fritz could be involved in cilia formation in different tissues. Also, I found direct evidence suggesting that Fritz function is necessary for the cilia formation and thereby critical to neural tube closure. Finally, the subcellular localization of Fritz has been determined using the fusion of Green Fluorescent Protein (GFP) to Fritz protein revealing possible mechanisms of Fritz function of cilia formation.

Materials and Methods

Embryo collection

All experiments were done using embryos from *Xenopus laevis*, also known as the African clawed frog. *Xenopus* embryos were used because of the ease in which to obtain these vertebrate embryos as well as their size, which makes them easy to visualize. Female *Xenopus laevis* were ovulated through injection with Human gonadotropic hormone. Eggs were fertilized in vitro, and once the embryos began cleavage, the jelly coat was removed using a 3% cysteine solution, and then embryos were grow in a 1/3X MMR (Marc's Modified Ringer's) solution. Once embryos reached a stage suitable for imagine, embryos were fixed using a MEMFA fixative.

In Situ Hybridization

Fritz DNA transformed into a culture of *Escherichia coli* bacteria. The Fritz DNA was then isolated from the bacteria, using a Quiagen miniprep, and invitro transcription was

done using a T7 RNA polymerase kit, Fritz DNA was made into RNA. Afterwards, another reaction was done where a complementary copy of the Fritz mRNA was made using artificial units, called oligonucleotides that were tagged with digoxigenin (a steroid molecule that is used as an immunohistochemical marker.) This copy, the antisense oligonucleotide probe, is what was used to determine where Fritz mRNA was localized in the embryo. When the embryo permeabilized suing Proteinase K, and then washed with this antisense oligonucleotide probe at a high temperature, the antisense oligonucleotide probe bound to the complementary Fritz mRNA on the embryo, allowing one to visualize Fritz mRNA expression. The bound probe was visualized through a reaction involving an anti-digoxigenin antibody that is tagged with alkaline phosphatase. After this antibody is bound to the digoxigenin tagged probe, the embryo is exposed to BM Purple, a chromogenic substrate for alkaline phosphatase which becomes purple when exposed to alkaline phosphatase. This results in the areas where Fritz mRNA is expressed in embryos to become purple, allowing one to visualize where Fritz can be found within the embryo.

Antisense Oligonucleotide morpholino

The antisense oligonucleotide, referred to as a morpholino, was artificially designed using oligonucleotides, or artificial units of genetic material. These artificial nucleotides are used to block translation by attaching to 5' untranslated region mRNA and prevent the initiation complex of ribosomes from attaching. These morpholinos were injected dorsally into stage 4 *Xenopus laevis* embryos to induce phenotypes that resulted in defective neural tube closure, and ventrally to induce phenotypes for defective cilia

formation on the *Xenopus laevis* epidermis. Morpholino injections varied between 20-40ng per blastomere.

Western Blot Analysis

Wild type and embryos injected with Fritz morpholino (20ng each to four blastomere in a four cell embryo), grown to stage 24. The embryos were lysed and proteins were extracted by homogenizing the embryos in a lysis buffer, and then centrifugating the solution to extract the protein layer. Sample buffer was added to the protein solution and this was ran through an SDS polyacrilimide gel. Afterwards, the protein samples from the gel were transferred onto nitrocellulose using a current, the paper was washed with a primary antibody against Fritz. The paper was then washed with a secondary antibody that bound to Fritz and was tagged with Horshradish peroxidase. The location and intensity of the protein was visualized when a substrate (Super Signal West Chemilumiescent Substrate) reacted with the horseradish peroxidase, which cause the substrate to give off light where the Fritz protein is on the paper. This emission of light was captured on film.

Histological Sections

Both wild type embryos as well as embryos dorsally injected with 30 ng morpholino were cut into 220 µm sections using a vibratome. Sections were fized in a MEMFA solution, then washed with Ptwn solution and then a TBST solution. Section were stained with acetylated tubulin antibody, and then cleared using a 2:1 Benzyl Benzoate Benzyl alcohol solution, and then imaged using confocal microscopy.

GFP Fusion Protein

GFP fusion proteins were synthesized by creating a GFP fusion construct. First, Fritz primers designed and used in PCR reactions to amplify the Fritz open reading frame. This was then ligated into a CS107-3S-Sp6 GFP vector using restriction emzymes. The CS107-3S-Sp6 GFP vector was linearized and used as a template for mRNA synthesis. mRNA was synthesized using an Ambion mMessgae mMachine transcription kit. mRNA was then injected into 4 cell embryos and grown until stage 25 (after multi ciliated cells in the epidermis had developed).

Imaging on Light Microscope

Embryos were imaged on Leica MZ16FA microscope. Embryos were mounted on clay or agarose plates and imaged.

Imaging using Confocal Microscope

Embryos were imaged in a Ptwn solution (for embryos injected with the GFP fusion protein) or Bensly Benzoate solution (for histological sections) on a Zeiss LSM5 Confocal microscope.

Results

In Situ Hybridization of Wild Type Embryos with Probe for Fritz

I performed in situ hybridization on several different stages (or ages) of embryo in order to see how the expression of the Fritz mRNA changed over time. In the staining of early stage embryos (stages 11-20) (Fig 1: A-E), the embryos had formed cell layers that would give rise to the nervous system, connective tissue, and gastrointestinal tissue.

In stage 11 embryos (Figure 1A), staining localizes to the dorsal portion of the embryo, which is appropriate because this portion of the embryo is what undergoes the convergent extension to form three germ layers, or layers of cells that will give rise to the gut, the central nervous system and connective tissue. This followed our hypothesis that Fritz affects convergent extension in tissues. Embryos at stage 15 and 17 (Figure 1B and 1C) have already begun to form the neural tube, and staining of Fritz mRNA continues to be localized to the neural tube itself as well as the neural folds, which is the folding of the ectoderm that becomes the closing edge of the neural tube.

Apart from staining the neural tube in stage 17 embryos, spots across the epidermis were stained in stage 17 embryos (Figure 2B). These spots are representative of ciliated cells located all over the epidermis of the embryo. The epidermis of *Xenopus laevis* embryos consists of goblet (mucous secreting) cells, small secretory cells, and ciliated cells. From this, we hypothesized that the stained puncta were cilia. Staining for stage 20 (Figure 2C) embryos also shows Fritz being found in ciliated cells all over the embryos.

Stage 22 embryos (Figure 1D), in which the neural tube has already formed, shows the expression of Fritz mRNA throughout the neural tube, as well as in the epidermis surrounding the neural tube. In later stage embryos, such as stage 34 and 40 (Figure 1E) during which organogenesis, or the formation of organs, is occurring staining is specifically localized the brain and spinal cord, which were derived from the neural tube.

It is also interesting to note that in both stage 34 and 40 embryos, staining is also localized to the ear, which is significant because many cilia are found within the ear

(Figure 2D). Furthermore, in stage 34 embryos, staining is found in three points behind the head of the embryo. These points make up the nephrostomes, which are a component of embryonic kidneys, and they are filled with cilia.

Overall the in situ data indicated that Fritz mRNA was expressed in tissues that would undergo convergent extension, within the neural tube, within epidermis near the neural tube, as well as areas where cilia were present.

Localization of Fritz GFP Fusion Protein to Basal Bodies

A GFP fusion protein was created in order to visualize the location of Fritz proteins within the *Xemopus* embryo. After injection of the Frtz-GFP fusion protein mRNA in stage 4 embryos, embryos were grown to stages after the development of multiciliated cells in the epidermis had occurred. Embryos were then imaged using confocal microscopy and images indicated the localization of GFP in the areas which looked like basal bodies of ciliated cells in the epidermis (Figure 3A). Fritz was not localized to the cilium itself, but in puncta at the base of the cilium, which looked similar to basal bodies. The basal body is a structure that is at the base of cilium and serves as the site for the beginning of ciliogenesis. Verification of this structure was done by also performing antibody staining on α-tubulin, a component of cilia, which showed that Fritz did not localize to the cilia axoneme (Figure 3B). Considering that the basal body is the site for ciliogenesis, this adds to the conclusion that Fritz function is necessary for ciliogenesis based on the fact that Fritz Protein localizes to this area

Analysis of Effectiveness of Fritz Morpholino

I specifically knocked down the protein expression level of Fritz using an antisense morpholino oligonucleotide (Fritz-MO) in order to explore phenotypes in which the Fritz

protein is knocked down. Fritz-MO has a complementary nucleotide sequence to the transcriptional start site of Fritz and thereby specifically blocks the formation of translational machinery on Fritz mRNA in vivo.

The morpholino was injected into embryos right after cell division began, and the embryos were grown to stage 24 (after the formation and folding of the neural tube would be complete). Embryos without anything injected into them were also grown to serve as a control.

After embryos injected with the Fritz morpholino and control embryos grown to stage 24, they were homogenized to extract protein from the embryos. The relative amounts of Fritz protein were examined for the embryos injected with the morpholino, which will be referred to as Frtz morphants, and wild type embryos using a Western Blot analysis.

The images from the Western Blot (Figure 4) indicated that in control embryos there was a large amount of Fritz protein and there was a little to no Frtz protein in embryos that had been injected with 30 ng of morpholino. In embryos that were injected with 40 ng morpholino, there was a large decrease in expression as compared to the wild type embryos. However, the gamma tubulin level was not changed, indicating that Fritz-MO specifically reduces Fritz protein only.

From this result it was apparent that injection with the Fritz morpholino did reduce Fritz protein expression in *Xenopus laevis* embryos

Defective Cilia in the Neural Tube of Fritz Morphants

The effects of Fritz morpholino on cilia of *Xenopus laevis* embryos were also observed. 30 ng of the Fritz morpholino was injected into the dorsal cells of the embryo and these

embryos were grown to stage 24 (after the formation of the neural tube). A set of control embryos (without morpholino injections) were also grown to stage 24. After the embryos were fixed, the embryos were sliced into 220 µm slices. This was done in order to look at the inside of the embryo, particularly the neural tube. In order to visualize structures within these slices, components of the slices were stained using antibody staining. The antibody used in this case stained for acetylated tubulin, which is a component of the hair-like portion of cilia. This antibody was visualized using a secondary antibody that was tagged to a green fluorescent molecule. Using a confocal microscope, I was able to observe where acetylated tubulin was located in the sections. This was important because I could now look at the amount and location of cilia within the neural tube for both embryos that were injected with Fritz morpholino, Fritz morphants, and control embryos. The neural tubes of control embryos were filled with cilia (Figure 5A), however cilia in the neural tubes of embryos injected with Fritz morpholino (Figure 5B) were either truncated (shortened) or not present. Therefore, Fritz function is involved in cilia formation in neural tube, which is a critical step for the proper neural tube development in vertebrate animals. Also, Tae Joo Park, who is the graduate student that I am working with, has shown that Fritz functions in multi-cilia formation in ciliated epidermal cells, supporting this conclusion.

Loss of expression of Hedgehog Target genes in Fritz Morphants

Considering that links have been shown between ciliogenesis and Hedgehog signaling, and that Fuzzy and Inturned knockdowns resulted in defective Hedgehog signaling, I wanted to see whether or not Hedgehog target genes would still be expressed embryos where the Fritz protein was knocked down. Hedgehog target genes are turned on as a

result of proper Hedgehog signaling, and reduced levels of Hedgehog target genes indicates that there may be defective Hedgehog signaling. The two different Hedgehog target genes that were tested were Vax1 and Nkx2.2. Vax1 is a forebrain maker and Nkx2.2 is a midline marker. Expression levels of these genes were examined in Fritz morphants by first dorsally injecting embryos Fritz morpholino. For determining Vax1 expression, embryos were injected with 30 ng Fritz morpholino, then grown up to stage 32, then fixed in MEMFA. Then in situ hybridization for Vax1 was done on Fritz embryos, as well as wild type embryos which were also grown up to stage 32. Embryos were imaged on a light microscope. Fritz morphants has significantly reduced levels of Vax1 expression (Figure 6D-6F) compared to control embryos (Figure 6A-6C) .

To look at expression levels of Nkz 2.2, embryos injected with 20 ng of Fritz morpholino were grown to stage 19. In situ hybridization was performed on Fritz morphants and wild type embryos. Embryos were then cleared using a Benzyl benzoate solution and then imaged on a light microscope. There were reduced levels of Nkx 2.2 expression in Fritz Morphants (Figure 7D-7E) compared to wild type embryos (Figure 7A-7B)

Lower levels of Vax1 and Nkx2.2 in Fritz morphants indicated that Fritz is necessary for the Hedgehog signaling pathway. Since these two genes are traditionally turned on by the Hedgehog signaling pathway, and are not expressed in environments without Fritz, we concluded that Fritz must be necessary for the Hedgehog signaling pathway to on transcription of its target genes.

Fritz Morphants have phenotypes characteristic of defective Hedgehog Signaling

Embryos with Fritz knocked down also displayed phenotypes that were characteristic of defective Hedgog signaling. Embryos that had been injected with 20 ng of Fritz

morpholino dorsally that were grown up to stage 43 showed small, close set hypoplastic eyes (Figure 8A), which are characteristic of defective Hedgehog signaling, and some embryos showed cyclopia, a classic defect associated with defective Hedgehog signaling (Figure 8B-8C) [26].

Knockdown of Fritz function causes defects in convergent extension

Fritz morphants that reached neurula stage consistently displayed a dorsal curvature, or bent back, phenotype which is characteristic of defective convergent extension (Figure 9B and 9D) [22]. This phenotype was quantified and 84% of embryos injected with 20-30ng Fritz morpholino displayed this phenotype.

Furthermore, embryos injected with Fritz morpholino also displayed defects in neural tube closure. The neural tube phenotypes were analyzed by Tae Joo Park, and embryos which were injected with Fritz morpholino showed defective neural closure (Figure 10B), indicating that the Fritz protein is necessary for the proper closure of the neural tube.

Discussion

My thesis is part of a larger project studying the function of the Fritz protein. As of today, this is the first research that has been done on the Fritz protein in vertebrates. So far, my data indicated that functional Fritz is necessary for the formation of cilia in the neural tube and thereby facilitates proper neural tube closure. Together the data from in situ hybridization, phenotypes that show a lack of cilia in Fritz morphants, and the localization of GFP fusion to Fritz protein suggests the Fritz also plays direct role in cilia formation in other tissues. Previously, it has been shown that other PCP genes, Inturned

and Fuzzy, also work in similar manner and control cilia formation in the neural tube and also ciliated epidermal cells [19]. Interestingly, Inturned and Fuzzy are also involved in convergent extension movements which is another critical step for neural tube closure [19]. Since Fritz had been identified as a PCP effector protein together with Inturned and Fuzzy, Fritz is most probably controlling both the cilia formation and the convergent extension aspect in neural tube closure.

Furthermore, like Fuzzy and Inturned, Fritz may also play a role in the Hedgehog signaling pathway which requires the formation of cilia to transduce the signal [18]. I have shown that this is the case my looking at transcription levels of Hedgehog target genes and observing phenotypes that are characteristic of defective Hedgehog singaling.

In the future, I will use cartilage staining to examine whether or not that Fritz morphants have jaw defects characteristic of defective Hedgehog signaling and using experiments, such as Keller explant assays, to further determine the role Fritz plays in convergent extension.

Much work is left to determine exactly what role Fritz plays in PCP signaling, convergent extension, neural tube closure, and Hedgehog signaling. Work is being done in the Wallingford lab to determine what proteins Fritz associates with, and electron micrography is being conducted on Fritz morphants to determine the exact role Fritz plays in cilia formation. However, from the data shown in this thesis, Fritz does seem to be playing a role in neural tube formation and Hedgehog cilia and cilia formation, the only question is how.

Overall this shows that by being a member of these two signaling pathways, the Fritz protein is essential for the proper closure of the neural tube as well as Hedgehog

signaling. With this information, future genetic screens can be implemented to prevent birth defects like Spina bifida and defects associated with Hedgehog signaling from occurring. Further more, information from Fritz and a better understanding of the Planar Cell Polarity Pathway can also give rise to gene therapies can be implemented with this gene to fix neural tube defects and give a better quality of life to thousands of children worldwide.

References

1. Wallingford, J.B., *Neural tube closure and neural tube defects: studies in animal models reveal known knowns and known unknowns.* Am J Med Genet C Semin Med Genet, 2005. **135C**(1): p. 59-68.
2. Wallingford, J.B., *Vertebrate gastrulation: polarity genes control the matrix.* Curr Biol, 2005. **15**(11): p. R414-6.
3. Simons, M. and M. Mlodzik, *Planar cell polarity signaling: from fly development to human disease.* Annu Rev Genet, 2008. **42**: p. 517-40.
4. Lawrence, P.A. and P.M. Shelton, *The determination of polarity in the developing insect retina.* J Embryol Exp Morphol, 1975. **33**(2): p. 471-86.
5. Adler, P.N., *Planar signaling and morphogenesis in Drosophila.* Dev Cell, 2002. **2**(5): p. 525-35.
6. Lawrence, P.A., J. Casal, and G. Struhl, *Cell interactions and planar polarity in the abdominal epidermis of Drosophila.* Development, 2004. **131**(19): p. 4651-64.
7. Strutt, D., *Frizzled signalling and cell polarisation in Drosophila and vertebrates.* Development, 2003. **130**(19): p. 4501-13.

8. Myers, D.C., D.S. Sepich, and L. Solnica-Krezel, *Convergence and extension in vertebrate gastrulae: cell movements according to or in search of identity?* Trends Genet, 2002. **18**(9): p. 447-55.

9. Mlodzik, M., *Planar cell polarization: do the same mechanisms regulate Drosophila tissue polarity and vertebrate gastrulation?* Trends Genet, 2002. **18**(11): p. 564-71.

10. Seifert, J.R. and M. Mlodzik, *Frizzled/PCP signalling: a conserved mechanism regulating cell polarity and directed motility.* Nat Rev Genet, 2007. **8**(2): p. 126-38.

11. Wallingford, J.B. and R. Habas, *The developmental biology of Dishevelled: an enigmatic protein governing cell fate and cell polarity.* Development, 2005. **132**(20): p. 4421-36.

12. Satir, P. and S.T. Christensen, *Structure and function of mammalian cilia.* Histochem Cell Biol, 2008. **129**(6): p. 687-93.

13. Simons, M., et al., *Inversin, the gene product mutated in nephronophthisis type II, functions as a molecular switch between Wnt signaling pathways.* Nat Genet, 2005. **37**(5): p. 537-43.

14. Guo, N., C. Hawkins, and J. Nathans, *Frizzled6 controls hair patterning in mice.* Proc Natl Acad Sci U S A, 2004. **101**(25): p. 9277-81.

15. Wu, J., et al., *Frizzled-Dishevelled signaling specificity outcome can be modulated by Diego in Drosophila.* Mech Dev, 2008. **125**(1-2): p. 30-42.

16. Ross, A.J., et al., *Disruption of Bardet-Biedl syndrome ciliary proteins perturbs planar cell polarity in vertebrates.* Nat Genet, 2005. **37**(10): p. 1135-40.

17. Gilbert, S.F., *Developmental biology*. 8th ed. 2006, Sunderland, Mass.: Sinauer Associates, Inc. Publishers. xviii, 817 p.

18. Eggenschwiler, J.T. and K.V. Anderson, *Cilia and developmental signaling.* Annu Rev Cell Dev Biol, 2007. **23**: p. 345-73.

19. Park, T.J., S.L. Haigo, and J.B. Wallingford, *Ciliogenesis defects in embryos lacking inturned or fuzzy function are associated with failure of planar cell polarity and Hedgehog signaling.* Nat Genet, 2006. **38**(3): p. 303-11.

20. Collier, S., et al., *The WD40 repeat protein fritz links cytoskeletal planar polarity to frizzled subcellular localization in the Drosophila epidermis.* Genetics, 2005. **169**(4): p. 2035-45.

21. Wong, L.L. and P.N. Adler, *Tissue polarity genes of Drosophila regulate the subcellular location for prehair initiation in pupal wing cells.* J Cell Biol, 1993. **123**(1): p. 209-21.

22. Wallingford, J.B., et al., *Dishevelled controls cell polarity during Xenopus gastrulation.* Nature, 2000. **405**(6782): p. 81-5.

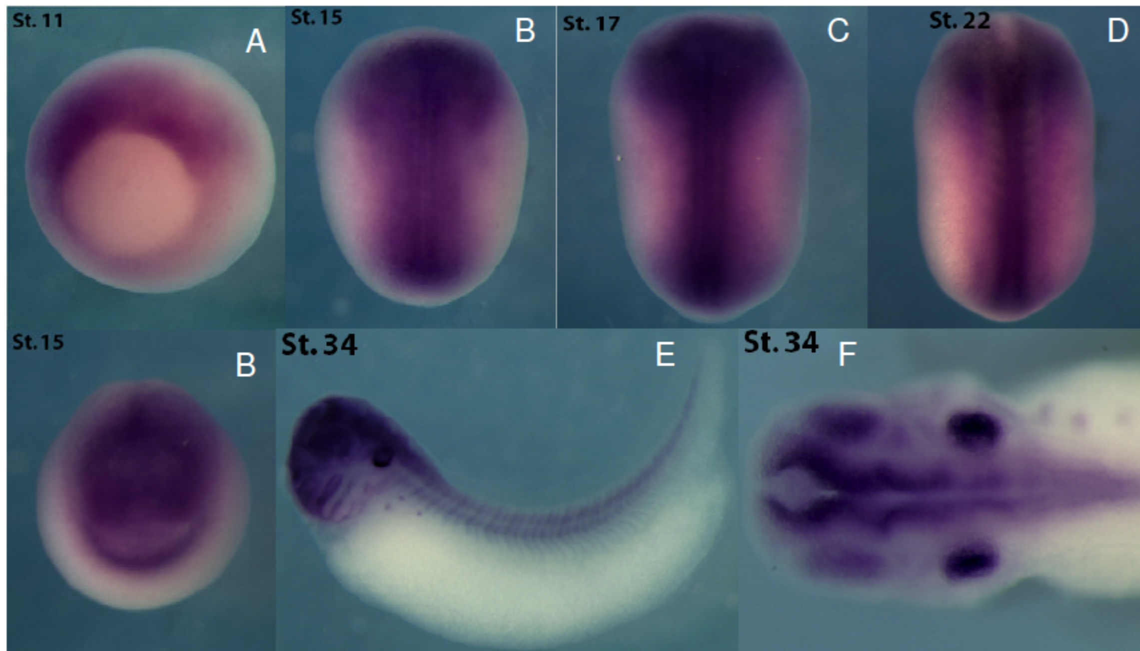
23. Gubb, D. and A. Garcia-Bellido, *A genetic analysis of the determination of cuticular polarity during development in Drosophila melanogaster.* J Embryol Exp Morphol, 1982. **68**: p. 37-57.

24. Adler, P.N., J. Charlton, and W.J. Park, *The Drosophila tissue polarity gene inturned functions prior to wing hair morphogenesis in the regulation of hair polarity and number.* Genetics, 1994. **137**(3): p. 829-36.

25. Collier, S. and D. Gubb, *Drosophila tissue polarity requires the cell-autonomous activity of the fuzzy gene, which encodes a novel transmembrane protein.* Development, 1997. **124**(20): p. 4029-37.

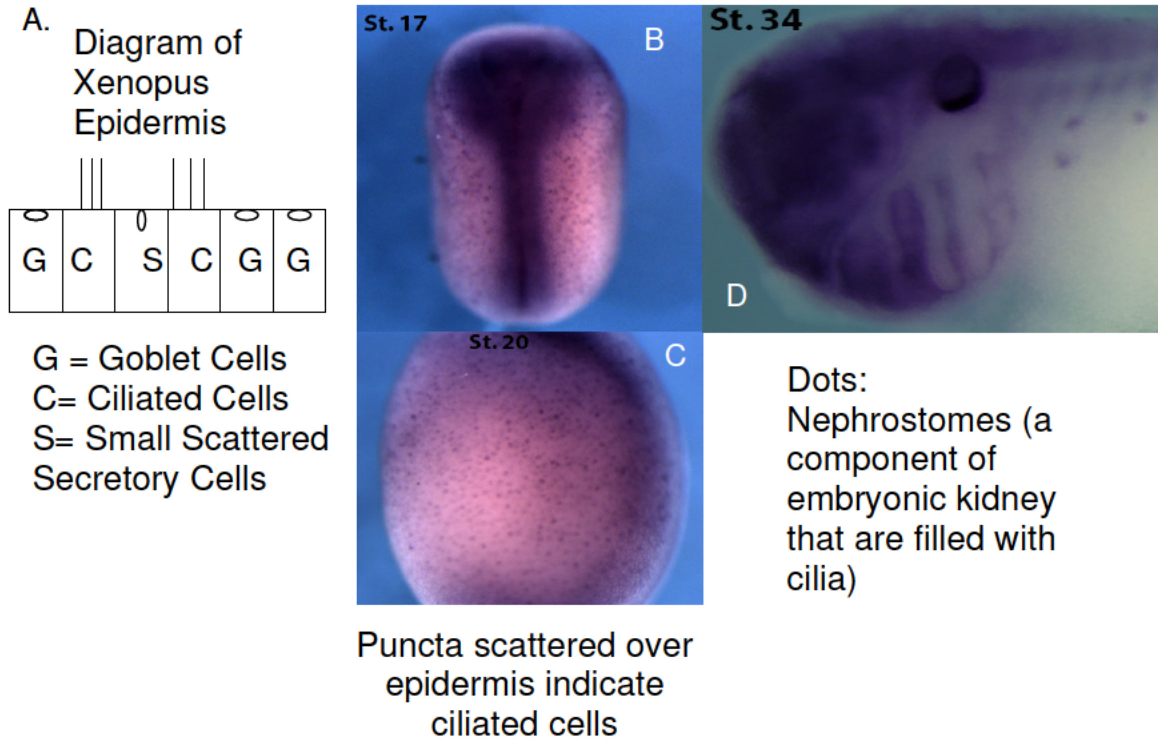
26. Cordero, D., et al., *Temporal perturbations in sonic hedgehog signaling elicit the spectrum of holoprosencephaly phenotypes.* J Clin Invest, 2004. **114**(4): p. 485-94.

Figure 1: Fritz is expressed in tissues that undergo convergent extension, neural tube closure, or arise from neural tube closure



Fritz is expressed in tissues undergoing convergent extension in St. 11 embryos (A). Fritz is also expressed in the neural folds and neural tube in embryos at stages 15, 17, 22 (B-D). Fritz is expressed in the brain and spinal cord of embryos at stage 34 (E anF).

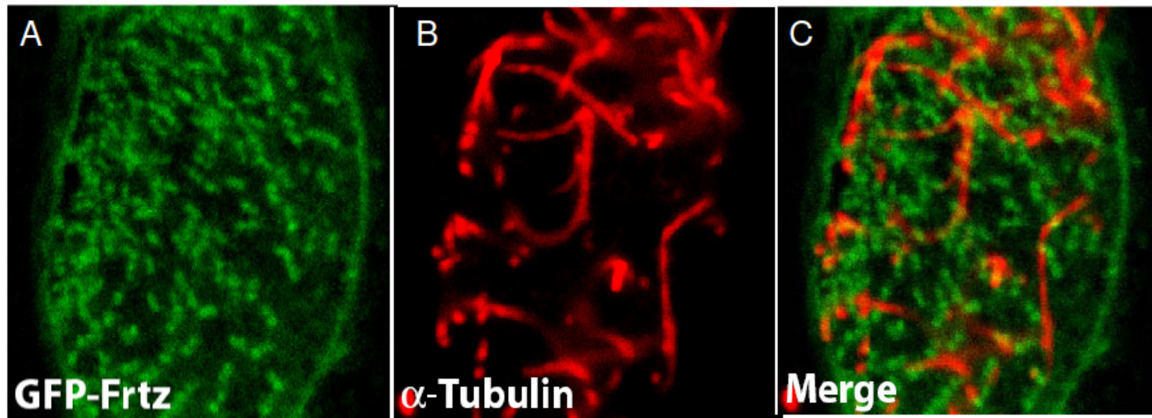
Figure 2: Fritz is expressed in ciliated cells.



Fritz is expressed in cilated cells on the epidermis of *Xenopus laevis* embryos (A-C).

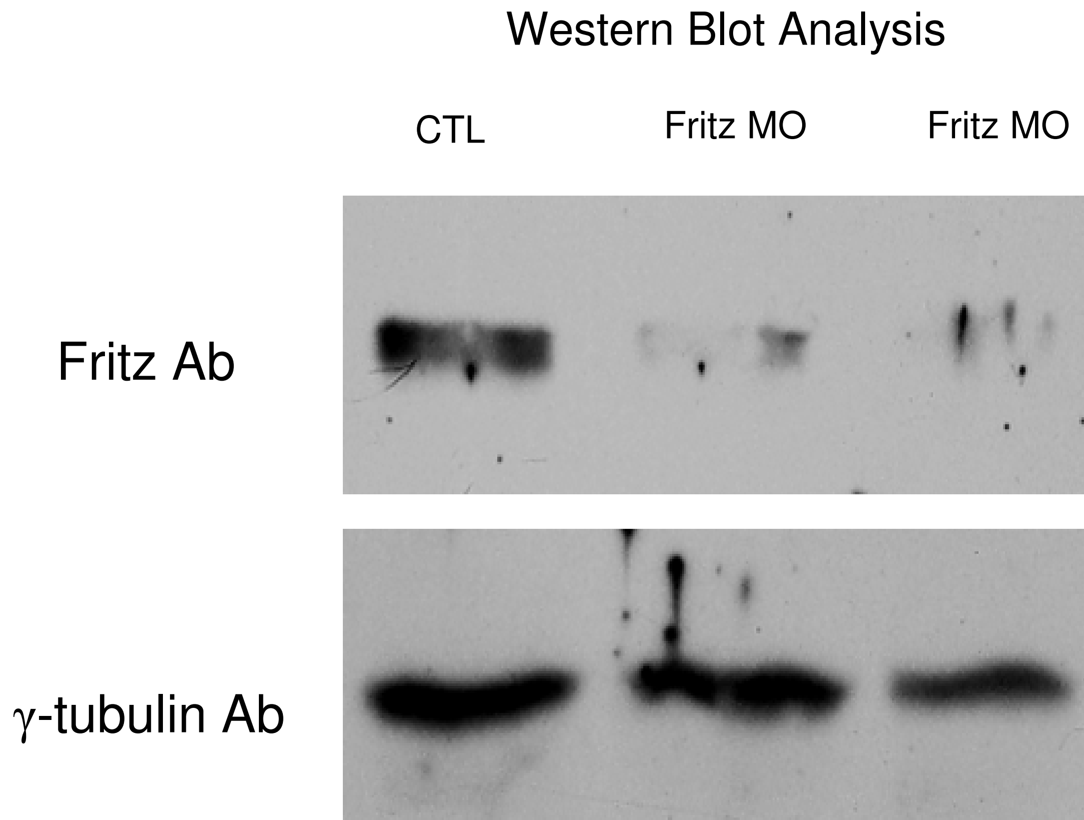
Fritz is also expressed in the nephrostomes and ear of *Xenopus laevis* embryos, which are structures that are filled with cilia (D).

Figure 3: Localization of Fritz Protein using a GFP fusion Construct: Fritz localizes to puncta below cilia



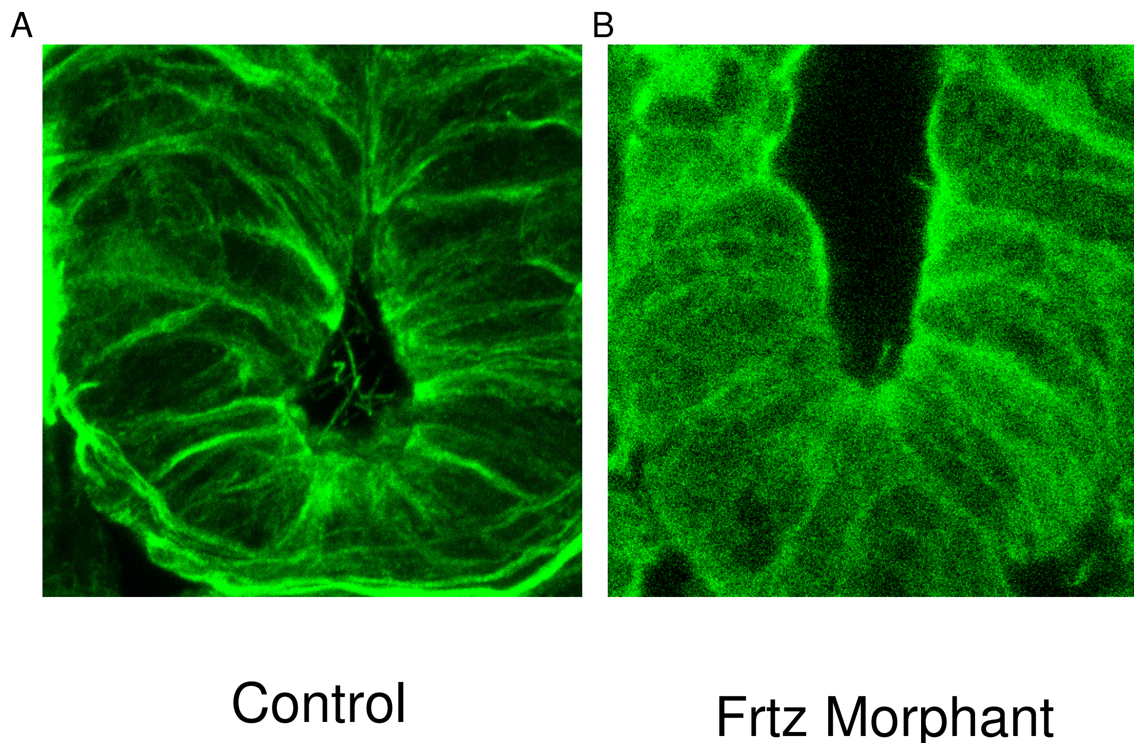
Fritz does not localize to the cilia axoneme (B), but rather to punta beneath the cilia (A).

Figure 4: Fritz morpholino knocks down Fritz protein



Fritz protein is present in ctl (or wild type) embryos, whereas Fritz protein has been knocked down in embryos that have been injected with Fritz morpholino. A ganna tubulin antibody was used on this same blot to verify that total protein concentration of the Fritz morpholino samples was not reduced, and thus a false positive did not result from the Fritz morpholino bands.

Figure 5: Reduced Amounts of Cilia in the Neural Tubes of FritzMorphants

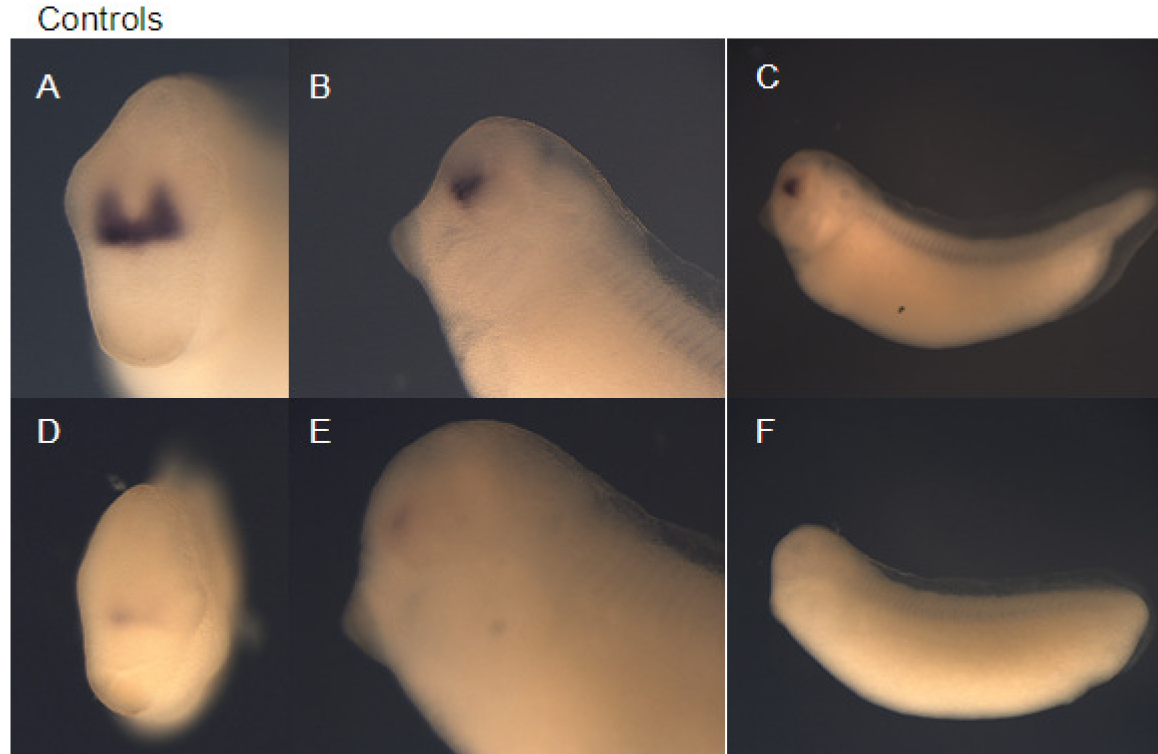


Control

Frtz Morphant

Histological sections were taken on wild type and Fritz morpholino injected embryos and acetylated tubulin was used to stain for cilia. In the control embryos (A), the neural tube is closed and the tube is filled with cilia, wheras in the morphant (B) the neural tube is open and cilia are missing or are truncated.

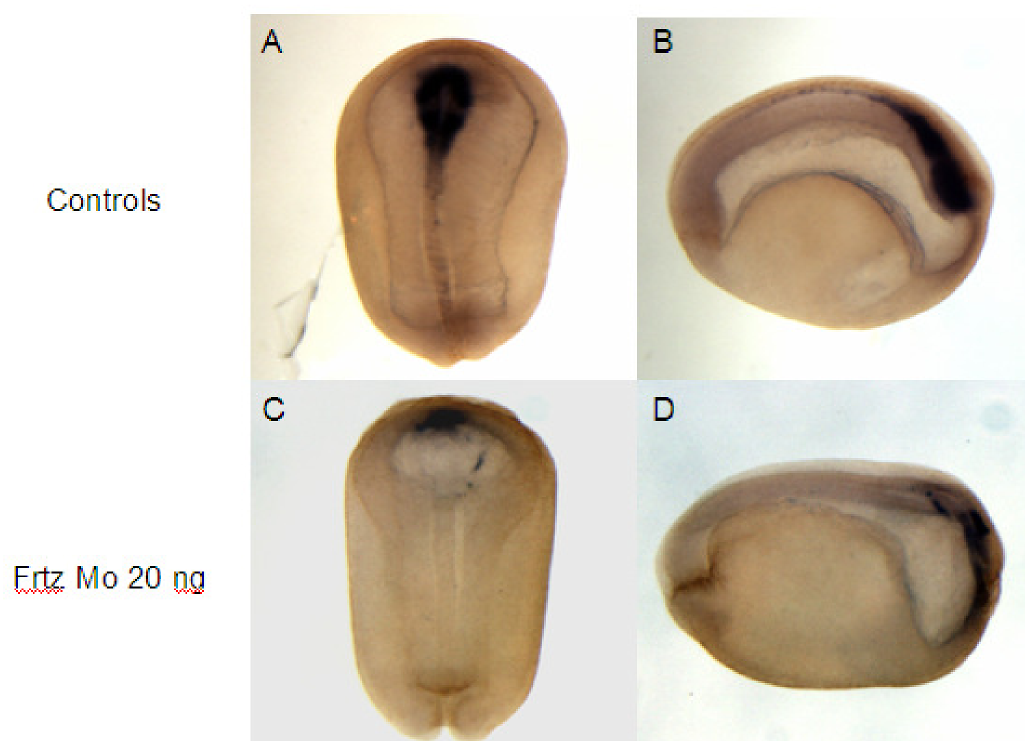
Figure 6: Reduced Expression of Hedgehog Target Genes: Vax1



Fritz Morphants

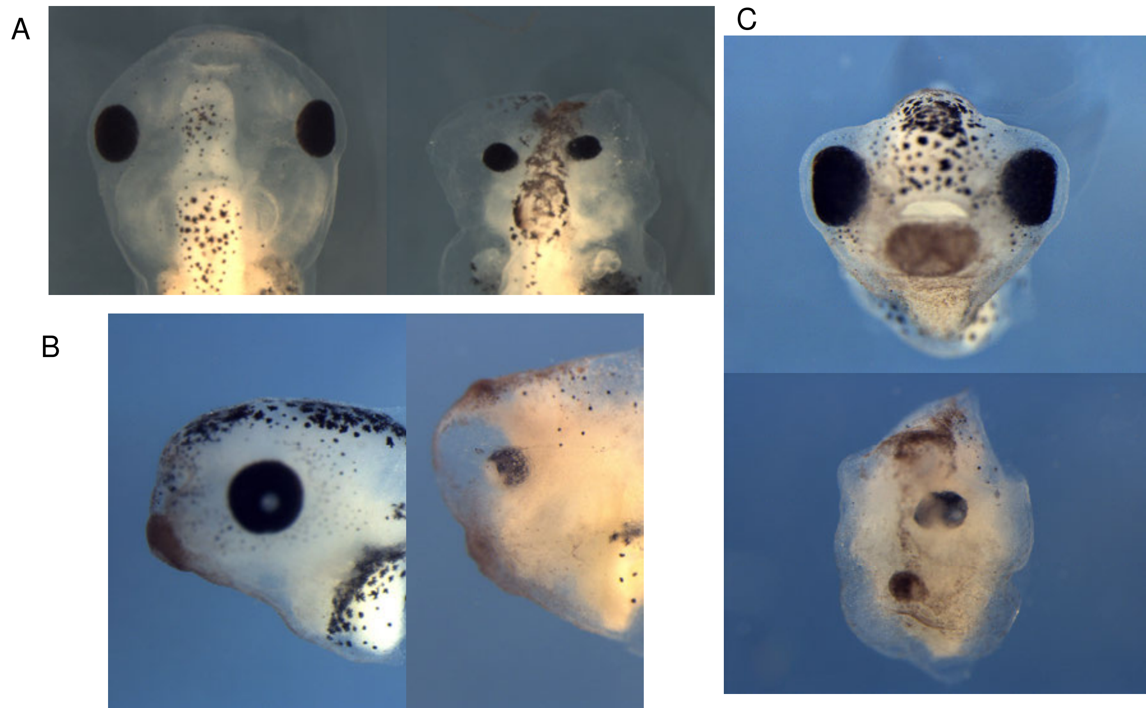
In situ hybridization for Vax 1 was done on wild type (A-C) and Fritz morpholino (D-F) injected embryos, and reduced levels of Vax 1 are evident in the Fritz morphants (shown on the lower row) as opposed to the wild type embryos (upper row.)

Figure 7: Reduced Expression of Hedgehog Target Genes: Nkx 2.2



In situ hybridization for Nkx 2.2 was done on wild type (A-B) and Fritz morpholino injected embryos (C-D), and reduced levels of Nkx 2.2 are evident in the Fritz morphants (shown on the lower row) as opposed to the wild type embryos (upper row.)

Figure 8: Fritz mophants display phenotypes indicative of defective Hedgehog Signaling

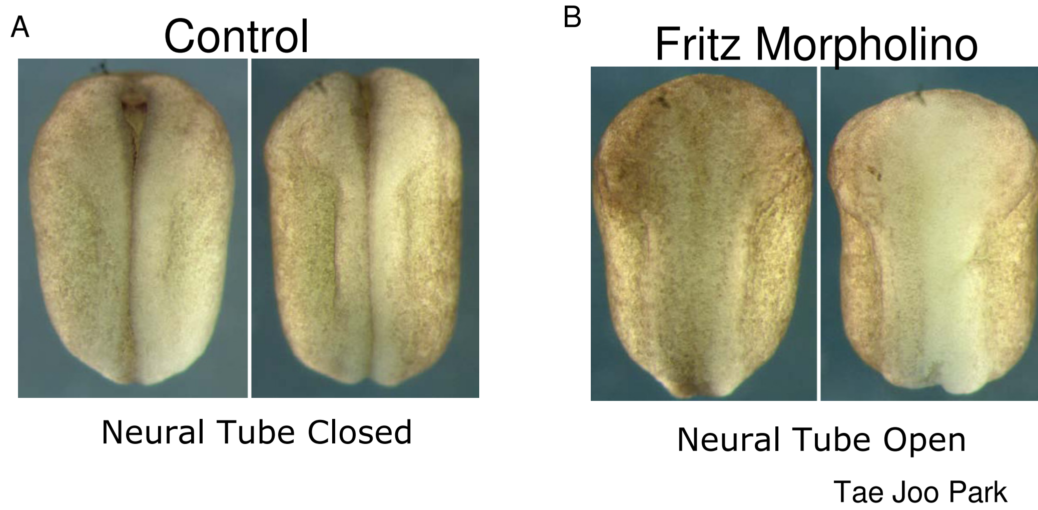


Embryos injected with Fritz morpholino display craniofacial defects characteristic of defective Hedgehog signaling. (A) shows narrow close set hypoplastic eyes, and (B) and (C) show cyclopia, a classic phenotype of defective Hedgehog signaling.

Figure 9: Fritz Morphants display dorsal curvature – a phenotype of defective convergent extension

84% of embryos injected with Fritz morpholino exhibited dorsal curvature, or a bent back phenotype (B and D), which is characteristic of defective Hedgehog signaling.

Figure 10: Embryos injected with Fritz morpholino cannot undergo neural tube closure.



Work done by Tae Joo Park shows that embryos that have been injected with Fritz morpholino (B) do not undergo neural tube closure.