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Investigating the Roles of Lhx1a, Lhx1b, and Lhx5 in Zebrafish Spinal Cord Interneuron Specification and Function

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Abstract

Located in the vertebral column, the human spine is responsible for regulating body movements and receiving sensory input about pain and touch. Currently, few treatments for neurological diseases and spinal cord injuries exist, partly because we know little about how a fully functioning spinal cord is constructed. As such, studying spinal cord development, specifically neuronal specification and patterning, should be useful for developing better treatments for people with spinal cord injuries and diseases. Zebrafish are a prime model organism for studying neuronal specification because their transparent embryos develop outside the mother, allowing us to easily examine gene expression, cell movements and cell morphology during development . Furthermore, the zebrafish spinal cord has few types of interneurons compared to mammals, and each interneuron type can be recognized by its distinct morphology.

I focused on V1 cells, which form in the ventral spinal cord and are functionally similar in all vertebrates. In zebrafish, V1 cells develop into CiAs, or **Ci**cumferential **A**scending interneurons, which control movement and sensory gating. Several transcription factors are expressed consistently in all vertebrate V1 cells, and my research focused on Lhx1a, Lhx1b and Lhx5. As a result of findings in mice, I predicted that knocking down these transcription factors would result in neurotransmitter deficits and potentially compromise movement ability. Additionally, a main focus of my project became assaying various experimental strategies for knocking-down Lhx1a, Lhx1b and Lhx5. These included injecting

reagents into 1-4 cell stage embryos and taking advantage of a *lhx1b* mutant fish line. For the injections, I used morpholinos (MOs), antisense agents that either interfere with RNA transcription to protein or with RNA splicing. I also used RNA constructs which should act as either dominant activators or dominant repressors. Using *in situ* hybridization, I then tried to assess the impact on neurotransmitters throughout the spinal cord and specifically within CiAs.

I successfully identified a PCR and restriction enzyme digest method for identifying *lhx1b* mutants. This was exciting as it was a completely novel method for identifying these fish. Furthermore, my results demonstrated that homozygous *lhx1b* mutants are viable which was previously unknown. My injection results demonstrate that *lhx1b* and *lhx5* splice-blocking MOs are also an effective tool for knocking-down the function of these two genes. In contrast, the *lhx1a* MOs that I tried were not effective. My RNA injection results were inconclusive and I determined that a higher concentration was probably needed to impact Lhx1 and Lhx5 function.

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Acknowledgements

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Introduction

Located in the vertebral column, the human spine is responsible for controlling many bodily functions that healthy individuals take for granted. In addition to regulating standard body movements and organ operations, the spinal cord receives sensory input about pain and touch from the rest of the body. Various neurological conditions including neuro-degeneration as well as spinal cord injuries result in deficits in these functions. These types of ailments are particularly common. A recent survey estimated that 183,000-230,000 people in the United States alone suffer from spinal cord injuries (NIH Medline Plus). Furthermore, approximately 11,000 new cases of spinal cord injury present each year in the United States (NIH Medline Plus). Oftentimes these injuries are the result of accidents, violence, and sports-related injuries and happen to people in the prime of their life (NIH Medline Plus). Currently, there are few treatments for these conditions, partly because we know very little about how a fully functioning spinal cord is constructed. As such, studying spinal cord development, specifically neuronal specification and patterning, can ultimately be useful for developing better treatments for people with spinal cord injuries and diseases.

Zebrafish are a powerful model organism in which to study neuronal specification for a number of reasons. For example, zebrafish are a relatively inexpensive choice when compared to other vertebrates and a large quantity of embryos can be easily obtained from adult fish without harming the parents. In addition, zebrafish have transparent embryos that grow rapidly outside of the mother (**figure 1**). These characteristics allow us to examine central nervous system (CNS) development, including spinal cord development, in live embryos. In some instances, being able to visually examine CNS development in live embryos becomes particularly imperative. For instance, some mutations cause characteristic morphological phenotypes that can

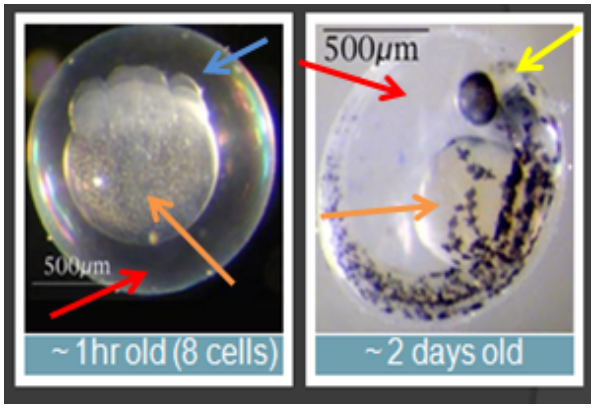

Figure 1. Depicting rapid development of a zebrafish embryo. Blue arrow indicates cells in early embryo. Red arrows indicate chorion which surrounds embryo during early development. Orange arrow indicates yolk which provides nutrients to embryo during early development. Yellow arrow indicates zebrafish head. Based on figure by Dr. Lewis.

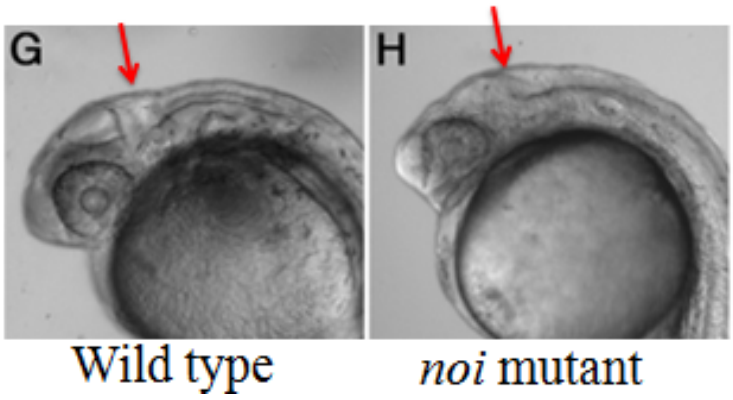

Figure 2. Absence of mid-brain hindbrain boundary in *noi* mutants at 28 hours post-fertilization. Red arrows shows midbrain/hindbrain boundary (or absence in H). Figure taken from Molina et al. (2007).

be readily viewed during embryonic development. The transparency of zebrafish embryos enables us to identify mutants and separate them from wild-type embryos. An example of this is *noi* (no-isthmus) mutants, in which the mid-brain hindbrain boundary does not form. As shown in **figure 2**, the mutants can readily be identified because of their characteristic lack of this boundary.

Zebrafish are also a useful model organism because interneuron morphology can be examined in live embryos. This can be accomplished by labeling specific cell types with fluorescent proteins such as GFP. It is difficult to observe embryonic interneurons in some vertebrate, particularly mammals. This is because mammalian embryos develop inside the mother and are not transparent. It is easier to study interneurons and identify them as a particular cell type with a specific function in zebrafish. This is because, in addition to being able to

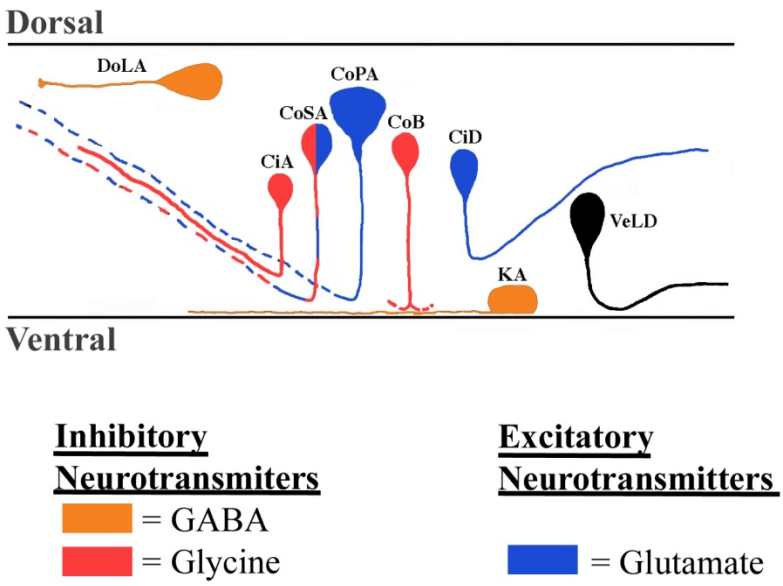

Figure 3. Schematic showing the unique morphologies and neurotransmitter fates of zebrafish interneurons. Anterior is to the left. Dotted line= contralateral axons. Solid line= ipsiliateral axons. Figure based on a figure from Lewis and Eisen (2003) and information from Higashijima (2004).

visualize individual interneurons in the externally-developing transparent zebrafish embryos, the zebrafish spinal cord has relatively few different types of interneurons in comparison to mammals. Furthermore, each interneuron type can be easily recognized by its particular morphology, as seen in **figure 3**. For example, the interneuron type I studied, **Ci**rcumferential **A**scending (CiA) interneurons, have a distinct interneuron shape which distinguishes them from the others as seen in **figure 3**. In addition to the unique morphologies, each interneuron type has a distinct neurotransmitter fate. Neurotransmitters are the chemicals within the central nervous system that help neurons to communicate. Finally, it is easy to knock down individual gene functions with mutants and other methods in zebrafish which was particularly important for my project. A primary method used in this project was injections into 1-2 cell stage embryos, where I injected various reagents into an embryo via a thin needle. The transparency and rapid development of zebrafish embryos enables this experimental technique to be utilized relatively easily.

This project focused on V1 neurons which form in the ventral spinal cord. During spinal cord development, cells originate in distinct dorsal-ventral

Cell Population	Function
V0	Control side-to-side motion during movement
V1	Regulate/enable fast movements
V2	Alternation during rapid movement, rhythm generation
V3	Rhythm during locomotion

Figure 4. Table listing the different functions of ventral cell populations. Figure created based on information in Goulding (2009).

progenitor domains and migrate laterally to specific dorsal-ventral post-mitotic domains (**figure 5**). In both regions, individual cells take on a particular identity depending on their dorsal-ventral location in the spinal cord. This dorsal-ventral identity then leads cells to develop into distinct interneuron types with unique characteristics functions. For instance, distinct ventral interneurons control different aspects of movement as indicated in **figure 4**.

In zebrafish, V1 cells, which regulate/enable fast movement, develop into CiAs or Circumferential Ascending interneurons (Goulding, 2009). CiAs have several functions including motor control and sensory gating. Sensory gating is the ability to screen for irrelevant sensory input and prevent it from interfering with other neurological processes (Burgess and Granato, 2007). Various projects have already been conducted with V1 cells in other organisms such as frogs and mice. Results from tadpoles demonstrated that the cells correlating to CiAs and V1 cells, called aINs in frogs, are implicated in swimming behaviors and sensory

gating (Li, et al., 2004). Similarly, V1 cells in mice are required for fast movements. When V1 cells were ablated, mice had difficulty moving at faster speeds although movement was still possible (Gosgnach et al., 2006). The CiA cells in zebrafish are functionally similar to the V1 cells seen in mammals (Higashijima, 2004), which makes them an appropriate choice for study considering my goal of aiding in the treatment of spinal cord injuries in humans.

Each interneuron type expresses a distinct combination of transcription factors (**figure 5**). Transcription factors are proteins that bind to DNA and either promote or inhibit transcription of other genes. Therefore, understanding the roles of

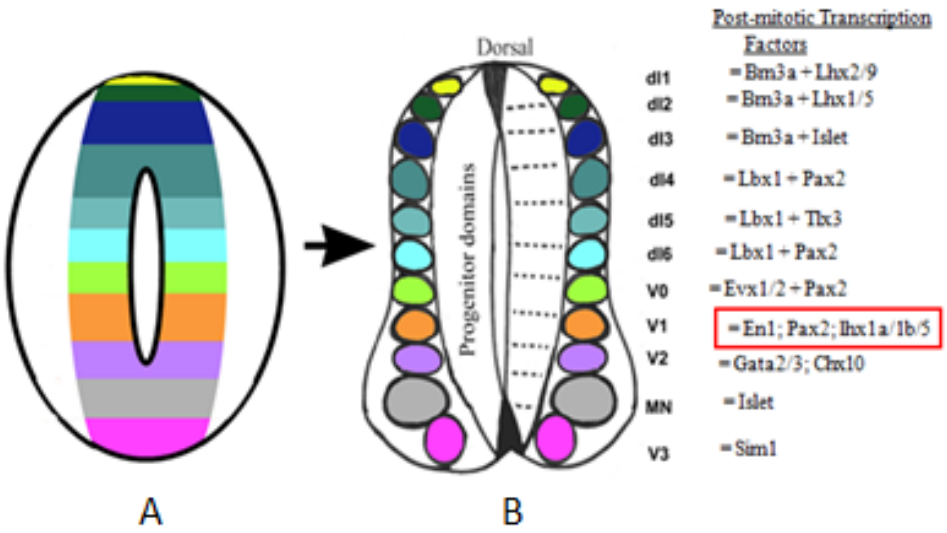

Figure 5. Each cell population in the spinal cord expresses unique transcription factors. (A) represents the spinal cord during early development when cell populations are still in the progenitor domains. (B) indicates spinal cord when cells have migrated to their post-mitotic domains. The post-mitotic transcription factors listed beside (B) are only a subset of the transcription factors expressed. Dl= dorsal interneuron. V= ventral interneuron. MN= motoneuron. Figure kindly provided by Dr. Lewis.

transcription factors is key to better understanding how neuronal characteristics

are genetically specified. Several transcription factors are expressed consistently in all vertebrate V1 cells and my research focused on a few of these, namely Lhx1a, Lhx1b, and Lhx5 (referred to here as Lhx1a/Lhx1b/Lhx5).

A unique characteristic of Lhx transcription factors is that they are LIM-homeodomain (LIM-HD) proteins. Similar to other homeodomain proteins, the LIM-HD transcription factors have been "well preserved throughout evolution" (Hobert and Westphal, 2000). LIM-HD proteins are also quite similar to one another, suggesting a shared ancestor. LIM-HD proteins can be categorized into smaller groups based on common ancestors and the Lhx1/5 transcription factors I study fall into the Lin-11 group (Hobert and Westphal, 2000). Found in both vertebrates and invertebrates, LIM-HD proteins are characterized by LIM domains, that have a zinc-finger formation, which are protein binding domains located upstream of the homeodomain as shown in **figure 6** (Hobert and Westphal, 2000). These zinc fingers allow the proteins to bind to other transcription factors in different combinations and might, therefore, enable the LIM-HD proteins to help orchestrate a variety of developmental phenomena (Hobert and Westphal, 2000). Although other Lhx family members exist as shown in **figure 7**, my project focused on Lhx1/5. Lhx1/5 are probably more closely related to one another than to other Lhx family members because they share a more recent ancestor (Hobert

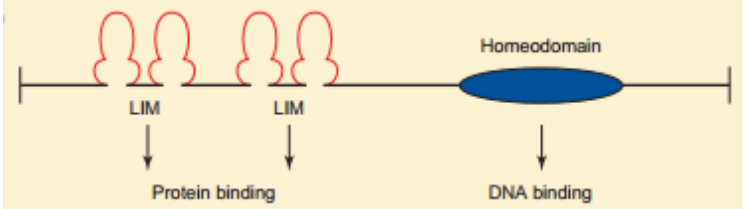

Figure 6. LIM-HD Protein Structure (Hobert and Westphal, 2000).

and Westphal, 2000). Furthermore, in the zebrafish embryonic spinal cord they are co-expressed in the same cells, suggesting that they might act redundantly (Cerda-Moya, 2011).

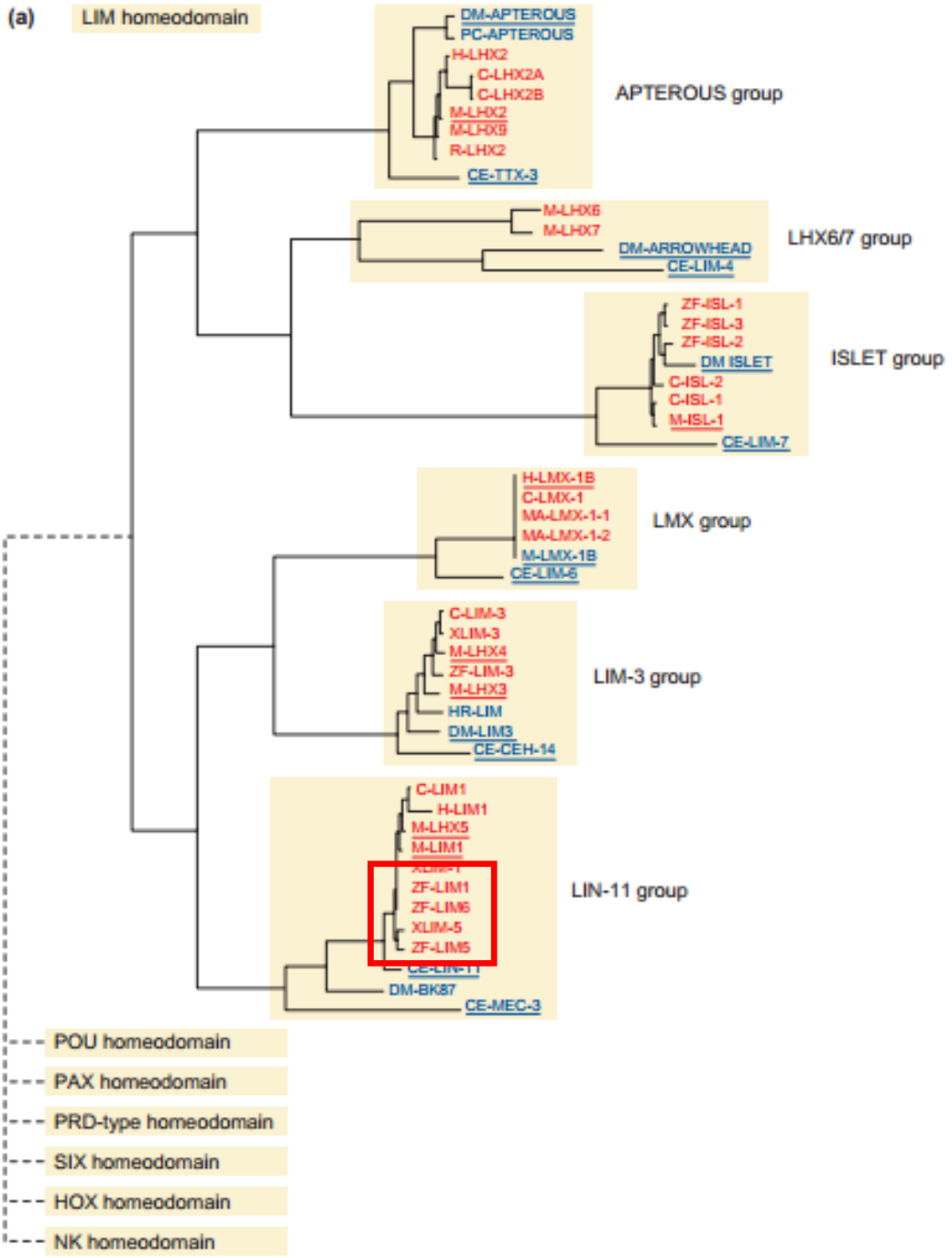

Figure 7. An evolutionary tree of the LIM-HD proteins. The red box indicates where Lhx1/5 are located in the Lin-11 group. Other Lhx genes are located in other more distant groups. Lhx1 and Lhx5 used to be called Lim1 and Lim5. Lhx1b used to be called Lim6. (Hobert and Westphal, 2000).

Experiments investigating the functions of the Lhx1/5 transcription factors have been conducted in mouse. In these experiments, gene expression in mutant mice lacking Lhx1 and Lhx5 was compared to WT mice in order to assess the effects of knocking-down Lhx1/5 in the mouse spinal cord (Pillai et. a, 2007). Pillai and colleagues found that Lhx1 and Lhx5 were co-expressed early in neurogenesis. However, these expression patterns diverged over time (Pillai et. al, 2007). One reason for the early identical expression might be that Lhx1 and Lhx5 act redundantly initially, but then develop independent roles. In terms of their specific functions, Lhx1/5 help to maintain inhibitory neurotransmitter fates in mouse, but these transcription factors are not required to initially specify inhibitory fates. Furthermore, in the absence of Lhx1/5, the cells do not adopt an excitatory neurotransmitter identity. Additionally, Lhx1/5 are required to maintain Pax2 expression in dorsal inhibitory interneurons, which is important because Pax2 is also known to be required for inhibitory neurotransmitter fates (Pillai et al., 2007; Batista and Lewis, 2008). The fact that Lhx1/5 are required to maintain Pax2 expression suggests that Lhx1/5 are upstream of Pax2 and that both are required to specify the inhibitory neurotransmitter fate. This shows that different families of transcription factors interact in the specification of neurotransmitter fates (Pillai et al., 2007).

The fact that transcription factor families might interact in interneuron specification is relevant to my project because it means that Lhx1/5 might interact closely with another group of transcription factors. Therefore, the absence of Lhx could potentially impact that secondary group and vice versa. For instance, the

phenotype of mice that lack Lhx1 and Lhx5 resembles that of mice that lack Pax2 and also that of *pax2a/pax2b/pax8* triple knockdown zebrafish embryos (Pillai et al., 2007; Batista and Lewis, 2008). Previous work in the Lewis Lab showed that Pax2/8 are redundantly required for the glycinergic and GABAergic phenotypes of both CiAs and other neurons that express these Pax2/8 proteins (Batista and Lewis, 2008). Furthermore, although Pax2/8 are required to specify inhibitory neurotransmitter fates, their absence does not result in neurons becoming excitatory (Batista and Lewis, 2008).

Recent work by Gustavo Cerda-Moya in the Lewis Lab at University of Cambridge, UK demonstrated that *lhx1a* (used to be called Lim1), *lhx1b* (used to be called Lim6), and *lhx5* (used to be called Lim5) are co-expressed in cells in the zebrafish spinal cord (Cerda-Moya, 2011). As these transcription factors have very similar sequences, this co-expression suggests that these transcription factors might act redundantly in these cells. Therefore, it might be necessary to knockdown all three Lhx1/5 genes in order to investigate their function.

As mentioned before, the loss of Lhx1/5 in mouse and the loss of Pax2 in mouse or Pax2/8 in zebrafish procedures a similar spinal cord phenotype which suggests that these transcription factors might act together to specify inhibitory neurotransmitter fates. However, the exact details of this mechanism of neurotransmitter fate specification are unknown. It was also unknown what the effects of eliminating the Lhx1/5 transcription factors would be on zebrafish embryonic spinal cord, as this had never been tested. As discussed above, Lhx (Lim homeodomain) transcription factors are involved in the regulation of

inhibitory neurotransmitters in the dorsal spinal cord in mice (Pillai et al., 2007). Therefore, I predicted that they would have a similar function in zebrafish. I hypothesized that knocking-down the functions of these transcription factors would result in neurotransmitter deficits and potentially compromise movement ability. Moreover, Pax2 is also thought to maintain Lhx1/5 expression in mouse, suggesting that the Lhx1/5 and Pax2/8 might act in a reciprocal fashion to maintain each other's expression (Burrill et al., 1997; Cheng et al., 2005; Gross et al., 2002; Morales and Hatten, 2006; Pillai et al., 2007; Zhao et al., 2000; Batista and Lewis, 2008). As such, another aim of my research was to investigate the connections between Lhx1/5 transcription factor function and Pax2a, Pax2b and Pax8 expression.

Therefore, the main questions for my research were as follows:

1. Can we identify *lhx1b* mutants by genotyping? Is the *lhx1b* mutation embryonic lethal?
2. What are the best reagents to test Lhx1a/1b/5 function in zebrafish?
3. What is/are the function/s of Lhx1a/1b/5 in the zebrafish spinal cord?
4. How are the Lhx and Pax families of transcription factors related? Do they maintain one another?

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Materials and Methods:

The methods utilized for my research can be summarized as follows:

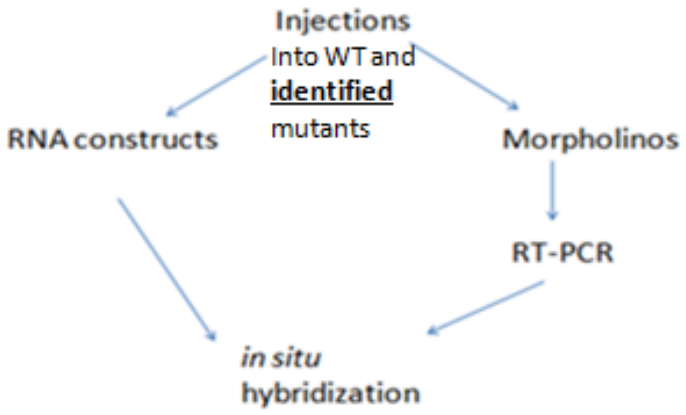

Figure 8. Outline of Utilized Methods.

As depicted in **figure 8,** I used three distinct tools to investigate the function of the Lhx1/5transcription factors: mutant fish, RNA fusion constructs, and MOs.

Zebrafish lines

Zebrafish (*D. rerio)* embryos were obtained from mating crosses of wild-type (AB or TL) adults (Picker et al., 2002). Embryos were staged according to hours post-fertilization at 28.5°C and/or morphological criteria as elucidated in Kimmel et al. (1995). The Lewis lab was already in possession of a *lhx1b* mutant allele fishline from the Sanger institute. Mutants are useful because it means that fewer potentially toxic substances must be introduced into the embryos in order to knock down potentially redundant genes. Morpholinos (MOs) and RNA constructs can have nonspecific effects, meaning that they have the potential to yield phenotypes unrelated to the gene knockdown. Furthermore, in some

instances, the concentration necessary to fully knock down a transcription factor might be too high to inject either individually or when combined with other knock-down reagents. If all of the embryos die before they can be studied, then it is impossible to understand the effects of a knock down. The *lhx1b* mutant was useful because it eliminated the need to inject the *lhx1b* morpholino. However, the homozygous or heterozygous mutants had to first be identified using a fin-clip genotyping procedure.

The *lhx1b* mutation is a nucleotide change from a T to an A, resulting in an amino acid change from a tyrosine (TAT) to an ochre (TAA) stop codon. As seen in **figure 9**, the mutation (indicated by the highlighted "Y") alters amino acid 110. The total sequence for this gene is 402 amino acids, meaning that the last 292 amino acids are lost when this mutation occurs. The LIM domain is unaffected by this mutation because it consists of amino acids 3-54 (as shown in **figure 9**); however, the homeodomain is lost as a result of this mutation as it consists of amino acids 178-240. The LIM domain is responsible for binding to proteins whereas the homeodomain binds to DNA. As such, it is possible that if a truncated protein is produced in this mutant line it may act as a dominant negative because it would bind partner proteins, but would be unable to bind to Lhx1/5 DNA target sites. In this way, it could titrate out WT binding partners.

MV*HCAGCERPILDRFLLSVLDRAWHAKCVQCCDCKCSLT*
*DRCFSREGRLYCKND*FFRRYGKCGGCAQGISPSDLVRKARSKVFHL
NCFTCIMCNKQLSTGEELYILDEYKFVCKEDYLNSNGKDTNLLSITTCSDP
SLSPESQDPQDDYKDSESGPMSDKETCNNENDEQNLGGKRRGRTTIK
AKQLETLKAAFAATPKPTRHIREQLAQETGLNMRVIQ
VWFQNRRSKERRMKQLSLGARRHMFFRSPRRMRALGDRMEPG
ELMANGHFSFYGDYQSEYYGPGSNYDYFPQGPPSQAHTPGDLGFMPSSG
PAGTPLGNMDPHHGAHHPSNDTQCFSEMISHHPGDSPSPEPSAPSIHSISTD
MCDSTPPFTSLNSLSANGYSNQLSSEMNEGTVW

Figure 9. Sequence of Lhx1b LIM and Homeodomains in a WT. The larger italicized print indicates the amino acids of the LIM domain. The larger underlined print indicates the amino acids of the homeodomain. The red letters indicate the beginning of each new exon. Black lettering indicates the first exon with the subsequent blue lettering indicating the start of the second exon, and so forth. The yellow highlighted "Y" refers to the amino acid tyrosine and is the amino acid that is changed as a result of this mutation.

Genotyping Lhx1b Mutant Fish

To genotype the *lhx1b* fish line, I cut off ("clipped") the caudal fins of individual fish and used them to prepare DNA. Two small glass beakers with 70% ethanol and distilled water were prepared to sterilize the scissors and tweezers. 300 mL of 0.016% Tricaine solution diluted with water was put into a clean tank. Fish were transferred to Tricaine solution using a net, with no more than 2-4 fish anesthetized at one time. As soon as the fish stop swimming, they were collected onto a plastic spoon. Using thumb and index finger, the fish was secured on the spoon with the tail exposed over the edge of the spoon. One half to two thirds of the caudal fin was removed using the sterilized scissors. The piece of fin was then placed in a microcentrifuge tube containing 100 µL of the DNA extraction buffer. DNA extraction buffer was made the day of the fin-clip. The extraction buffer was made in a volume of 3.0 mL with 100µLof the buffer needed per fin clip. The

3.0 mL consisted of 30µL10 mM Tris (pH 8.2), 60 µL 10 mM EDTA, 600 µL 200 mM NaCl, 75 µL 0.5% SDS, 60µL200µg/µL proteinase K, and 2175µL ddH_2O. The fish was then placed in an individual tank containing fresh fish water which rinsed off the Tricaine solution. Fish were kept separate or in pairs of 1 male and 1 female so that they could be readily identified and correlated with their respective DNA. Fish were kept separate until they were genotyped. These steps were repeated until all fish had been fin-clipped. Scissors and forceps were sterilized between fish by washing them in the aforementioned ethanol and water.

After the fin-clips, the DNA was extracted. The microcentrifuge tubes, each containing individual fin-clipped samples in DNA extraction buffer, were vortexed thoroughly and incubated for 2-3 hours at 55°C. Tubes were vortexed periodically during this 2-3 hour span in order to mix contents. Proteinase K was inactivated by incubating for 10 minutes at 100°C in a hot block. Tubes were then centrifuged for 20 minutes at 13000 rpm at room temperature. This spun down cell debris. The supernatant was then removed and transferred to clean, sterile microcentrifuge tubes. The genomic DNA was precipitated by adding 200µL of 100% ice-cold RNAse-free EtOH. Tubes were mixed or briefly centrifuged and then placed at -20°C for at least 1 hour. Genomic DNA was then precipitated by centrifuging for 20 minutes at 13000 rpm at 4°C. Next, ethanol supernatant was carefully removed and the pellet was washed with 500µL of fresh 70% EtOH. Tubes were centrifuged for an additional 3 minutes at 13000 rpm at 4°C and supernatant was again removed. The resulting pellets were air-dried for 5 minutes

at room temperature before being re-suspended in 100µL of H_2O. The re-suspended DNA was then stored at -20°C.

Forward primer: GCGAAGAACTGTACATCCTAGATGAATT

Reverse primer: TTGGCGGGTCTTTATGAAAATATATGAC

For PCR the following reactions were assembled:

	[Stock]	[Final]	Volume
Nuclease-free H_2O			5.351 µl
NEB Thermo Pol Reaction Buffer	10x	1x	1.0 µl
dNTPs	10 mM each	209 µM	0.209 µl
NEB Taq polymerase			0.04 µl
Forward primer	10 µM	0.2 µM	0.2 µl
Reverse primer	10 µM	0.2 µM	0.2 µl
Genomic DNA	variable	variable	3.0 µl
Total Volume			10 µl

The samples were subjected to the following PCR conditions:

94°C 180 sec

92°C 20 sec
65°C 30 sec } 18 cycles
72°C 60 sec

92°C 20 sec
56°C 30 sec } 15 cycles
72°C 60 sec

72°C 180 sec

16.0°C held until samples removed

Following the PCR, a digest reaction was performed. The following digest reaction was assembled in a 20µL volume: 5.875µL Nuclease-free water, 2.0µL NEBuffer 4, 2.0µL Acetylated BSA, 0.125µL *DraI*, 10.0µL PCR reaction product. The reaction was incubated for 2 hours at 37°. While the digests were incubating, a 2.5% TBE agarose gel was prepared. 4µL DNA loading dye was added to each of the digest reactions (giving a total volume of 24 ul). Then 10-15µL of each sample was loaded into a different individual well on a 2.0% TBE agarose gel. The gel was run at 130 volts for 40 minutes. This was a change from the usual protocol because I found that running a TBE gel for a long period of time at a slower voltage resulted in a clearer demarcation of DNA sizes.

Injections into zebrafish embryos

A Sutter Instrument Company glass-puller (model P-2000; program #8) and glass capillary tubes (10 cm length, O.D 1.2 mm, I.D. 0.94 mm) were used to make the injection needles. Injection plates were made using a specific mold that creates grooves in a 1% agarose gel in which the embryos rest. The plates themselves were made in standard 100 mm petri dishes using 1% agarose in embryo medium. 3L of a 60x stock solution of embryo medium requires 51.6g 5mM NaCl, 2.3g 0.17mM KCl, 8.7g 0.33 $CaCl_{2.2}H_2O$, and 14.7g 0.33 mM $MgSO_{4.7}H_2O$. Next, embryo medium is buffered to pH 7.8 with hepes (1g for

approximately 100 mL of 60x stock solution). Then 166 mLs of 60x stock solution with hepes is diluted with 20 mL 0.01% Methylene Blue in dH_2O to make 10L. Molds did not touch the base of the petri dish, but were deep enough so that embryos were not touching the surface of the liquid as seen in **figure 10**. Once the agarose set, the molds were removed using forceps and the dishes were filled with embryo medium to keep them moist. Adult fish were set up the night before in tanks with dividers separating males from females to ensure that the embryos were laid only once everything was set up for the experiment.

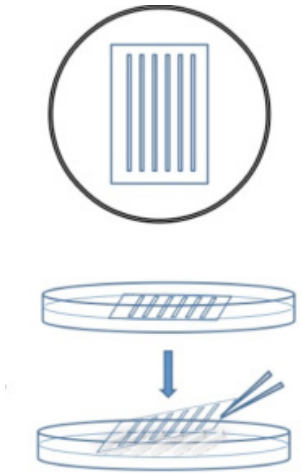

Figure 10. Injection mold set up. Based on figure by Dr. Lewis.

The morning of injections, a needle holder jar containing a small amount of distilled water in the bottom (to stop the needles from drying out) was used to hold loaded needles. Needles were loaded using a P2 or P20 pipette and filtered tips. Phenol red was mixed with the MOs or RNA so that injected liquid was visible in the embryo. If injection plates were made the night before,

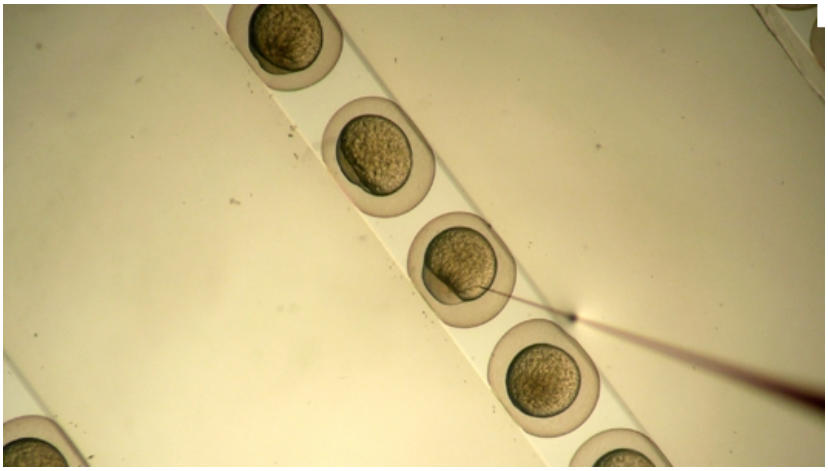

Figure 11. Injection of 1-cell embryo. Kindly provided by Dr. Lewis.

they were warmed to room temperature so as not to temperature shock the embryos. Once laid, embryos were collected from tanks and transferred to injection plates using plastic or glass pipettes. Forceps were used to guide embryos into the appropriate grooves in the plates. A General Valve Corporation picospritzer model II was used for the injections. Needles were secured into the picospritzer which forced compressed air down the capillary tube and controlled how much of the injected material was released. As illustrated in **figure 11**, 1-cell stage embryos were injected in the yolk region of the embryo. After injecting, embryos were inspected to ascertain that none were visibly harmed during injections. Damaged embryos were removed. Embryos were placed into a 28.5°C incubator. Wild-type uninjected controls were maintained for each pair of fish that laid embryos.

Several hours later, injected embryos were inspected. If phenol red remained in the yolk, these embryos were separated or discarded as the injected material had not fully permeated the embryo. Infertile embryos were also removed at this stage. The rest of the embryos were removed from the injection plates and pipetted into clean petri dishes with fresh embryo medium. These dishes were then placed back in the 28.5°C incubator overnight. Embryos were fixed at 24-27 hours depending on the nature of the experiment. Staging was performed based on a staging chart, pigmentation, and somite numbers. Embryos were fixed in 4% paraformaldehyde in phosphate-saline buffer (PBS) and put overnight at 4.0°C.

RNA fusion construct injections

RNA fusion constructs were injected in order to determine the function of

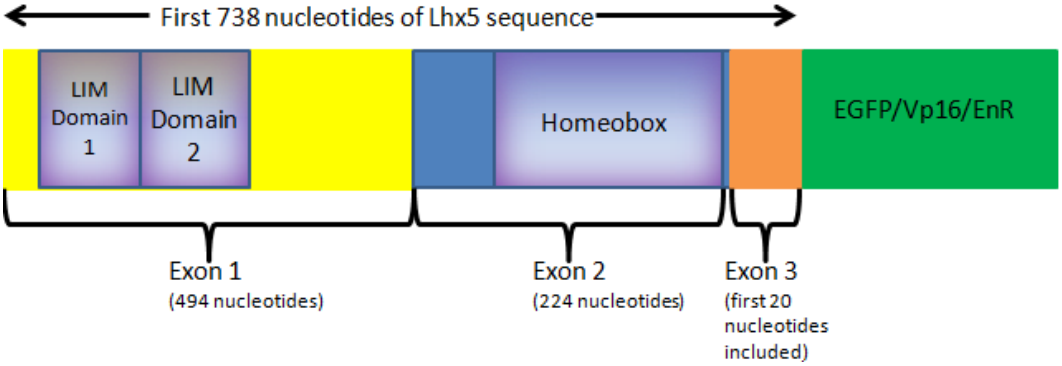

Figure 12. Diagram of RNA Constructs and protein domains that they encode for. The first 738 nucleotides of the *lhx5* sequence were used. The yellow box represents the first exon (494 nucleotides) in the *lhx5* sequence. The blue box represents the second exon (224 nucleotides) in the *lhx5* sequence. The orange box is the portion of the third exon (20 nucleotides) that was included from the *lhx5* sequence. The green box represents where the EGFP,Vp16, or EnR sequence was attached. The first LIM domain is encoded by nucleotides 3-61 and the second LIM domain is encoded by nucleotides 62-125, both fall within exon 1. The homeobox domain is encoded by nucleotides 180-239 and falls within exon 2.

the Lhx1/5transcription factors. Four different RNA constructs were injected: Lhx5VP16, Lhx5GFP, Lhx5EnR (Lewis Lab), and Lhx5EnR (Westerfield Lab). Each of these constructs contained the first 738 nucleotides of the Lhx5 sequence which includes 2 LIM domains and the homeobox (**figure 12**). Additionally, these constructs each had either a Vp16, a GFP, or a EnR sequence attached after the Lhx5 coding sequence (**figure 12**). The Lhx5VP16 was expected to be a dominant active version of the desired protein while Lhx5EnR was hypothesized to be a dominant negative version of the desired protein based on research using a Lhx5EnR construct in the Westerfield lab (Peng and Westerfield, 2006). In order to prepare the RNA, plasmid DNA was linearized with SacII. The reaction was assembled in a 20µLvolume consisting of 2µLrestriction enzyme, 1 ug of DNA,

0.5µLrestriction enzyme buffer, and the rest was dH_2O. The reaction was placed at 37°C for 2 hours. DNA was run on a 1% TAE gel to make sure it was cut. If cut,1µL of proteinase K was added and the reaction was placed at 37°C for 30 minutes. DNA was then extracted using phenol/chloroform and chloroform. Finally, DNA was precipitated by adding 1µL glycogen (as an inert carrier), 1/10 volume of 4M NaCl, and 2x volume of EtOH and placing at -20°C overnight. After spinning the reaction at 13,500 rpm, the supernatant was removed and the pellet was washed with 80% cold EtOH. Then the DNA was re-suspended in 14µLdH_2O. RNA was then made using an sp6 Ambion mMessage mMachine kit.

Splice-Blocking Morpholino injections and controls

Morpholinos (MOs) are antisense oligonucleotides that interfere with RNA transcription to protein or RNA splicing. Although they are synthetic molecules, MOs mimic the design of actual nucleic acids and bind to the usual complementary sequences (Gene Tools). Both types of MOs, ATG and splice-blocking (S-B) MOs, were utilized. ATG MOs are oligonucleotides that bind to the 5'-untranslated region of mRNA and therefore interfere with movement of the ribosomal initiation complex. The initiation complex never reaches the start codon and consequently translation can never take place.

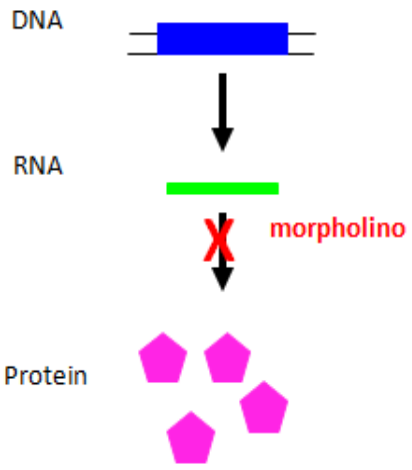

Figure 13. Schematic of how ATG MOs work. Figure kindly provided by Dr. Lewis.

Because translation never occurs and the protein is absent (or reduced) as demonstrated in **figure 13**, we can determine the function of the unmade protein (Gene Tools). Splice-blocking MOs prohibit the removal of introns so that the translated product no longer codes for the same protein (Gene Tools). Splice-blocking MOs were primarily used because the effectiveness of these MOs can be checked by reverse transcription polymerase chain reaction (RT-PCR) to check if the RNA has been correctly spliced. Generally speaking, by inhibiting or reducing protein production, MOs enable us to determine the function that each gene is necessary or required for.

MOs were injected into 1-2 cell stage embryos from a cross of wild-type AB fish. The original ATG and S-B MOs had been used in a pilot experiment in a previous Lewis lab study. Another MO was used successfully in Peng and Westerfield (2006). Sequences (Cerda-Moya, 2011) were as follows:

Lewis *lhx1a* S-B: TGCAAGCTGTAACTGAATAGGGAAA

Lewis *lhx1a* ATG: AGCCCGCACAGTGGACCATCGTCTT

Lewis *lhx1b* S-B: TGCAGGTGGTAACTGTAAAAAAGCA

Lewis *lhx1b* ATG: TCTCGCATCCAGCACAGTGGACCAT

Lewis *lhx5* S-B: CGACACTGTGGTATGATAAAGAACC

Lewis *lhx5* ATG: TGCACCATCATTCCGCCCTGGAGGG

Peng and Westerfield *lhx5*: GTGCGTTGTTCTCACCTGAATCACC.

Lewis ATG MOs were injected at 2.1µg/µL, 3.02µg/µL and 3.32µg/µL. These concentrations were chosen based on previous work in the Lewis lab. p53 MO was also injected in a mix with the other MOs at 4.0µg/µL to inhibit any

nonspecific apoptosis effects. p53 reduces the possibility that the phenotypes observed in the MO injections are the result of non-specific toxicity or apoptosis (Robu et al., 2007). Lewis S-B MOs were originally injected at 1µg/µL and 2µg/µL each. It appeared that there was an effect with these concentrations only on Lhx5 splicing (see results section). However, Lhx1a and Lhx1b splicing did not seem affected by these concentrations. Concentrations of all three MOs were increased several times to 2.5µg/µL, 3.0µg/µL, 3.5µg/µL, and 4.5µg/µL. No visible knock-down of RNA splicing was apparent at any of these concentrations by PCR assay. Although Lhx5 had originally shown an effect at the 1 µg/µL and 2 µg/µL concentrations, it was later shown via RT-PCR that this result was anomalous (see results).

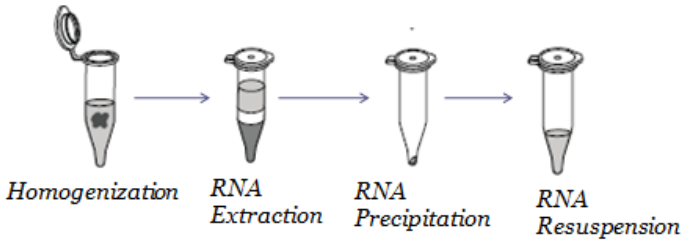

Figure 14. RT-PCR Protocol Illustrated. Figure based on Qiagen One-Step RT-PCR Kit.

To examine whether the absence of a knock-down was the result of an ineffective MO, a second *lhx5* MO was injected that had been used successfully by Peng and Westerfield (2006). Based on the concentrations suggested by Peng and Westerfield (2006), the new *lhx5* MO was injected originally at 4.0µg/µL, 5.0µg/µL, and 6.0µg/µL. The concentrations were subsequently increased to 6.5µg/µL, 7.5µg/µL, and 8.3 µg/µL. In this case, there appeared to be an effect on RNA splicing (see results).

Wild-type embryos obtained from each pair of zebrafish that laid were compared to injected embryos from that same pair to ensure that morphological changes were in fact the result of injections. Furthermore, these control embryos were also used in the subsequent RT-PCR experiments to maintain a precise control throughout.

RT-PCR

After injecting splice-blocking MOs, I performed a reverse transcription polymerase chain reaction (RT-PCR). RT-PCR was utilized to see if the splice-blocking MOs had fully blocked RNA splicing. To prepare for the RT-PCR, 25 injected embryos were collected per tube. All of the liquid (embryo medium) was removed and the RNA was extracted using 250µLAmbion TRI Reagent®. Next, the procedures for homogenization, RNA extraction, RNA precipitation and wash, and RNA solubilization were performed according to the protocol listed in the Qiagen One-Step RT-PCR kit (**figure 14**). RNA was re-suspended in 20µLof water. 2.4µLof DNAase Buffer (RNase-free DNase) and 2µLof DNase were added and the reaction was incubated for 15 minutes at 37°C and then for 10 minutes at 65°C. 0.5µL RNAsin was then added and the RNA was stored at -20°C.

The RT-PCR procedure and reagent volumes were performed according to the Qiagen OneStep RT-PCR kit. Primers used were as follows:

lhx1a rtpcr SB2-3FW TGATGTGTAACAAGCAGCTTTC
lhx1a rtpcr SB2-3RV AATAACTCGCATGTTGAGTCC
PCR product= spliced 395bp versus un-spliced 985bp

lhx1b rtpcr SB2-3FW ACGTTATGGCACTAAATGTGG
lhx1b rtpcr SB2-3RV CTGCTTGGCTTTGATTGTAGT
PCR product= spliced 397bp versus un-spliced 691bp

lhx5 rtpcr SB2-3FW GTTTCATCTCAACTGTTTCACG
lhx5 rtpcr SB2-3RV ATGTTAAGGCCTGTCTCCTG
PCR product= spliced 411bp versus un-spliced 3010bp

The following program was set up on the PCR machine:

30min 50°C
15min 95°C
35X [30sec 94°C
45sec 57°C
60sec 72°C]
10min 72°C

Samples were then run on a 2% TBE agarose gel at 120 volts for 40 minutes.

in situ

in situ hybridization was performed as described in Concordet et al. (1996). To determine neurotransmitter phenotypes, I focused on genes corresponding to proteins which transfer or produce specific neurotransmitters (Batista and Lewis, 2008; Higashijima, 2004). I used the following probes: *glyt2a, glyt2b*, and *pax2.1*. *glyt2a* and *glyt2b* were used to test whether the knock-down of Lhx5 resulted in a change in neurotransmitter fate in these cells. *glyt2a* and *glyt2b* are markers of glycinergic cells which would enable me to see whether the CiAs retained their inhibitory neurotransmitter fate in the absence of Lhx. p*ax2.1* was used because of previous work conducted in mice which suggested a relationship between the Lhx and Pax families of transcription factors (Pillai,). I

also decided to use *pax2.1* because it was one of my probes that had worked the best in previous *in situ* experiments.

Results

Genotyping lhx1b Mutants

Developing a method to identify *lhx1b* mutants was important to my project because it not only was more cost effective and time efficient than sending fish to sequencing, but also eliminated the number of constructs I had to inject in order to knock-down all three Lhx1/5 genes. After examining the sequence of the mutation, I determined that there was no restriction enzyme site created or removed by the mutation; however, by creating PCR primers that amplified the region around the mutation and altered one nucleotide, I was able to introduce a restriction enzyme site so that an enzyme would cut in the WT but not in the mutant DNA. Creating a primer that did not end on a "T" nucleotide was important because it would have been less stringent. Furthermore, I did not want the last nucleotide to be the one that changed. The enzymes DraI and PsiI both had the potential for introducing their cutting site into the WT DNA. Both PCR primers that introduced a nucleotide change were designed to be 28 base pairs in length. The G/C ratio and melting and annealing temperature were also comparable between both. Therefore, I tested both options. By introducing a restriction enzyme site, I eliminated the need to send the DNA from the PCR to be sequenced. Instead, I could do a restriction enzyme digest on PCR products and run the DNA on a gel. This method is much cheaper and faster than sending the DNA to sequence from each *lhx1b* mutant fish.

Table 1. Primer sequences (compared to wild-type) used to identify Lhx1b mutants.

Name	Sequence
Wild-type	GGCGAAGAACTGTACATCCTAGATGAATATAAATTTGTCTGCAAG GAGGATACTTGAATAACAGCAATGGAAAAGACACAAACCTTCTGT CAAgtaagtcggtgattttgggccttgtgggcctacatgagataaaatatttacattagacactgaggatacaa tcaagagtattagtttttcccatctccgtgttcgttaactgcaggcaattggcgggtctttatgaaaatatatgac
PsiI	G<u>GCGAAGAACTGTACATCCTAGATGA</u>*T*<u>TA</u>TAAATTTGTCTGCAAG GAGGATTACTTGAATAACAGCAATGGAAAAGACACAAACCTTCTG TCAAgtaagtcggtgattttgggccttgtgggcctacatgagataaaatatttacattagacacatgaggatac aatcaagagtattagtttttcccatctccgtgttcgttaactgcaggcaattggcgggtctttatgaaaatatatgac actatttattaaaatgtattagtgatacaaaatgcgttgtctgtgtagaatggcattgcatttcgatagacaataggc ctacatgataatttacgcatgatatttaagtggcctacataacagcttctaaatgtgctttttacag
DraI	G<u>GCGAAGAACTGTACATCCTAGATGAAT</u>*T*TAAATTTGTCTGCAAG GAGGATTACTTGAATAACAGCAATGGAAAAGACACAAACCTTCTG TCAAgtaagtcggtgattttgggccttgtgggcctacatgagataaaatatttacattagacacatgaggatac aatcaagagtattagtttttcccatctccgtgttcgttaactgcaggcaattggcgggtctttatgaaaatatatgac actatttattaaaatgtattagtgatacaaaatgcgttgtctgtgtagaatggcattgcatttcgatagacaataggc ctacatgataatttacgcatgatatttaagtggcctacataacagcttctaaatgtgctttttacag
Reverse primer sequence	gtaagtcggtgattttgggccttgtgggcctacatgagataaaatatttacattagacacatgaggatacaatcaa gagtattagtttttcccatctccgtgttcgttaactgcaggcaa<u>ttggcgggtctttatgaaaatatatgac</u>actattt attaaaatgtattagtgatacaaaatgcgttgtctgtgtagaatggcattgcatttcgatagacaataggcctacat gataatttacgcatgatatttaagtggcctacataacagcttctaaatgtgctttttacag

* Primer sequence is underlined. Red indicates mutation site. Italics indicates nucleotide change site. WT=T; mutant=A. Fragment should be 241 bp.

All primers were custom DNA oligos from Integrated DNA Technologies. In order to see which 5' primer was more effective at introducing a restriction enzyme site, both possible combinations of primers were tested on wild-type genomic DNA. The enzymes were tested on wild-type genomic DNA because the restriction enzyme site introduced meant that the enzyme would cut in the wild-type and not in the mutant. DraI was determined to be the better enzyme because the differences between the cut and uncut DNA on an agarose electrophoresis gel, while subtle, were more apparent than the DNA cut with PsiI. Based on the results from wild-type tests, it was determined that DraI was the better enzyme to identify the *lhx1b* mutants (**figure 15**). The experiment was then repeated using DraI and the fin-clipped DNA. Initially, 4 genotyped fish were also sent to sequencing at Cornell University to confirm that the method could correctly identify heterozygous and homozygous mutants.

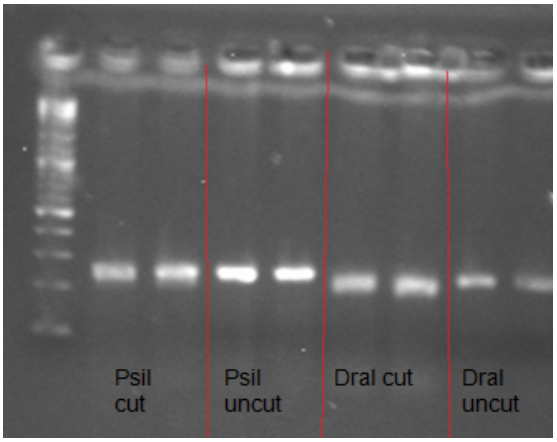

Figure 15. Demonstrating the effectiveness of PsiI vs. DraI.

As discussed in the materials and methods section, I designed a protocol to identify *lhx1b* mutants. This PCR-based genotyping procedure that used specially designed primers that introduced a restriction enzyme site into WT, but not mutant DNA, was successful in identifying *lhx1b* mutants. Known wild-type fish were used as a control to ascertain that the procedure was working. The shorter

band is the cut WT DNA (you do not see the second fragment because it is too small). The longer band is the uncut mutant DNA. On the gel, two bands

Figure 16. Results of Lhx1b Genotyping. Two bands (both the long and the short) correspond to heterozygous fish (lane 1, 4, & 9). Lanes appearing like lane 2 were WT, as they only had the shorter or weaker band. This could be because of an incomplete digest. In this case, it is safer to assume they are WT as opposed to heterozygous. Lanes with only the longer band (land 7) were homozygous.

corresponds to a heterozygous fish (**figure 16**). Heterozygous fish yield two bands because one band corresponds to the uncut mutant DNA (the longer one) and the second band corresponds to the cut WT DNA band (the shorter one). The protocol resulted in clearly distinguishable bands of two different sizes on the gel. This protocol was successful because the mutant and WT fish could be differentiated from one another, which was confirmed by sequencing.

In total, 6/22 fish (27.3%) were WT, 3/22 fish (13.6%) were homozygous for the Lhx1b mutation, and 13/22 fish (59.1%) were heterozygous for the mutation. This is approximately what was expected because of Mendelian genetics where ¼ should be homozygous, ¼ should be wild-type, and ½ should be heterozygous. A chi squared test equals 1.545 with 2 degrees of freedom which gives a p value of 0.4618. The two-tailed P value for this data indicates that the

difference between the observed and expected percentages of WT/het/hom fish is not statistically significant.

Obtaining a *lhx1b* homozygous mutant fish was unexpected as fish sent by the Sanger Institute are usually the progeny of an outcross. As explained by Mendelian genetics, it is impossible to obtain a homozygous mutant from an outcross of fish. Therefore, to be sure that the protocol really yielded a homozygous mutant, I confirmed the findings from this genotyping procedure by sequencing 4 fish at Cornell University (results in **figure 16**). At first, we were concerned that the band on the gel indicating a homozygous mutant could have been because the restriction enzyme did not cut properly. If

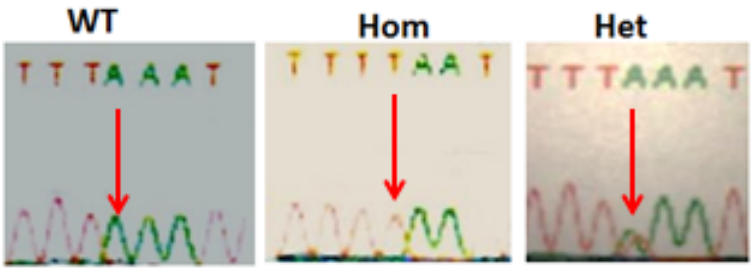

Figure 17. Sequencing results for 3 separate fish.

this was the case, the absence of the shorter band on the gel may have been an experimental error as opposed to an indication that the fish was a homozygous mutant. The WT sequencing picture (seen farthest left in **figure 17**) shows residues corresponding to only the A nucleotide as indicated by the red arrow. This was what I expected given that a WT fish should be homozygous for the WT allele. The homozygous (hom) fish sequencing indicates a T nucleotide in this position, consistent with a homozygous mutation. Finally, the heterozygous fish has both the homozygous and wild-type nucleotide (both A and T). The differences between the sequences for the WT, homozygous mutant, and

heterozygous mutant demonstrate that these fish were indeed heterozygous and homozygous *lhx1b* mutants.

Identifying a homozygous mutant adult fish was a major result because it shows that the mutant is homozygous viable. Prior to my work, it was unknown whether a *lhx1b* homozygous mutant fish would be viable. Initially, we were concerned that perhaps this mutation did kill some of the fish because the number of identified homozygous mutants was lower than the expected number (13.6% rather than 25.0%). However, a chi square test comparing homozygous mutants to the rest of genotyped fish equals 1.515 with 1 degree of freedom which gives a p value of 0.2184. The two-tailed P value for this data indicates that the difference between the observed and expected percentages of mutants versus non-homozygous-mutant siblings is not statistically significant. Moreover, the chi square test comparing all mutants (both heterozygous and homozygous) and wild-type fish equals 0.061 with 1 degree of freedom which gives a p value of 0.8055 which is not statistically significant so this low frequency of homozygous mutants could be due to chance. To test this, I would need to identify more fish and increase the numbers. This p value indicates that the difference between the observed and expected percentages of homozygous and heterozygous mutants versus wild-type is not statistically significant. Based on this data, it does not seem that homozygous mutants preferentially die. However, they might preferentially die, but I would need a larger sample size to see if the difference between the observed and expected numbers of *lhx1b* homozygous mutant fish becomes statistically significant. It is possible that this mutation is not

homozygous viable in all individuals perhaps because the mutation automatically triggers the DNA for degradation or because the mutation eliminates the homeodomain which is lethal to the embryos.

RNA injection results

Three constructs were initially injected: Lhx5Vp16, Lhx5GFP, and Lhx5EnR. Each of these constructs was injected several times as described in

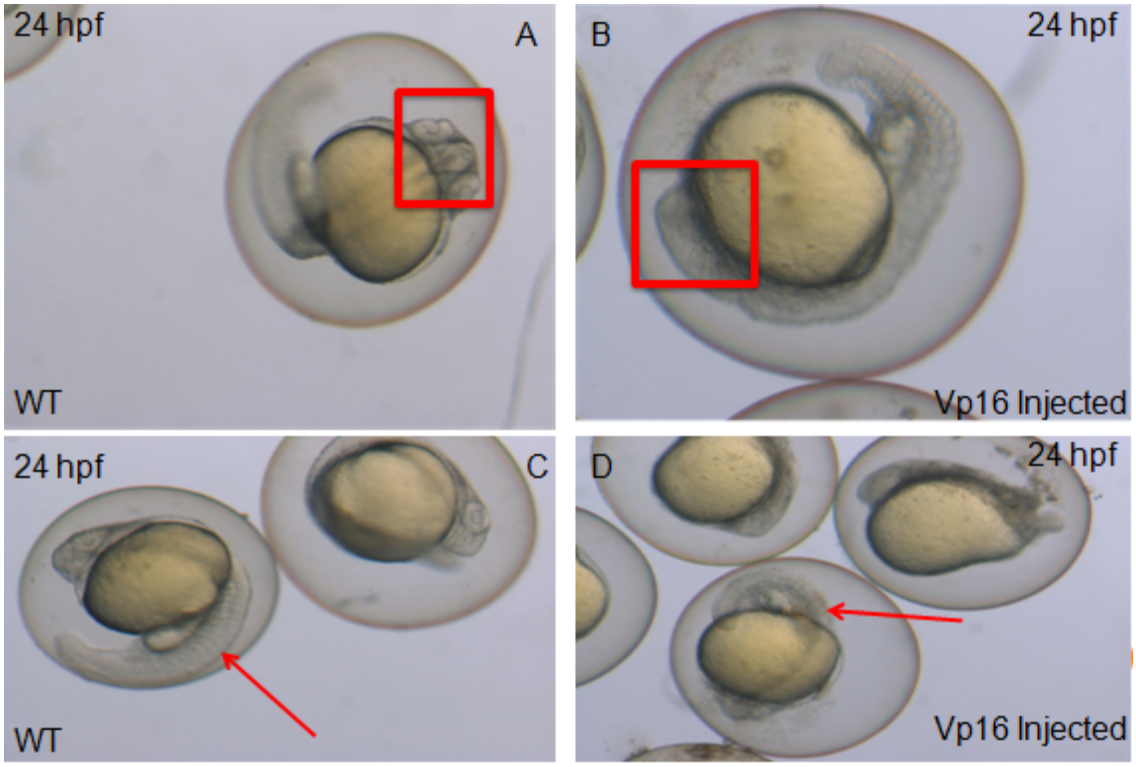

Figure 18. Phenotypic effects of Lhx5EnR RNA Injections. In (A and B) red boxes indicate morphological differences in the eyes. In (C and D), red arrows indicate misshapen tails and somites.

table 2 at different concentrations with the hopes of optimizing the results. As mentioned in materials and methods, I expected Lhx5EnR to act as a dominant active construct, Lhx5Vp16 to act as a dominant negative construct, and Lhx5GFP to show the endogenous expression pattern (Peng and Westerfield,

2006). Furthermore, since the DNA binding domains for Lhx1a, Lhx1b, and Lhx5 are similar, I expected that these RNA construct might interfere with the activity of all three of these transcription factors. The major advantage of RNA constructs was that we thought they might affect all three Lhx activities so we could potentially knock down all transcription factors using just one reagent. During these experiments, I noticed several significant morphological phenotypic changes (**figure 18**). In the injected embryos, the eyes were clearly less pronounced and not well defined. Furthermore, the midbrain/hindbrain boundary was not clearly differentiated. Moreover, the somites in the spinal cord of the embryo appeared less distinct. Finally, the tail of the embryo was more rounded and not as well defined as in the wild-type, un-injected embryos. All of these phenotypes were in comparison to a wild-type, un-injected control obtained from the same pair of zebrafish that laid the injected embryos. **Table 2** below indicates the abnormalities seen in the injected embryos as compared to the wild-type controls. Each of the constructs appeared to result in similar morphological phenotypes.

Table 2. Injection results with the original RNA fusion constructs.

Construct Injected	Concentration & volume	Number Injected	Total Dead	Phenotype	Control
Lhx5EnR Lewis	8.42µg/µL; 3-4 nL	70	58.5 % (41)	23 had misshapen bodies, distorted somites, and misshapen head structures. 5 were WT looking.	1 dead; 1 with just head and yolk; rest look WT
Lhx5EnR Lewis	8.42µg/µL; 3 nL	54	48.1 % (26)	17 had misshapen heads, bodies, and tails. Some tails had kink. Some tails were short and stubby. One embryo was cyclopic.	8 infertile; some had small chorions
Lhx5EnR Lewis	8.42µg/µL; 4 nL	75	20.0 % (15)	9 were misshapen and distorted. 3-4 were WT, though 2 had weird eyes. Many had small chorions, mushed heads, stubby trunks, and missing eyes. Some embryos had just a tail *short body phenotype	15 infertile N= 20
Lhx5EnR Lewis	21.4µg/µL; 3nL	30	43.3 % (13)	8 were misshapen, distorted, and mostly yolk. 3 were very misshapen with malformed heads. 1 had a larger head and no distinct eyes.	3 infertile; otherwise look WT N= 20
Lhx5Vp16	3.22µg/µL; 2-3 nL	70	38.5 % (27)	20 were misshapen and distorted. 8 had malformed trunks and/or heads. 1 was very thin. Some embryos exhibited trunks that appeared to zigzag.	1 dead; 1 mush; 1 deformed N= 20

Lhx5Vp16	3.22µg/µL; 2-3 nL	50	38% (19)	15 were misshapen, distorted, and mostly yolk. 5 were misshapen with malformed heads, stubby tails, and no distinct eyes. 1 appeared WT.	4 infertile; otherwise look WT N= 20
Lhx5Vp16	3.22µg/µL; 3-4 nL	45	57.8 % (26)	12 were misshapen and distorted and mostly yolk. 1 was very small with a narrow body. 3 were mush. 3 were WT (but two moving significantly less so). 1 had a curly tail. 2 had weird eyes or were cyclopic.	WT looking N= 20
Lhx5Vp16	3.22µg/µL; 2-3 nL	50	54.0 % (27)	18 were misshapen and distorted and mostly yolk. 1 was very misshapen and disturbed. 6 were WT looking.	WT looking N= 20
Lhx5Vp16	8.17µg/µL; 4-5 nL	55	65.5 % (36)	11 were misshapen and/or distorted and mostly yolk. 8 were funny with some being very disturbed. Others had just unusual body parts. 1 had very strange boxy tail and odd somites. Finally, 1 appeared WT.	WT looking N=20
Lhx5GFP	3.94µg/µL; 3-5 nL	50	42.0 % (21)	Many had misshapen heads and exhibited necrosis. Some had no defined heads, stubby tails, and misshapen eyes.	9 died (after removing infertiles). 3 mush; 1 with funny head N= 20
Lhx5GFP	3.94µg/µL; 4 nL	107	7.5 % (8)	Some had weird heads. The tails were also either absent or kinked/stubby.	9 with small yolks. N= 20

Lhx5GFP	3.94µg/µL; 3 nL	56	25.0 % (14)	6 were misshapen, but many were WT looking.	WT looking N=20
Lhx5GFP	3.94µg/µL; 3 nL	76	7.9 % (6)	17 had funny heads and several with smaller bodies and stubby tails.	1mush; rest WT looking N=20

After working with these RNA constructs, I discovered that the wrong enzyme for linearizing the template DNA, BssHI, had been entered into the

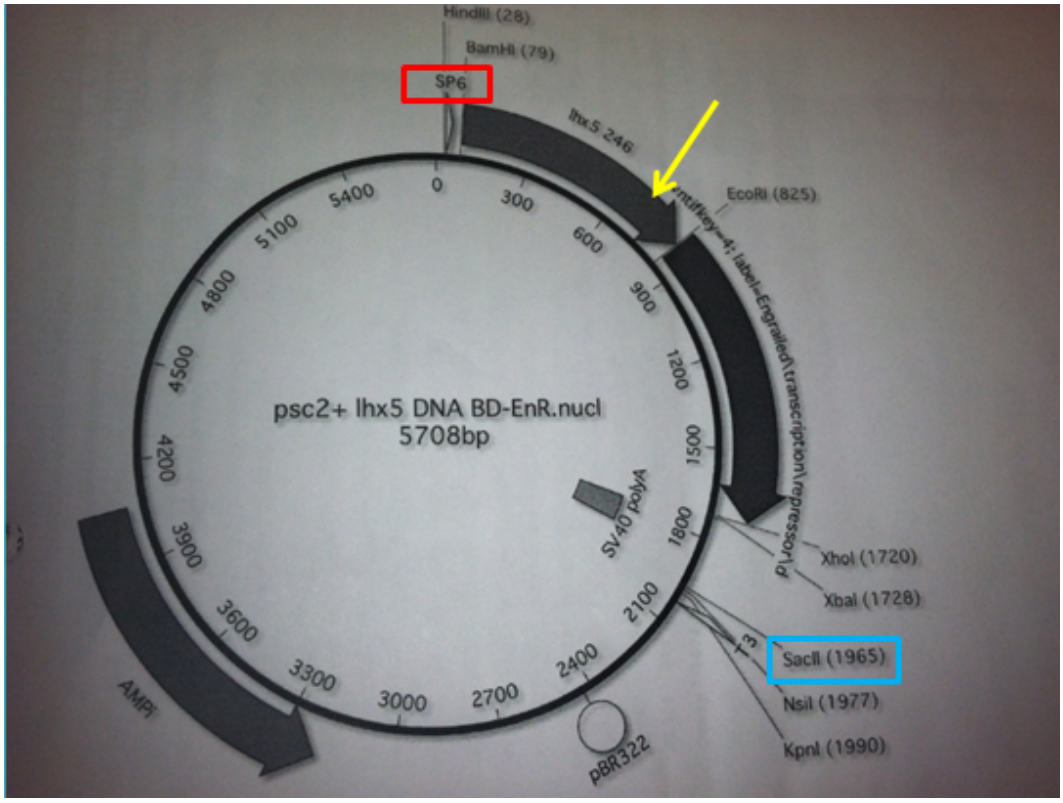

Figure 19. Plasmid map for Lhx5EnR. Red box indicates the sp6 RNA polymerase start location. The yellow box indicates approximately where a BssHII site exists within the Lhx5 sequence. As shown in the picture, the distance between the sp6 start location and the BssHII cut site is shorter than the full length of the insert and does not include the end of the Lhx5 coding sequence or the EnR sequence. The blue box indicates where the new enzyme I used, SacII, cuts.

database by a previous student. Consequently, the RNA injected into the embryos

did not correspond to the intended RNA fusion constructs. All three RNA constructs that I initially used were made as a BamHI-Lhx5-EcoRI-EGFP/EnR/Vp16-Xhol cassette cloned into pCS2+. Only the first 738 nucleotides of the Lhx5 coding sequence were used which is equivalent to the first 246 amino acids. These 246 amino acids contain two LIM binding domains which are amino acids 3-61 and amino acids 62-125. The LIM homeobox is also included in these 246 amino acids and is amino acids 180-239. Unfortunately, the BssHII site is present in the Lhx5 sequence used (**figure 19**). The BssHII site occurs at nucleotides 238-243 of the Lhx5 coding sequence meaning that the last 495 nucleotides of the Lhx5 sequence in the construct as well as the EGFP/EnR/Vp16 portions are excised when BssHII is used (**figure 19**). Therefore, when I was making sense RNA to inject, I only was only transcribing nucleotides 1-243. This means that I was only including the first LIM binding domain and was not including the second LIM domain or the LIM homeobox. As such, the resulting RNA I made could have been unstable because it did not include a stop codon or a PolyA tail in addition to missing the other aforementioned portions. Although it may also have acted as a dominant negative, as the remaining LIM domain may have still been able to bind to partner proteins.

After discovering this mistake, I relinearized the DNA with the correct enzyme, which is SacII. However, after linearizing with this enzyme and injecting RNA made from these newly cut template DNAs, only wild-type-looking embryos were seen, suggesting either that there was a problem with the initial DNA preparation, or that the correct construct does not produce morphological

phenotypes. In order to distinguish between these possibilities, I performed a new midi-prep as per the Qiagen kit instructions from a glycerol stock to obtain a fresh DNA preparation.

Following the midi-prep, I remade and injected the RNA. My initial round of injections yielded an apparent phenotype in that the embryos were distorted with misshapen heads and bodies. However, my second injection round with this new RNA yielded different results (**table 3**). The third time I repeated the experiment the results were the same as in round two, suggesting that either there was no effect or that the effect had no visible morphological phenotype.

Table 3. New RNA injection results.

Injection Round	RNA concentration and volume injected	Number injected	Number dead	Comments/Phenotypes	Control
#1	189.9ng/µL 3nL injected	40	40.0% (16)	17 all yolk/mush; unable to be dechorionated; 7 infertile	7 infertile; WT looking N= 20
#2	189.9ng/µL 3nL injected	45	0.0% (0)	10 were necrotic, 15 WT-looking	2 infertile; WT looking N=20
#3	189.9ng/µL 3nL injected	70	2.85% (2)	WT-looking	WT looking; N=20

Using ATG Morpholinos to knock-down Lhx1a/1b/5 function

Initially the ATG MOs were injected at two concentrations. Mix#1 consisted of 2.1µg/µL each of Lhx1a, Lhx1b, and Lhx5 as well as 4.5µg/µL of

p53 MOs. Mix#2 consisted of 3.32µg/µL each of Lhx1a, Lhx1b, Lhx5 and 3.84µg/µL p53 MO. In the initial injection round, mix #1 was not concentrated enough to yield an effect while mix #2 was too toxic to the embryos. In mix #2: the embryos were either dead or too sick to use in an *in situ*. However, when the concentration of each of the *lhx1a*, *lhx1b*, and *lhx5* MOs was reduced to 3.02µg/µL and the p53 MO was kept at 3.84µg/µL, the ATG MOs produced seemingly promising results in that the embryos survived and showed a morphological phenotype. As mentioned above, p53 MO was also added to the MO mix in the case of the ATG MO injections. Embryos injected with p53 as part of the ATG MO mixture did not appear morphologically different from those injected with MO in the absence of p53.

Table 4. ATG Morpholino Results.

What injected	Concentration Injected	Mush	Dead	Funny/ Misshapen	WT-like	Total	Control
MO Mix#1	Lhx1a, Lhx1b, Lhx5 @ 2.1µg/µL each and p53 @ 4.0 µg/µL (~2nL)	1.92% (1)	3.84% (2)	46.2% (24)	48.1.% (25)	52	WT looking; N= 20
MO Mix#1	Lhx1a, Lhx1b, Lhx5 @ 2.1µg/µL each and p53 @ 4.0 µg/µL (~4 nL)	2.63% (1)	0.0% (0)	47.4% (18)	50.0% (19)	38	WT looking; N=20
MO Mix#2	Lhx1a, Lhx1b, Lhx5 @ 3.32µg/µL each and p53 @ 3.84 µg/µL (~3nL)	75.6% (34)	15.6% (7)	8.9% (4)	0.0% (0)	45	WT looking; N=20
Middle Concentration	Lhx1a, Lhx1b, Lhx5 @ 3.02µg/µL each and p53 @ 3.84 µg/µL (~2nL)	13.1% (14)	19.6% (21)	67.3% (72)	0.0% (0)	107	WT looking; N=20
Middle Concentration	Lhx1a, Lhx1b, Lhx5 @ 3.02µg/µL each and p53 @ 3.84 µg/µL (~4nL)	27.5% (11)	22.5% (9)	50.0% (20)	0.0% (0)	40	WT looking; N=20

Determining whether splice-blocking MOs completely eliminate RNA splicing

Initially S-B MOs were injected at 1.0µg/µL and 2.0µg/µL. The initial concentrations were chosen based on injections conducted previously in the Lewis lab with the same constructs. I injected the S-B MOs into 1-4 cell stage embryos, incubated them until they were ~24 hpf, removed their chorions, and put them in TRI Reagent®. Next, I followed the RT-PCR protocol detailed in the materials and methods section and depicted in **figure 13**. After extracting the RNA, I performed an RT-PCR reaction using specific primer sets. The primers were also used on WT cDNA to test that they were working. Furthermore, for each sample, two different sets of primers were used: the correct primer set to test for the blocking of RNA splicing and a second primer set to test that the RNA was viable. For example, if I injected *lhx5* S-B MO I extracted the RNA from this sample and ran an RT-PCR with two tubes of this sample. In one of the tubes, I used *lhx5* primers and in the other I used either *lhx1a* or *lhx1b* primers. If the RNA was viable, the *lhx1a* or *lhx1b* primers should always yield a band because that transcription factor gene should have been unaffected by the injections. By testing the primers in this way, I was checking that the template RNA was viable.

The initial results shown in **figure 20** indicate that the embryos injected

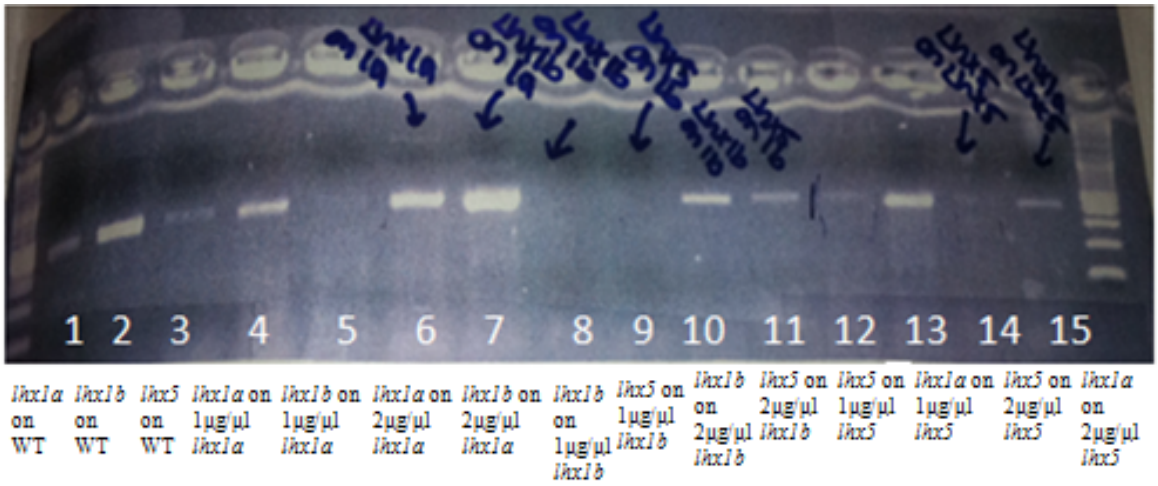

Figure 20. RT-PCR #1 Lewis S-B MOs. Each lane is labeled with the S-B MO injected and the primers used. Example: lane 2 is *lhx1b* primers on WT DNA. Lanes 1-3 were run to check that primers were working as this was the first time they had been used. Lanes 5, 7, 9, 11, 13, and 15 are controls for lanes 4, 6, 8, 10, 12, 14, respectively.

with1µg/µL of *lhx1b* S-B MOs did not produce a band with the *lhx1b* primers. The absence of a band could suggest that splicing has been blocked and the RNA is unstable. However, an alternative possibility is that the cDNA synthesis did not work possibly due to contamination with RNases during the RNA prep or cDNA synthesis. Both samples injected with 1µg/µL of *lhx1b* demonstrated similar problems as neither the control nor the experimental showed up on the gel. This suggests that the lack of bands was indeed due to a problem in the cDNA synthesis. In samples from embryos injected with 2µg/µL of *lhx1a* and *lhx1b* MOs there was also no significant blocking of RNA splicing. However, this conclusion was made only by comparing the bands from samples of differing concentrations to one another because in these cases the controls did not show up on the gel. This may be due to the PCR conditions used. Finally, the samples from embryos

injected with 1µg/µL and 2µg/µL of *lhx5* MO suggested that the RNA from this gene might not be being completely spliced. This was because when the *lhx1a* primers were used on both samples, the bands were clear which indicated that the cDNA synthesis had worked and the primers were working. However, in comparison, the *lhx5* primers yielded weaker bands, with the 2µg/µL band appearing weaker than the 1µg/µL band. This trend suggested that as the concentration was increased the splice blocking became more severe.

The same injection concentrations were repeated for Lhx5 to confirm the

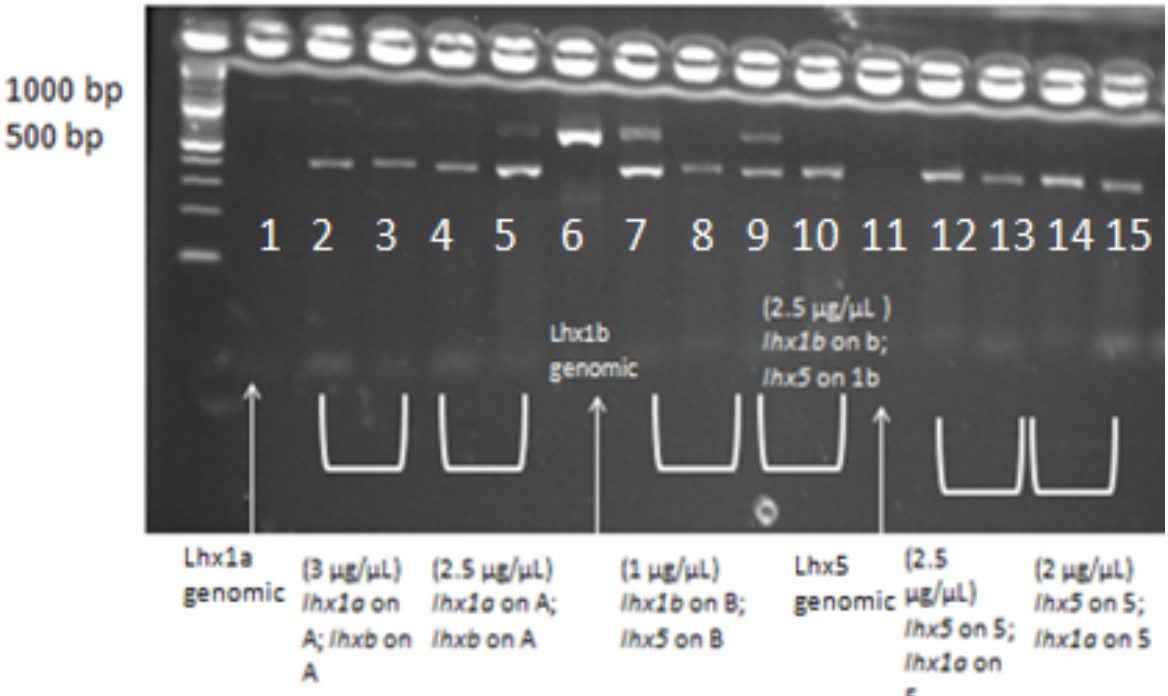

Figure 21. RT-PCR for injection round two with Lewis S-B MOs. Each lane is marked with the primers that were used and which sample type they were used on. For example, lane 2 is *lhx1a* primers on *lhx1a* S-B MO cDNA. Lanes 1, 6, and 11 are genomic controls for *lhx1a, lhx1b,* and *lhx5*, respectively. Genomic samples did not show up for *lhx1a* or *lhx5* (lanes 1 and 11). This may be due to the PCR conditions used.

blocking of splicing. The concentrations injected were also increased for all genes

because I had not seen a complete knock-down in any case (**figure 20**). I hoped that the results (**figure 21**) would confirm the original results and that a higher concentration might result in a more complete knock-down of Lhx5. Additionally, a genomic control was added where primers were run on fin-clipped genomic DNA samples as opposed to WT cDNA. This control tested for whether the PCR conditions were able to generate a product of the size of the unspliced RNA product as well as whether there was genomic contamination in the samples. The gel suggested that some splice-blocking might have occurred with the *lhx1a* MO 2.5µg/µL and 3µg/µL injections. A second band started to appear that was the same size as the un-spliced genomic control. However, there were also similar second bands in the controls with *lhx1b* primers suggesting that the secondary bands seen in both the experimental and control might be the result of genomic contamination. Genomic contamination can occur when preparing the RNA before performing the RT-PCR. In this case, some genomic DNA might have been carried through to the final RNA extraction and then used in the RT-PCR. As such, a secondary band corresponding to the unspliced genomic control would appear. With regards to the Lhx1b samples, secondary bands appeared in both the 1.0µg/µL and 2.5µg/µL injected cDNA samples. Since the control samples appeared normal (did not have bands at the size of the genomic DNA), this suggested splicing was being impacted in the samples injected with 1.0µg/µL and 2.5µg/µL of *lhx1b* MOs. The Lhx5 genomic control was not visible on the gel, suggesting my PCR conditions were not correct to a get a band at the correct length for the genomic DNA. Most importantly, the Lhx5 samples did not show

the same reduction in band strength as was seen in the initial injection. This suggested that either the original results or these results were incorrect perhaps because of a difference in injection size, an error in extracting the RNA for the RT-PCR, or a problem with the RT-PCR itself in either the first or second round of injections.

Therefore, I performed a third injection experiment to see if increasing the concentration of *lhx1a* MO would result in a more significant knock-down and to test which of the different results from injecting *lhx5* MO was more likely. The

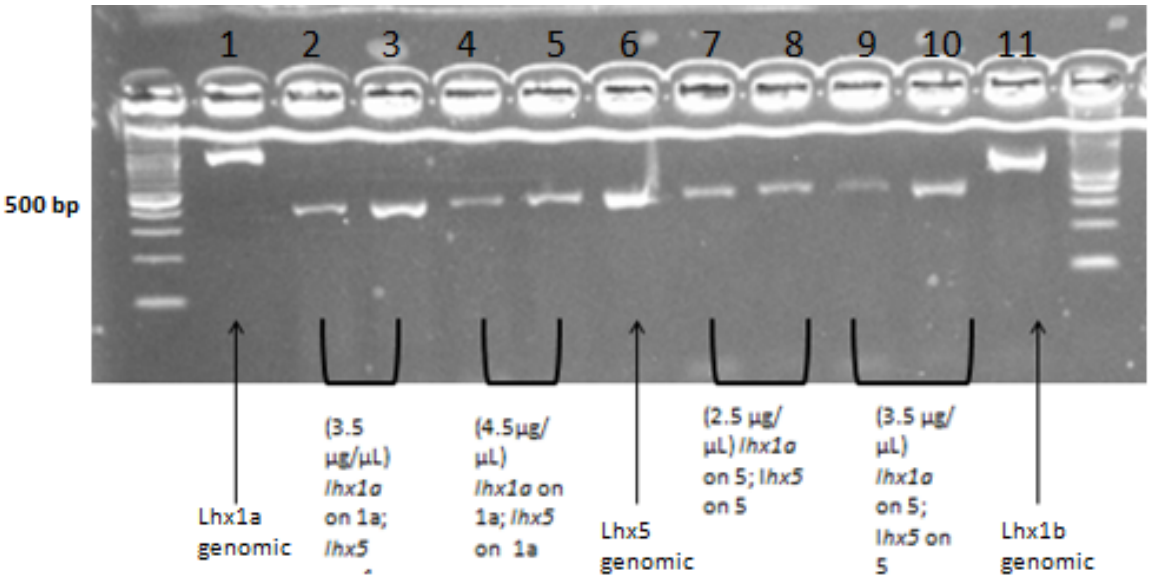

Figure 22. Third and final injection results with Lewis MOs. Lanes 1, 6, and 11 indicate genomic controls. Lanes 3 and 5 indicate control lanes for lanes 2 and 4, respectively. This is because lane 3 is *lhx5* primers on *lhx1a* S-B MO injected which essentially tests the viability of the cDNA. Lanes 7 and 9 are control lanes for lanes 8 and 10, respectively.

results are depicted in **figure 22**. By increasing the extension time, the *lhx1a* genomic control band became stronger. Unlike in the second experiment, the *lhx1a* injected samples did not demonstrate any knock-down. In this round of RT-PCR, the Lhx5 genomic control was visible. However, the spliced versus genomic

unspliced bands were almost identical in size which would make it hard to tell whether splicing had been blocked in an experimental sample. Finally, the results of the Lhx5 injections were consistent with what had been seen in the second experiment, namely that no significant knock-down was visible. Lhx1b injections were omitted in this round because the *lhx1b* mutant was successfully genotyped around this same time, eliminating the need to knock-down the transcription factor via injections.

As I hypothesized that Lhx1a/Lhx1b/Lhx5 might act redundantly and I might need to knock down all 3 genes to see a phenotype, I injected *lhx5* and *lhx1a* S-B MOs together to test whether the combination of the two at their highest previously injected concentrations would be lethal to the embryos. Since I had not seen a loss of splicing at these concentrations, I knew that I would have to increase the concentrations of each of these MOs individually; however, if the embryos died when the MOs were combined at their current concentrations then there was no point in continuing to increase the dosage. This is because I wanted to ultimately inject the MOs together to see a complete knock-down of Lhx1/5. In these injections (done twice) 95.0-100.0% of embryos died (see **table 5**).

Table 5. Injection results when *lhx1a* and *lhx5* MOs were injected together.

MO Injected	Concentration	Number Injected	Number Dead
lhx1a and *lhx5*	*lhx1a:* 4.5µg/µL *lhx5:* 3.5µg/µL 3 nl injected	35	100% (35)
lhx1a and *lhx5*	*lhx1a:* 4.5µg/µL *lhx5:* 3.5µg/µL 3 nl injected	40	95.0% (38)

Because those injections killed the embryos, I tried a new *lhx5* morpholino. This was a MO that had previously been used by Peng and Westerfield (2006). They found that when this *lhx5* S-B MO was injected the

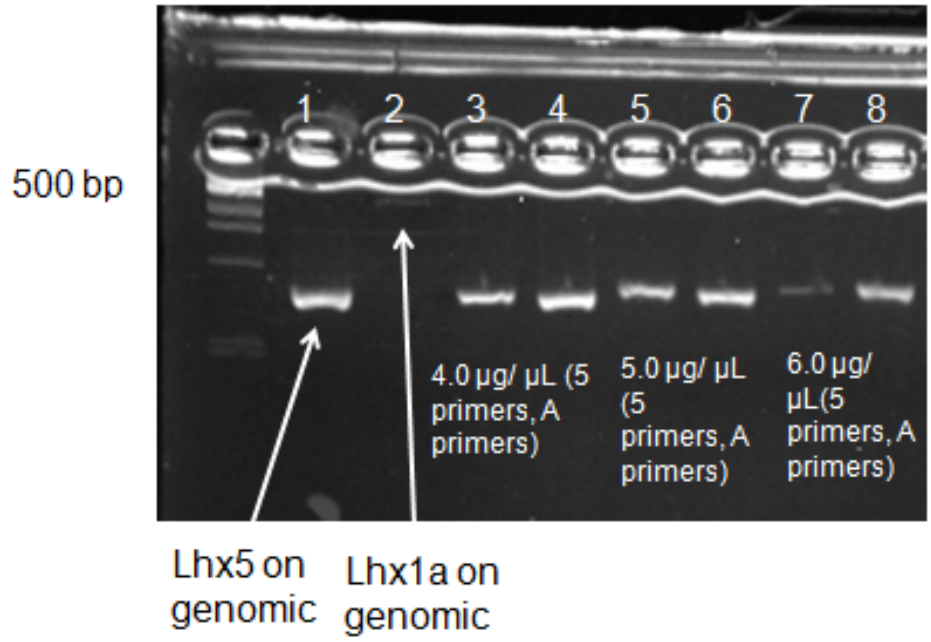

Figure 23. Initial injection results with Westerfield *lhx5* MO. Lanes 1 and 2 are genomic controls. Lane 2 is weaker because this sample requires a longer extension time. Lanes 4, 6, and 8 are control lanes because they have *lhx1a* primers on *lhx5* S-B MO injected samples. Lanes 3, 5, and 7 are experimental and have *lhx5* primers on *lhx5* S-B MO injected. As indicated by lanes 3,5 and 7, the bands become weaker as the concentration is increased. This suggests that splicing is being blocked.

embryos did not have rostral head structures and had misshapen posterior head

structures (Peng and Westerfield, 2006). In my previous injections, I had never seen any morphological phenotypes. Therefore, I thought that this Peng and Westerfield S-B MO which had previously resulted in such visible effects might be a better choice for my research. Based on Peng and Westerfield 2006, the initial concentration that I injected was slightly higher than that of the other MOs. In the first injection, both genomic controls appeared visible and identical to those previously seen in the other injection experiments. Furthermore, as the concentration of injected MO increased from 4.0µg/µL to 6.0µg/µL, there appeared to be a visible change in the band strength, suggesting that the levels of spliced RNA were being reduced (**figure 23**). In addition to the PCR results from this round of injections, I observed some morphological phenotypes in injected embryos at 24 hours post-fertilization. The embryos were necrotic with misshapen heads and eyes which was similar to Peng and Wsterfield who saw that *lhx5* MO injected embryos have smaller eyes and heads (Peng and Westerfield, 2006). This necrosis was most significant in the heads of the embryos. Retrospectively, it may have been a good idea to inject the new *lhx5* S-B MO in combination with a p53 MO; however, I did not initially do this as Peng and Westerfield had only injected the *lhx5 S-B* MO by itself. The somites in the tails were also not well-defined. Most notably, the tails were visibly kinked backwards. Finally, and interestingly, the embryos were hardly moving as compared to the wild-type controls.

To confirm my findings, I conducted a second round of injections with this new MO. This time, the highest concentration (6.0 µg/µL) used previously was reinjected as well at two higher concentrations, 6.5µg/µL and 7.5µg/µL. The results as shown in **figure 24** confirmed the initial findings: the splicing of *lhx5* seemed to be at least partially blocked. The complete absence of longer bands in **figure 24** might be because the PCR conditions were not ideal for the longer, unspliced PCR product. A second possibility is that when splicing is blocked this can make some RNAs less stable. In these cases, the RNA is present transiently and is unstable, possibly because this longer length is targeted for degradation.

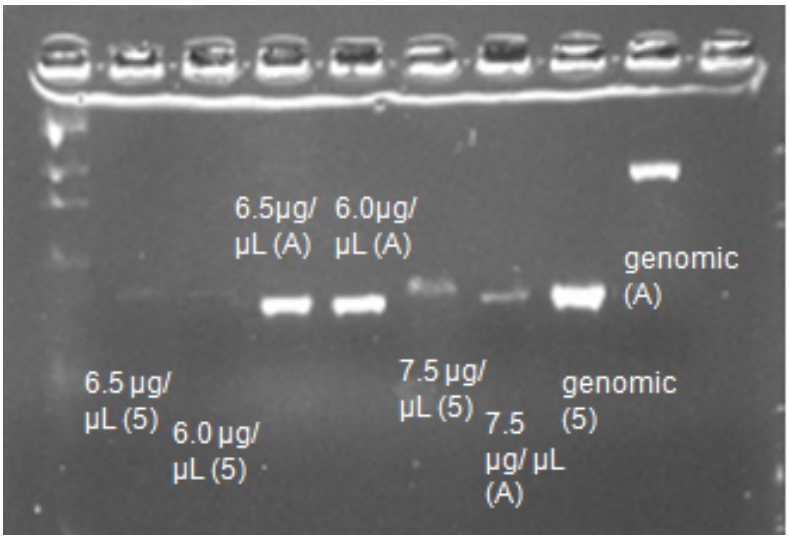

Figure 24. Second injection results with Westerfield *lhx5* MO. All samples were injected with *lhx5* S-B MO. The letter in parentheses indicates the primers used. For example, 6.5µg/uL (5) means that *lhx5* S-B MO was injected and *lhx5* primers were used.

As with the previous injection round, I observed morphological phenotypes. The embryos appeared identical to those in the previous injections with the new *lhx5* MO. Again, the most notable phenotypic changes were necrosis and misshapen heads along with the tail kinked backwards (**figure 25**).

Figure 25. Morphology of WT vs. *lhx5* S-B MO injected. Embryos at 24 hpf. The *lhx5* S-B MO injected embryo has a stunted, kinked tail and misshapen head structures.

When I injected the *lhx5*S-B MO a fourth time, I repeated the 7.5µg/µL concentration and also increased the concentration to 8.3µg/µL in order to see if the knock-down would be more severe. **Table 6** shows the results of my fourth round of injections.

Table 6. Results of fourth round of *lhx5* S-B MO Injections.

MO Injected	Concentration	Number Injected	Number Dead	Phenotypes/Comments	Control
lhx5 MO	7.5µg/µL 3 nL injected	55	14.5% (8)	85.5% (47) had misshapen heads and somites, had kinked tails.	N=20; looked WT
lhx5 MO	8.3µg/µL 3 nL injected	44	63.6% (28)	36.4% (16) had misshapen heads and somites, had kinked tails.	N=18; looked WT

I also conducted an RT-PCR in order to assess whether the *lhx5* MOs knocked-down Lhx5 (**figure 26**). My results from this RT-PCR suggest that when injected with higher concentrations of *lhx5* S-B MO that splicing is completely blocked. As seen in **figure 26**, the lanes with samples from injected embryos have no bands while those acting as positive controls still have bands. This shows that spliced RNA was eliminated in the experimental lanes. I used some of these injected embryos in an *in situ* to assess the impact of injections on neurotransmitters throughout the spinal cord and within the CiA cells in particular. However, the *in situ* was unsuccessful.

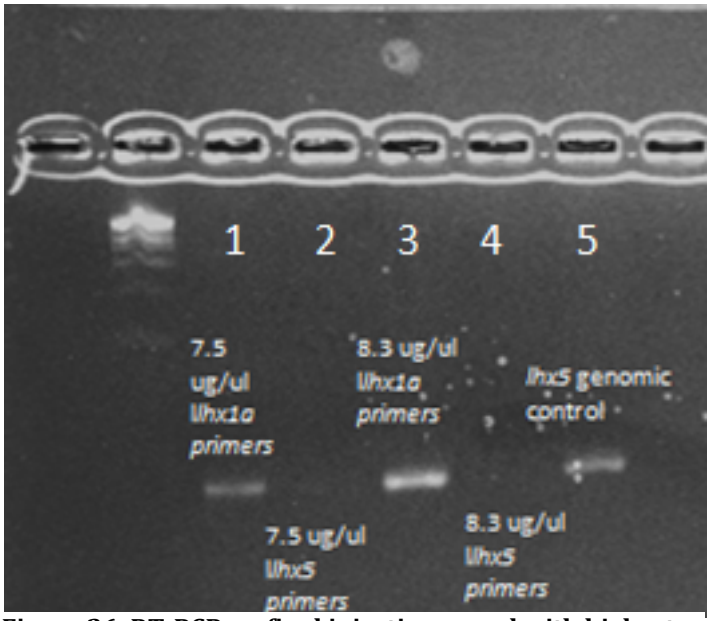

Figure 26. RT-PCR on final injection round with highest *lhx5* MO concentrations. In (control samples in lanes 1 and 3) where *lhx1a* were used on *lhx5* S-B MO injected samples there are still bands representing splicing. However, in (2 and 4) which are *lhx5* S-B MO injected samples with *lhx5* primers, there is a visible reduction (an absence in this case) of the bands, suggesting that splice was blocked. Finally, the genomic sample (lane 5) shows up normally, demonstrating that the *lhx5* primer is working appropriately.

After the *in situ* was unsuccessful, I decided to inject a final time in order to conduct another *in situ*. In this round, I again injected 8.3µg/µL of *lhx5* S-B MO. However, unlike in previous injection rounds, I injected into the *lhx1b*

homozygous mutant which finally laid embryos in addition to WT embryos. Although the *lhx1b* mutant finally laid embryos that I could use in an experiment, all of these injected embryos subsequently died. It should be noted that I only saved two of these *lhx1b* mutant embryos to use as a control. Because only 24 embryos were laid (**table 7**), I was eager to use as many as possible for my actual experiment. Although these two control embryos survived, I cannot be completely sure whether the death was due to my injections or because these mutant embryos were sick prior to my injections. I could only be sure if I had more embryos as a control. Furthermore, many of the WT injected embryos also died. In the previous round of injections with 8.3µg/µL of *lhx5* S-B MO, 63.6% (**table 6**) of the embryos died compared to the 88.2% (**table 7**) that died this time. The increased number of dead embryos could be due to the fact that I injected more embryos during this last injection round or that the concentration was actually a bit higher than I thought. The surviving embryos from this injection were used in an *in situ*.

Table 7. **Results of final round of injections with *lhx5* S-B MO.**

MO Injected and fish line	Concentration	Number Injected	Number Dead	Phenotypes/Comments	Control
lhx5 MO into *lhx1b mutant*	8.3µg/µL 3 nL injected	22	100.0% (22)	All embryos died.	N=2; appeared normal
lhx5 MO into WT	8.3µg/µL 3 nL injected	102	88.2% (90)	Embryos displayed the same phenotypes as my other injected embryos (kinked tails and misshapen heads).	N= 40; all appear WT

Analyzing expression of Pax2 and Neurotransmitter Markers in embryos with reduced Lhx1/5 function

I did an *in situ* on the embryos injected with 3.02 µg/µL each of *lhx1a, lhx1b,* and *lhx5* S-B MO and 3.84 µg/µL p53 MO. Unfortunately, these embryos were rather fragile and did not make it through the procedure. This is likely because they were in proteinase K too long. Proteinase K increases the permeability of the membranes in the embryo so that the probes can enter the cells and bind to RNA. However, if left too long, the proteinase K can make embryos fragile. The amount of time I used was appropriate for WT embryos, but if the injected embryos were more fragile to begin with, it may have been too long for them. When I completed the *in situ* and went to analyze the results, I found that none of the embryos had made it through the protocol. Instead, I found pieces of the embryos and yolks. This is why I think that the proteinase K time was too long.

I also performed an *in situ* on my embryos injected with 7.5µg/µL *lhx5* S-B MO as well as on the embryos from my most recent RNA injection (**table 3**). In this *in situ*, I used two glycinergic markers, *glyt2a* and *glyt2b,* which correlate with transporters that are required to reuptake glycine (Higashijima et al, 2004). Based the aforementioned work in the mouse (Pillai et al., 2007), I hypothesized that Lhx1/5 might be required to specify an inhibitory neurotransmitter fate in CiAs which could be tested using the *glyt2a* and *glyt2b* as they are inhibitory markers. Additionally, I utilized a *pax2.1* probe which encodes for the *pax2.1*

transcription factor (Pfeffer et al., 1998). However, the staining that appeared in my *in situ* was nonspecific and primarily background. This meant that the probes had not worked and consequently I had no results from this *in situ*.

As such, I performed a final *in situ* hybridization with a *pax2.1* probe that had been kindly prepped and provided by another student in the Lewis lab, Gisella Rodriguez-Larrain. I chose to use this probe because I knew that it had worked for Gisella in the past. I also knew this would be the last *in situ* I would have time to perform. I hope that using a *pax2.1* probe would enable me to answer the question of how the Lhx1a/1b/5 and Pax2/8 families of transcription factors interact in the zebrafish spinal cord. Furthermore, I was attempting to answer whether Lhx1/5 and Pax2/8 maintain one another. As such, I expected to see a difference in the number of *pax2.1* expressing cells in the WT as compared to the control. The small dots, most clearly seen in **figure 27C**, are actually individual cells that express Pax. **Figure 27C** demonstrates that the *in situ* was successful because specific staining can be seen in individual cells throughout the spinal cord.

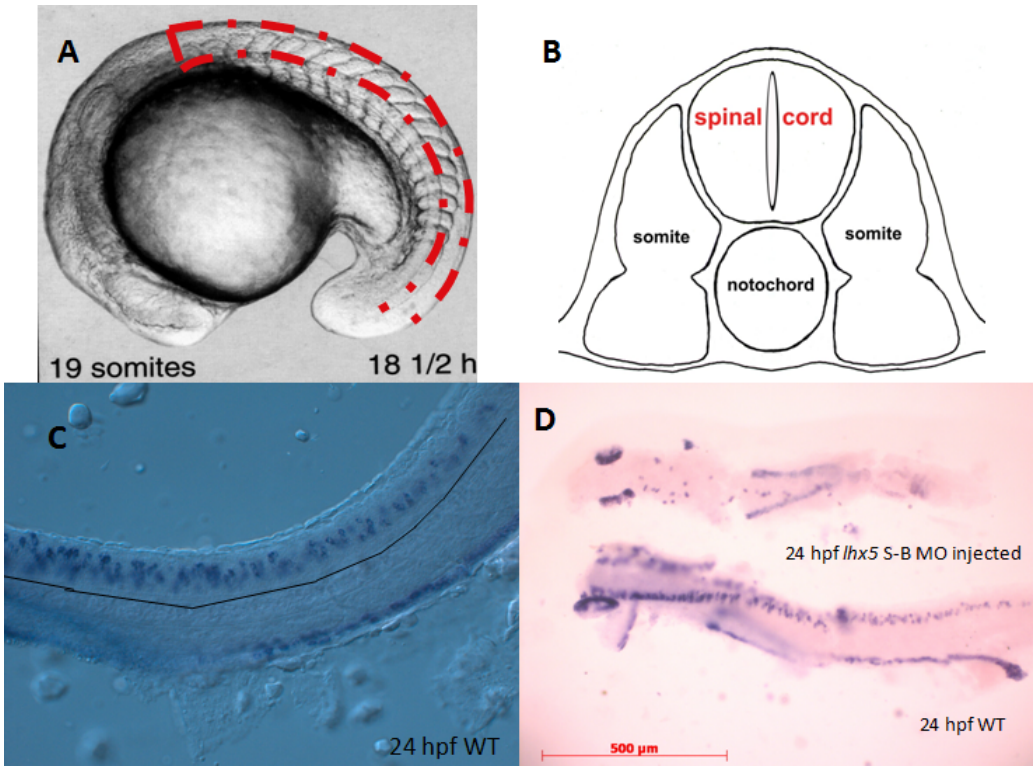

Figure 27. *pax2.1 in situ* results on WT compared to injected embryos. (A) and (B) were kindly provided by Dr. Lewis. (A) indicates the portion of the spinal cord that our lab analyzes. The red dotted lines surrounds the midline and extends upwards towards the most ventral cell populations. (B) is a cross-section of the spinal cord and indicates the location of the spinal cord relative to the somites and notochord in the zebrafish. In pictures (C) and (D), embryos from the *in situ* I performed are situated with their heads towards the left and their tails to the right. (C) is a picture of a WT embryo I used in my *in situ* with the *pax2.1* probe. The black line indicates the midline of the embryo. This picture was taken at 40x magnification. This picture was included to demonstrate that the probe had in fact worked and to show what an ideal picture of an *in situ* looks like. If my injected embryos had been less fragile, I would have taken a similar picture with them. (D) is a comparison between a *lhx5* S-B MO injected embryo and a WT both with the *pax2.1* probe. The orientations of the embryos are slightly different, but I included this picture so that the obvious differences in the amount of cells expressing *pax2.1* could be visualized. The orientation was unable to be changed because the injected embryo continued to deteriorate as I tried to reorient it. (D) was taken at 10x magnification.

The embryos I used in this *in situ* were particularly fragile as they had been injected with 8.3µg/µL *lhx5* S-B MO and their heads and tails were misshapen, as shown in **figure 25**. As such, these embryos made it through the *in*

situ protocol, but when I was removing the yolk to take pictures, I found that the embryos deteriorated and were especially hard to mount to take a picture. Consequently, **figure 27D** is not ideal, but still shows that the number of cells expressing Pax in the *lhx5* S-B MO injected appears to be significantly less than in the WT control. This was the case for all five *lhx5* S-B MO injected embryos that made it through the *in situ*. The results from my *in situ* suggest that Lhx1/5 are related to Pax2/8 in the zebrafish spinal cord which is a novel finding. Based on the fewer Pax2/8 expressing cells seen in **figure 27D**, it is possible that Lhx1/5 help to maintain Pax2/8 expression in the spinal cord. This experiment will need to be repeated so as to obtain a more ideal picture and to conduct actual cell counts. Counting the cells within the spinal cord will enable us to quantify the difference that I believe can be seen in these pictures (**figure 27D**). Although this experiment would need to be repeated and cell counts would need to be done, this is an exciting result because the effects of knocking-down Lhx5 on Pax2/8 in the zebrafish spinal have never been studied and it was unknown whether Lhx1/5 and Pax2/8 were related in the zebrafish spinal cord.

Discussion

lhx1b Mutant

Initially, we were unaware that the *lhx1b* fish sent to us from the Sanger institute were the progeny of a heterozygous incross as the usual practice is to send an outcross of a heterozygote. Therefore, it seemed surprising when my PCR results suggested that one of the adult fish from this line that I identified was a homozygous mutant. We were concerned that this result might have been due to a false positive, with the restriction enzyme not cutting the DNA (because WT DNA should cut and mutant should not). However, sequencing revealed that this fish was indeed a homozygous mutant. This is an exciting result as it shows that the *lhx1b* mutation is embryonic viable.

By creating a PCR and restriction enzyme protocol to identify the *lhx1b* mutants, I established a way to identify *lhx1b* mutants in the future and accurately separate homozygous and heterozygous fishes from wild-type, without having to send DNA from each fish to sequence. However, given the risk of false positives (if the DNA does not completely cut), it still might be worth sequencing identified heterozygous and homozygous fish. This mutant line is especially valuable in that it eliminates the need to inject *lhx1b* MOs or RNA constructs, making the knock-down of all 3 Lhx1/5 genes in zebrafish much easier to perform without nonspecific toxic effects.

Some of the fish that I identified as wild-type might actually be heterozygous for the *lhx1b* mutation as they have a weak longer ("mutant like") band (example lanes 6 and 11 **figure 16**). Fish that had any ambiguity were compared to the known WT control. In several instances the WT control fish also demonstrated weak longer bands (as seen in the last lane with a WT sample in **figure 16**). This could be because of an incomplete digest in the reaction which might occur if there is an excess of DNA, leading to the presence of a second uncut band. Therefore, fish with a weaker longer band were considered WT and fish were only identified as heterozygous if they had two distinct bands, with both exhibiting equal brightness. In the case where a "WT" fish had a second band, the shorter band was clearly more distinct than the longer, consistent with the idea that a small amount of DNA did not cut. Interpreting the results in this way has the advantage that we are less likely to wrongly identify a wild-type as a heterozygous mutant, which could have dire consequences for our experiments. If the bands are not equally strong, then I identified the fish as a wild-type. Misidentifying wild-type fish as heterozygous would be much more detrimental that misidentifying a heterozygous fish as WT because if we use a WT fish in an experiment where we thought we were using a *lhx1b* heterozygous mutant then *lhx1b* would not actually be knocked-down. While misidentifying a heterozygous fish as a WT fish would be a loss because it would mean I have fewer mutant fish to work with, I would not be compromising my experiments. In addition, the sequencing results as well as the results of the chi square test demonstrate that this

rationale for distinguishing between WT and heterozygous fish was probably accurate.

RNA fusion constructs as a method of impacting Lhx1/5expression

This portion of the experimentation was perhaps the most frustrating in that the injections appeared to be yielding significant results, but then I found out that the enzyme used initially, BamHII, was cutting in the incorrect location. The fact that I still saw phenotypic effects from the injections can be attributed to a variety of possible causes. It is possible that the injected RNA still had either partial function or dominant active or dominant negative function and, therefore, was managing to have some effect on the embryos. However, it is more likely that the RNA injected was simply sickening the embryos and had no specific effect on any of the Lhx1/5 transcription factors. At times, injections can be toxic to embryos simply on the basis that too much foreign material is being introduced.

When the correct enzyme was used to linearize the DNA, the embryos mainly appeared morphologically WT. This suggested that either there might be an issue with the RNA itself or there might not be any morphological effect from interfering with Lhx1/5 activity. However, given that the literature suggested that injecting fusion constructs did result in phenotypic changes to the embryos, I remade the RNA. Unfortunately, the injection results from the newly prepped RNA contradicted one another. In one round, there appeared to be a morphological phenotype whereas the second injection round yielded no morphological phenotype. It is possible that these findings actually recapitulate

the earlier findings, with the first RNA synthesis with the correct restriction enzyme, that the RNA fusion construct had no morphological effect, meaning that there was nothing "wrong" with that RNA after all. However, it is also possible that the second injection experiment was anomalous and that there is a real effect. A reason for the anomaly could be if I introduced an RNAse (RNA-degrading enzyme) into the RNA injected thereby degrading the RNA. Furthermore, it is theoretically possible that I pipetted inaccurately and therefore injected a different amount of RNA than calculated. The results from the third injection round (**table 3**) were consistent with the second round in that the embryos did not exhibit a morphological phenotype. Based on these results, I concluded that either these injected RNA concentrations do not cause a morphological phenotype and the effect can only be seen via *in situ* hybridization or that the concentration must be increased to achieve a phenotype. Although I performed an *in situ* on the RNA injected embryos to assess whether the former or the latter was the case, the *in situ* was unsuccessful and failed to yield any specific staining. As such, the injections will need to be performed again and another *in situ* conducted in order to assess the effects of injecting this RNA construct on Lhx1/5.

ATG MOs as a tool for knocking-down Lhx1/5function

The embryos obtained from the ATG MO injections were quite fragile. Even once the middle concentration was injected, the surviving embryos were visibly disturbed and exhibited distorted head structures, misshapen tails, and slight necrosis. However, I dechorionated them and put them through the *in situ*

protocol. Despite my best efforts, the embryos did not make it through the *in situ* and only the yolks were left at the end of the experiment. This suggested that the proteinase K time should have been reduced so as to allow the probes to permeate, but reduce the impact on the embryos themselves. Although I did not have time to return to the ATG MOs before completing my thesis, this area is certainly one that should be addressed by another researcher in the future.

S-B MOs as a method for impacting Lhx1/5expression and function

When injecting the original S-B MOs, as these had not been used before, I chose to inject at higher concentrations and titrate down so as to more easily discover the maximum effect that the morpholino might have. If I had initially tried a low concentration and had not seen splice blocking it would have been necessary to increase the concentration until all of the embryos died in order to determine the maximum dose and most severe effect. Furthermore, I injected each of the three MOs (*lhx1a, lhx1b, lhx5*) individually in order to be sure how much was required to inhibit splicing of each individual transcription factor gene before combining them. However, I also injected the two MOs together knowing that the amount required to knockdown the function of one transcription factor might be too toxic when combined and injected with the other two MOs. This was in fact the case when two splice-blocking MOs (*lhx1a* and *lhx5*) were injected together at the maximum single injection experimental concentrations that I had tried. As the mix of all two was lethal to the embryos and yet for *lhx1a* and *lhx5* I had not seen an effect on splicing at these concentrations, I tried switching to

another *lhx5* S-B MO that had been reported to work (Peng and Westerfield, 2006). I tried this MO initially at concentrations suggested by Peng and Westerfield and therefore did not try the method I had used for the other S-B MOs where I titrated the concentration.

In my RT-PCR experiments I had very variable results. There are several possibilities for these inconsistencies. With regards to the morphological variation, one possible reason is that knocking down a single transcription factor might have variable or even imperceptible results because the other two transcription factors act redundantly and could compensate for the loss. However, this applies only to morphological phenotypes. Other reasons may account for the differences in splice-blocking PCR results. For example, I hypothesized that the PCR band for the RNA in question might get longer or a second unspliced band might appear if RNA splicing was blocked. This is because splice-blocking means the introns are not removed and the RNA product is therefore longer. Consequently, the product would run at a different position on the gel. Alternatively or in addition, the WT band might appear fainter, or disappear completely if inhibiting of splicing caused the RNA to be unstable. Another possibility was that the gel might appear blank. This could have been the result of several possibilities. First, the primers might not have worked in the PCR, resulting in a lack of product. Second, one of the reagents in the RT-PCR might not have worked or splicing was blocked and the RNA was targeted for degradation. To try and distinguish between these possibilities, I ran a positive control in parallel to each experimental sample in order to fully understand the

results. In order to control for the first possibility in particular, I ran two separate reactions with each cDNA sample. In each case, the corresponding primer set was used (if I injected *lhx5* MO then I used *lhx5* primers), but a second reaction was also run in which another set of Lhx primers was used as a control to test that cDNA was present. I expected that a band would always appear on the gel in the lane representing where the non-corresponding primer set was used as this region should be able to be amplified and should never be knocked down in the absence of the corresponding MO.

My initial MO injection results seemed promising, suggesting a partial knock-down of *lhx5*. However, the second and third experiments were not consistent with this finding. It is possible that the results seen in the first injection round were actually the result of some contamination that impacted cDNA synthesis. If something had damaged the sample, the band on the gel might appear weaker even if the MO was not knocking-down *lhx5*. However, arguing against this explanation, the control bands appeared normal and the effect seemed to become more significant as the concentration was increased. These anomalies can perhaps be attributed to some other error in the PCR. The absence of any apparent knock-down in the subsequence two experiments suggested that the possible knock-down observed in the first injection was not a true reflection of the effect of the MO. This demonstrated the importance of repeating experiments to check that the results are reproducible.

With regards to *lhx1a*, it looked like when I injected a higher concentration in the second round of injections a knock-down might be occurring.

However, when the 3.5μg/μL concentration was repeated in addition to a higher (4.5μg/μL) concentration, the results showed otherwise. This suggests that the results seen in the second injection round were in fact not accurate. It is possible that some of the samples were not completely inserted into the wells of the gel and instead settled in neighboring wells. This might also explain the presence of secondary bands for the control samples in the second injection round. Moreover, it is possible that these samples were contaminated with genomic DNA. As such, it was concluded that the *lhx1a* MOs did not have a significant effect on Lhx1a splicing.

Finally, when I increased the concentration of Lhx1b in the second round of injections, it appeared a knock-down was occurring. This was perhaps the strongest instance of a possible knock-down as the second bands were appearing at the un-spliced genomic size and no longer bands were seen in the control samples. The absence of longer bands in the control sample shows that there was no genomic contamination. Moreover, the band corresponding to the spliced *lhx1b* sample was more decreased in the 2.5 μg/μL injected sample than in the 1.0 μg/μL sample. This suggests that as the concentration of injected MO was increased the splicing was further inhibited. Furthermore, the secondary bands corresponding to the un-spliced *lhx1b* samples were at the same size as the un-spliced genomic control for *lhx1b*. Although the results looked promising, the *lhx1b* MO was not injected during the third round of injections because I had successfully genotyped the *lhx1b* mutant. Instead, I focused on finding the correct

concentrations of the remaining two MOs. Regardless, it appears that the 2.5 µg/µL injection of *lhx1b* was significantly impacting *lhx1b* RNA splicing.

Because the embryos all died when I injected a mix of *lhx1a* and *lhx5* S-B MOs at their highest tested individual concentrations (**table 5**), I decided to try a new MO. The reason that 2 embryos lived (**table 5**) might suggest that these embryos received a smaller injection or that they were on the side of the dish and had accidently not been injected at all or that less of the MO had traveled from the yolk into the embryo. Since all of the embryos died when I injected the mix of *lhx1a* and *lhx5* MOs, I tested a new *lhx5* MO which was used successfully by Peng and Westerfield (2006). The initial injection showed possible knock-down at the highest concentration of 6.0µg/µL. Furthermore, the control samples showed normal bands, indicating that the PCR reaction was working and the cDNA was present. The reason for the weaker *lhx1a* genomic band in **figure 19** is probably that this longer PCR product requires a longer extension time. However, I omitted the extended time because it had the potential to interfere with the results of the other samples. Although the band is weak, it is visible.

My second round of injections with the new *lhx5* MO confirmed my initial findings. As compared to the controls, the 6.0 µg/µL, 6.5 µg/µL, and 7.5 µg/µL experimental samples yielded weaker bands which indicated a knock-down effect. Consequently, Lhx5 can be at least partially knocked-down with a MO injection at a of concentration of 6.0-7.5µg/µL and a volume of 3 nl.

Furthermore, as mentioned earlier, I also observed a visible phenotype in these knock-down embryos which was a tail kinked backwards. Additionally, the embryos exhibited severe necrosis in their heads which were misshapen with the eyes and mid-brain hindbrain boundary not visible. The phenotype was significant in that it resembled the findings of Peng and Westerfield (2006) who found that Lhx5 S-B MO injections resulted in "embryos that lack the most rostral part of the head [and] posterior head structures". Moreover, MO injected embryos "later develop small heads with small eyes," a finding similar to my results (Peng and Westerfield, 2006). The reason that I decided to try a new MO was because, as stated previously, I thought it was key to knockdown all three genes together given that they are expressed by the same cells and may act redundantly like Pax2a/Pax2b/Pax8 (Gustavo Cerda-Moya, 2011; Batista and Lewis, 2008).

The results from my fourth round of injections (**table 6**) suggest that the phenotype continues to get more severe as the concentration of *lhx5* MO is increased. More embryos died in this round of injections as well, suggesting that I was approaching the upper limit for these injections. My results demonstrate that the splice blocking does get more pronounced as the concentration is increased. The bands corresponding to the WT spliced RNA in the injected embryos completely disappeared as shown in **figure 25** while the control samples yielded normal bands on the gel. This means that the MOs successfully affected Lhx5 RNA splicing.

In my final round of injections, I injected into *lhx1b* homozygous mutant embryos (**table 7**). Until this point, the *lhx1b* mutant had never laid any embryos for me to inject. Injecting into these embryos was exciting in that *lhx1b* was already knocked-down and I therefore expected that the phenotype might be more severe when I injected the *lhx5* S-B MO. I expected this because, as previously mentioned, Lhx1/5 are co-expressed in the same cells and therefore might act redundantly (Cerda-Moya, 2011). All of the *lhx1b* mutant embryos died as a result of my MO injections (**table 7**), suggesting that either the embryos were naturally less viable because it was the first time I had gotten the mutant to lay or that the concentration of injected MO was too high given that *lhx1b* was already knocked-down. However, it is also possible that fully knocking-down both Lhxlb and Lhx5 is embryonic lethal. This experiment would need to be repeated with varying *lhx5* S-B MO concentrations in order to fully assess the reason these embryos died.

Analysis of gene expression in embryos with reduced Lhx1/5 activity

Although my original intention was to investigate the function/s of the Lhx1/5 transcription factors, most of my time was spent testing various knock-down strategies. However, I did attempt to investigate the function/s of the Lhx1/5 transcription factors through *in situ* hybridization. The first two *in situ* experiments that I conducted were rather fruitless in their results. As mentioned, in the first *in situ* many of my embryos were lost, possibly as a result of subjecting them to proteinase K for too long which can damage already fragile embryos. In the second *in situ*, I found that my probes failed to yield any specific

staining which prohibited me from investigating gene expression in embryos with reduced Lhx1/5 activity. However, my most recent *in situ* had significant results. Although the pictures obtained were not ideal, I did see a reduction in the number of cells expressing Pax2/8. The differences between the embryos injected with 8.3µg/µL *lhx5* S-B MO and the WT embryos were drastic. This is exciting because the results have the potential to recapitulate findings in the mouse that indicated that Lhx1/5 help maintain Pax2/5/8 (Pillai, 2007). Furthermore, it suggests that Lhx1/5 might have a similar function in the zebrafish. Additionally, my findings suggest that there is a relationship between Lhx1/5 and Pax2/8 which has never been investigated or proven in the zebrafish.

Conclusion

My research has successfully assayed the tools available to knock-down Lhx1/5 in order to test the function of these transcription factors in the zebrafish spinal cord. One such tool was the *lhx1b* mutant line, which can now be successfully genotyped using PCR and specially designed primers as well as a restriction enzyme digest reaction utilizing the enzyme DraI. The ability to identify *lhx1b* mutants is a novel contribution as is my discovery that this mutation is not embryonic lethal. These discoveries have important implications for future research on this topic in that fewer knock-down reagents will need to be injected into the embryos in order to test the effects of knocking down all three of the Lhx1/5 transcription factors. Ultimately, injecting fewer reagents should reduce nonspecific toxic effects on embryos.

This project also significantly increased our understanding of how to knock down the Lhx1/5 transcription factors. Previously, it was unknown which were the best methods to employ. However, it now seems that Lhx1/5 MOs are the best option. Although the ATG MOs yielded promising results, this direction was not fully explored. However, with the results at hand, it currently appears that splice-blocking MOs are the most efficient choice for knocking-down Lhx5. In contrast, the RNA results at this point seem inconclusive. Experimentation with RNA fusion constructs will also be repeated in the future. Finally, *in situ* hybridization results suggest a relationship between Lhx1/5 and Pax2/8 in the zebrafish spinal cord and that Lhx1/5 might be necessary in order to maintain

Pax2/8. This result was novel as the exact relationship between Lhx1/5 and Pax2/8 had never been explored in the zebrafish.

Future Directions

Although my project yielded many useful results, there remains a great deal of work to be completed before the questions are fully answered. One potential direction would be to also utilize either GFP lines and/or *noi* (no-isthmus mutation) fish lines to investigate the effects of knocking-down *lhx1a/1b/5* on the zebrafish spinal cord. The GFP lines would enable the examination of morphological changes in specific interneurons within the spinal cord whereas the *noi* line would allow for the study of the effects of knocking down the Lhx1/5 in the absence of *pax2a*. To test whether Lhx1/5 and Pax2/8 have any independent functions in specifying neurotransmitter fates, it would be necessary to also knock down Pax2b and Pax8 and determine if the phenotype is more severe than either Pax2/8 triple knock-down or Lhx1/5 triple knock down embryos. This would be difficult to do without the embryos getting very sick as it would involve injecting four different MOs. This would be an important experiment because of the potential interactions of the Pax and Lhx transcription factor families. With regards to injections, the *lhx1a* ATG MO would be an excellent place to continue work as it is still not clear if this is an effective way of knocking down *lhx1a* or not. Although, as this is an ATG MO, it is difficult to test this without an antibody. Based on the findings mentioned briefly in this paper, there is the potential that *lhx1a* ATG and *lhx5* S-B MO injections into *lhx1b* mutants would yield novel findings. In order to target all three genes, the *lhx1a* ATG MO could be injected into the *lhx1b* mutant fish in combination with the *lhx5* S-B MO that

has already been shown to work. My results suggest that our existing *lhx1a* S-B MO is not effective, but new S-B MOs for this gene could be tried. In terms of the RNA fusion construct injections, the injection of Lhx5Vp16 and Lhx5GFP should be tried as this could lead to significant findings. These constructs were never re-prepped once the enzyme issue had been resolved as I ran out of time. Finally, the most recent *in situ* experiment should be repeated with embryos injected with 8.3μg/μL of *lhx5* S-B MO as compared to WT. This experiment will hopefully recapitulate the aforementioned findings (**figure 27C-D**).

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Capstone Summary

Located in the vertebral column, the human spine is responsible for controlling many bodily functions that healthy individuals take for granted. In addition to regulating standard body movements and organ operations, the spinal cord receives sensory input from the rest of the body about pain and touch. Various neurological conditions including neuro-degeneration as well as spinal cord injuries result in deficits in these functions. These types of ailments are particularly common. A recent survey estimated that 183,000-230,000 people in the United States alone suffer from spinal cord injuries (NIH Medline Plus). Furthermore, approximately 11,000 new cases of spinal cord injury present each year in the United States alone (NIH Medline Plus). Oftentimes these injuries are the result of accidents, violence, and sports-related injuries and happen to people in the prime of their life (NIH Medline Plus). Currently, there are few treatments for these conditions, partly because we know very little about how a fully functioning spinal cord is constructed. This means that studying spinal cord development, specifically neuron specification and patterning, the subject of my thesis research, should ultimately be useful for developing better treatments for people with spinal cord injuries and diseases.

Zebrafish are a prime model organism in which to study spinal cord development for a number of reasons. Most simply, zebrafish are a relatively inexpensive choice when compared to other standard vertebrate model systems like mouse and a large quantity of embryos can be easily obtained from adult fish

without harming the parents. In addition, zebrafish have transparent embryos that grow rapidly outside of the mother. These characteristics allow us to examine central nervous system (CNS) development, including spinal cord development, in live embryos.

Zebrafish are also a useful model organism because the cell shape (morphology) of individual nerve cells (neurons) can be examined in live embryos. Neurons are the communicating cells of the CNS. Interneurons are a type of neuron that helps to relay messages between other neurons and they are essential components of most CNS neuronal circuits. My project has focused on interneurons. In zebrafish embryos, interneuron morphology can be examined by labeling specific cell types with fluorescent proteins such as GFP. This is difficult to do in mammals because the embryos develop inside the mother and are not transparent. It is easier to study interneurons and identify them as a particular cell type with a specific function in zebrafish. This is because, in addition to it being easier to visualize individual interneurons in the externally-developing transparent zebrafish embryos, the zebrafish spinal cord has relatively few different types of interneurons in comparison to mammals. Furthermore, each interneuron type can be easily recognized by its particular morphology. For example, the interneuron type studied in my research, CiAs, have a distinct interneuron shape which distinguishes them from the others. In addition to the unique morphologies, each interneuron type has a distinct neurotransmitter fate. Neurotransmitters are the chemicals within the central nervous system that help neurons to communicate. Finally, it is easy to knock down individual gene functions with mutants and other

methods in zebrafish, which was particularly important for my project. Various reagents can be injected into an embryo via a thin needle in order to knock-down gene function. The transparency and rapid development of zebrafish embryos enables this experimentation to be conducted relatively easily.

My project focused on V1 neurons which form in the ventral (abdominal-side) spinal cord. In zebrafish, V1 cells, which regulate/enable fast movement, develop into CiAs or Circumferential Ascending interneurons (Goulding, 2009). During spinal cord development, individual cells take on a particular identity depending on their location in the spinal cord. Based on this cellular identity, the cells have unique characteristics and functions and later develop into distinct interneuron types. For instance, distinct ventral interneurons control different aspects of movement in the zebrafish

CiAs have several functions including motor control and sensory gating. Sensory gating is the ability to screen for irrelevant sensory input and prevent it from interfering with other neurological processes (Burgess and Granato, 2007). Various research projects have already been conducted with V1 cells in other organisms such as frogs and mice. Results from tadpoles demonstrated that the cells correlating to CiAs and V1 cells, called aINs in frogs, are implicated in swimming behaviors and sensory gating (Li, et al., 2004). Similarly, V1 cells in mice are required for faster movements. When V1 cells were ablated, mice had difficulty moving at faster speeds although movement was still possible (Gosgnach et al., 2006). The CiA cells in zebrafish are functionally similar to the V1 cells seen in mammals (Higashijima, 2004), which makes them an appropriate

choice for study considering my goal of aiding in the treatment of spinal cord injuries in humans.

Each interneuron type expresses a unique combination of transcription factors. Transcription factors are proteins that bind to DNA and either promote or inhibit expression of particular genes. Therefore, investigating the roles of transcription factors is key to better understanding how interneuron characteristics are genetically specified. Several transcription factors are expressed consistently in all vertebrate V1 cells and my research focused on a few of these, namely Lhx1a, Lhx1b and Lhx5 (often referred to here as Lhx1a/Lhx1b/Lhx5). My main questions were: What are the best reagents to test Lhx1a/1b/5 function? Can I identify ways to work with a new Lhx1b mutant line? and What is/are the function/s of Lhx1a/1b/5 in the zebrafish spinal cord? In order to answer these questions I had to use a variety of experimental techniques.

In my research project I primarily used morpholinos (MOs) and RNA fusion constructs to knock-down the function of specific transcription factors. Morpholinos are synthetic agents that mimic the actions of real RNA; however, whereas RNA is the template for creating proteins, MOs stop the production of protein. RNA fusion constructs are RNA that has been made from DNA that can be injected into an embryo. This RNA can either enhance or repress the expression of normal genes because it acts as the new template for creating proteins. Both MOs and RNA fusion constructs are reagents that can be injected into the zebrafish embryo to knock-down specific protein functions. To “knock-down” function in these cases, means to eliminate or reduce the presence or

activity of a particular protein. By knocking-down Lhx1a/1b/5, I aimed to assess what their functions were within the spinal cord because the processes and/or gene expression that they control should theoretically be stopped in their absence. I then used a specific experiment called reverse transcription polymerase chain reaction (RT-PCR) to determine whether the transcription factors had indeed been knocked-down. RT-PCR is a molecular biology tool that enables the creation and amplification of DNA from RNA and it can be used, as it was in this case, to assess the expression of certain RNAs (and therefore, by inference, the expression of the proteins encoded by those RNAs). Finally, I used *in situ* hybridization to examine the effects of reducing Lhx1/5 function in the spinal cord. *in situ* hybridization is an experiment that identifies which cells are expressing particular genes. I also utilized the molecular biology technique of polymerase chain reactions (PCRs) to identify *lhx1b* mutants, which are fish in which the *lhx1b* gene is mutated and therefore inactive. Similar to the RT-PCR explained above, PCRs are a molecular biology tool used to amplify specific regions of DNA. However, in this case, DNA, not RNA, is used as the initial template.

My research demonstrated that certain reagents, namely MOs, are more effective for knocking-down Lhx1/5 in the zebrafish spinal cord than RNA fusion constructs. I also developed a way to successfully identify *lhx1b* mutants. Developing a method to successfully identify *lhx1b* mutant fish is important because it eliminates the need to knock-down *lhx1b* with reagents such as morpholinos or dominant negative constructs, which are inherently more variable and more likely to have non-specific effects than a mutation. These results are

important because they will enable future researchers to perform more in depth experiments concerning the function of these transcription factors. Without a successful knock-down, neither I nor anyone else would be able to ultimately investigate the function of Lhx1/5. With a better understanding of the knock-down reagents comes the ability to determine the functions of Lhx1/5. Being able to better understand these transcription factors and the roles they play in the spinal cord has the potential to translate into important medical applications to help spinal cord regeneration. Preliminary data suggests that Lhx1/5 are involved in maintaining another family of transcription factors, Pax2/8. However, this relationship will need to be further investigated and other experiments will need to be performed to fully understand Lhx1/5 function.

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