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Analyzing the Roles of scl and gata3 in Zebrafish Spinal Cord Interneuron Specification and Function

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Analyzing the Roles of *scl* and *gata3* in Zebrafish Spinal Cord Interneuron Specification and Function

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

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Honors Capstone Project in Biology

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Abstract

My project focuses on the V2 cells of the zebrafish spinal cord. The V2 cells are an unusual class of spinal cells because they all originate from molecularly indistinguishable p2 progenitor cells in the spinal cord. However, as these cells become post-mitotic and differentiate, they start to express different transcription factor genes that allow them to initially develop into two sets of molecularly distinct cells. As differentiation continues, at least one more class of molecularly distinct cells develops.

Just as in other vertebrates, in zebrafish the V2 cells differentiate into at least two functionally distinct classes of cells, specifically, the *vsx1* (also called *chx10*) and *vsx2* expressing V2a cells and the *gata2, gata3,* and *scl* expressing V2b cells. The V2a and V2b cells in turn differentiate into excitatory Circumferential Descending interneurons (CiDs) and inhibitory Ventral Lateral Descending interneurons (VeLDs) respectively. Work on other model organisms also suggests that V2b cells may subdivide into V2b and V2c cells. Previous work by other authors suggests that two genes specifically expressed by V2c cells are *sox1* and *foxn4*.

My research has investigated whether transcription factor genes *scl* and/or *gata3* are necessary for proper V2b cell development. I have used *scl* and *gata3* mutants and GFP transgenic lines, to start to determine if the loss of function of *scl* and *gata3* affects:

- The expression of genes downstream of *scl* and *gata3*
- The morphology of VeLDs
- The number of V2b cells in the spinal cord

Additionally, I have investigated whether *sox1a, sox1b* and *foxn4* are:

- Expressed in the V2 domain
- Affected by the loss of *scl* or *gata3* function

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Thank you.

Introduction

The neuronal circuitry of the spinal cord is an integral component of the vertebrate central nervous system, because it coordinates sensory inputs as well as the movement of an organism. Proper formation and communication of the spinal cord neurons is vital because addition of new neurons or regeneration of existing neurons is almost impossible. However, little is known about the processes that regulate the formation and proper connections of spinal cord neuronal circuitry.

Neurons are a fundamental unit of the nervous system. A neuron is an electrically excitable cell that processes and transmits information through electrical and chemical signaling. There are various types of neurons. However, in this study I focus primarily on interneurons. Interneurons are a special group of neurons that usually form connections between other neurons. Unlike motor or sensory neurons whose soma (cell body) or axon may be outside the spinal cord, the entire length of the interneuron is contained in the spinal cord. Studying spinal interneurons is important because they compose the majority of neurons in the spinal cord. By understanding the processes that allow interneurons to form and function properly, we can gain a better understanding of how the neuronal circuitry of the spinal cord works.

Previous studies have demonstrated that neuronal circuitry is highly conserved in vertebrates, especially among zebrafish, xenopus, and mouse (Goulding and Pfaff, 2005; Higashijima et al., 2004b; Lewis, 2006; Li et al., 2004; Roberts, 2000). This is significant because it argues that data collected from zebrafish will be applicable to other vertebrates, such as humans. For my

research, zebrafish embryos were used for their oviparous and transparent qualities. Unlike mouse, whose embryos must be extracted from the mother and the mother euthanized, zebrafish embryos are easily accessible because eggs are laid outside of the mother's body. Additionally, the zebrafish has a functional spinal cord as early as 24 hours post fertilization. This fast development of the zebrafish spinal cord is another reason why zebrafish is an ideal model organism to study interneuron specification and function.

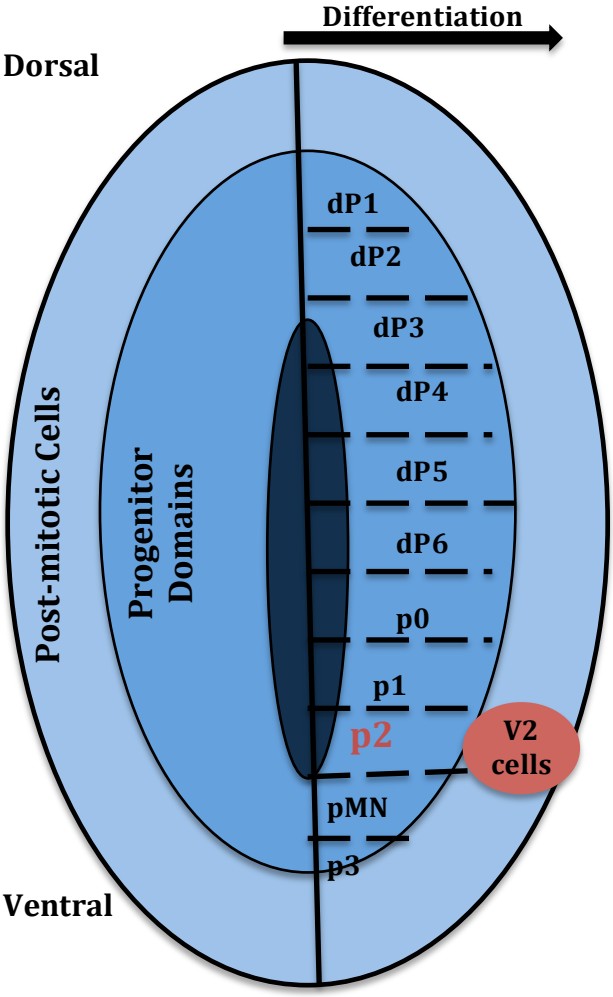


Fig 1 A general vertebrate schematic of a cross-section of the spinal cord with all the progenitor domains but only the post-mitotic V2 cells shown. Progenitor domains are medial. As cells stop dividing and start to differentiate they move lateral. dp1 to dp6 are different dorsal progenitor domains in the spinal cord. p0, p1, p2, and p3 are ventral progenitor domains. pMN is the progenitor domain for motor neurons.

Within the developing spinal cord, different dorsal/ventral progenitor domains in the medial region of the spinal cord give rise to distinct classes of post-mitotic cells **(Fig.1)**. As the progenitor cells become post-mitotic (stop dividing) and move towards the lateral edges of the spinal cord, they express different sets of transcription factors. Transcription factors are

proteins that bind to DNA and regulate the expression of other genes. Thus transcription factors are important players in spinal cord development and interneuron specification because they are thought to specify the neuronal characteristics that cells acquire as they differentiate (Lewis, 2006).

Typically, within the aminote spinal cord, each progenitor domain differentiates into one type of post-mitotic cell. However, the V2 cells are unusual because they do not follow this pattern. The V2 cells originate from molecularly indistinguishable p2 progenitor cells in the spinal cord. As the p2 progenitor cells become post-mitotic and differentiate, they start to express different transcription factor genes that allow them to initially develop into two sets of molecularly distinct cells, V2a and V2b cells (Batista et al., 2008; Del Barrio et al., 2007). As differentiation continues, at least one more class of molecularly distinct cells develop, V2c cells (**Fig. 2)** (Hargrave et al., 2000; Li et al., 2005; Panayi et al., 2010).

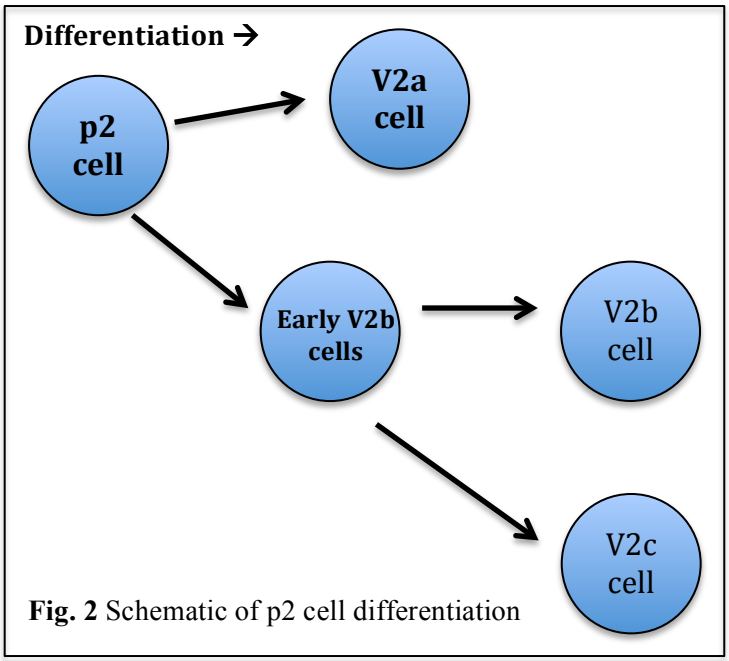


Fig. 2 Schematic of p2 cell differentiation

V2 cells have been shown to exist in mouse, chick and zebrafish (Batista et al., 2008; Del Barrio et al., 2007; Karunaratne et al., 2002; Muroyama et al., 2005; Smith et al., 2002; Zhou et al., 2000). In zebrafish and mouse, V2 cells differentiate into at least two functionally distinct classes of cells, specifically, the *vsx1* (used to be called *chx10*) and *vsx2* expressing V2a cells and the *gata2, gata3,* and *scl* expressing V2b cells (Batista et al., 2008; Del Barrio et al., 2007; Kimura et al., 2006; Karunaratne et al., 2002; Muroyama et al., 2005; Smith et al., 2002; Zhou et al., 2000). In zebrafish, V2a and V2b cells differentiate into excitatory Circumferential Descending interneurons (CiDs) and inhibitory Ventral Lateral Descending interneurons (VeLD), respectively (Batista et al., 2008; Bernhardt et al. 1990; Kimura et al., 2006). Both CiDs and VeLDs have a teardrop-shaped soma with an axon that runs ipsilaterally (extends on the same side of the embryo as the cell body) (**Fig. 3**). However, the VeLDs are, at least initially, more ventrally located in the spinal cord.

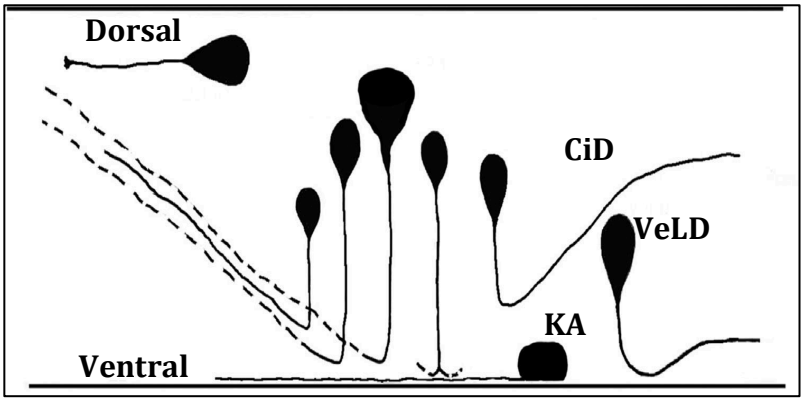


Fig. 3 Schematic of zebrafish embryonic spinal interneurons taken from Lewis & Eisen (2003). Solid lines show ipsilateral axons (axons that extend on the same side of the embryo as the cell body). Dashed lines show contralateral axons (axons that cross the midline and extend on the opposite side of the embryo to the cell body).

Within the zebrafish spinal cord, the excitatory CiDs contribute to escape movements whereas the exact role of VeLDs is unknown (Ritter et al., 2001; Kimura et al., 2006). The most fascinating aspect of the excitatory CiDs and inhibitory VeLDs is that they express different neurotransmitters even though they originated from molecularly identical p2 cells (Batista et al., 2008; Bernhardt et al. 1990; Kimura et al., 2006). By understanding how the p2 cell can differentiate into molecularly distinct interneurons with different functional characteristics, we can gain a better understanding of spinal cord development.

It is already known that zebrafish and mouse V2b cells express *gata2, gata3* and *scl* transcription factor genes (Batista et al., 2008; Karunaratne et al., 2002; Muroyama et al., 2005; Smith et al., 2002). Additionally, previous findings in zebrafish and mouse data suggest that *gata2* is expressed before *scl* and *gata3* (Batista et al., 2008; Peng et al., 2007*)*. However in the zebrafish, *gata2, gata3* and *scl* are also expressed by another set of interneurons, Kolmer-Agduhr (KA) cells (Batista et al., 2008). KAs are a type of interneuron that is located in the most ventral part of the zebrafish spinal cord and derived from the p3 domain (**Fig. 1)**. Consistent with work from Batista et al., 2008, work from Yang et al., 2010 has suggested that *gata2* is expressed upstream of *scl* and *gata3*, in the KA interneurons of zebrafish (**Fig. 4**). For this paper, it is important to note that V2bs and KAs express many of the same genes and genes that are expressed solely in V2bs were not known when I began my research.

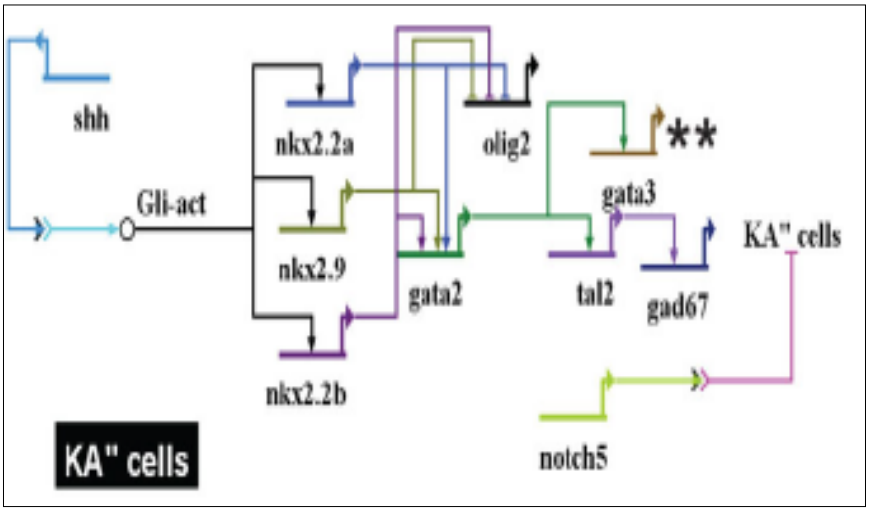


Fig. 4 Schematic outlining the regulatory interactions in zebrafish KA'' cells. There are two types of KA interneurons, KA' and KA''. KA' are more dorsal than KA'' in the spinal cord. Genes are expressed in bold font. The arrows demonstrate the order of expression. *shh* is not expressed by KA'' cells but plays a role in the induction of ventral cells in the spinal cord. Note that *gata2* is expressed before *tal1* (also called *scl*) and *gata3.* Scheme was taken from Yang et al., 2010.

Notch signaling is a necessary pathway for proper V2a and V2b cell generation (Batista et. al., 2010; Peng et al., 2007). In the absence of Notch signaling, inhibitory V2b cells do not form and excess excitatory V2a cells are produced (Batista et. al., 2010). In mouse, overexpression of Scl has been shown to be sufficient to bypass the loss of Notch signaling and specify the V2b interneuron cell fate (Peng et al., 2007). Essentially Peng et al., 2007 was able to overexpress Scl in Notch inhibited embryos (DN-MAML), which rescued the V2b cell-fate program (Peng et al., 2007). These data are fascinating because they suggest that Scl is sufficient, in this context, to drive the V2b cell fate.

In mouse, loss of function of Gata3 in *gata3* mutants leads to the onset of severe deformities in spinal cord development (Moriguchi et al., 2006). Yet the exact role of *gata3* in the mouse spinal cord is unclear. Even more uncertain is the role of *scl* and *gata3* in the zebrafish spinal cord. While *scl* zebrafish mutants exist and have been analyzed to determine the role of *scl* in the hematopoietic and

endothelial development of the zebrafish, the role of *scl* in the spinal cord is unknown (Dooley et. al., 2005). Furthermore, zebrafish *gata3* mutants were only recently isolated and they have yet to be used in the study of the spinal cord. In this project, I will use *scl* and *gata3* mutants to determine the roles of *scl* or *gata3* in the differentiation and speciation of zebrafish V2b interneurons.

With the use of *in situ* hybridization, work from Panayi et al., 2010 not only showed that *sox1* expressing cells were found in the V2 region, but that these cells do not co-express *chx10* or *gata3,* arguing that *sox1* expressing cells are a different type of V2 cell that they called V2c cells. Their results showed that in null $sox1^{GFP/GFP}$ embryos, there was a reduction of *sox1* expressing cells (V2c cells). More importantly, they demonstrated that the remaining *sox1* expressing cells moved closer to the p3 domain and expressed *gata3.* Thus, Panayi et al., 2010's data suggested that for at least some V2c cells, *sox1* is necessary to maintain its cell fate. Using *sox1* as a marker for V2c cells, Li et al., 2005 used Foxn4-Cre;R26R-YFP to determine that all *foxn4* positive cells co-expressed *sox1.* Thus, Li et al., 2005 showed that *foxn4* also labels V2c cells. Data from these studies support the hypothesis that p2 cells differentiate into multiple cell classes (Batista et al., 2008; Del Barrio et al., 2007; Li et al., 2005; Panayi et al., 2010).

Although there have been studies that analyzed *scl* and *gata3* roles in other model organisms, a study analyzing the roles of *scl* and *gata3* in the zebrafish spinal cord is novel. In this thesis, I will determine if *scl* and/or *gata3* are necessary for proper V2b cell development. With the use of *scl* and *gata3*

mutants and GFP transgenic lines, I will determine if the loss of function of *scl* or *gata3* effects the expression of genes co-expressed in V2b cells or in other cells, the morphology of VeLDs, and the number of V2b cells in the spinal cord. Additionally, I will determine if *sox1a, sox1b* or *foxn4* are expressed in the V2 domain and if they are affected by the loss of function of *scl* and/or *gata3*.

Materials and Methods

Zebrafish lines

Zebrafish (*Danio rerio*) embryos were obtained from wild-type (AB, TL, or AB/TL hybrids); identified heterozygous carriers for *scl*^{t21384} (also called *tal1*; Bussmann et al., 2007; kindly received from Marga Varga at UCL) or identified carriers heterozygous for *gata3*^{sa0234} (Stemple Lab, Sanger Institute). *scl* mutants have a nonsense mutation at amino acid position 183 (Bussmann et al., 2007). The *gata3*^{sa0234} allele was created with zinc finger nucleases (personal communication Steve Harvey and Derek Stemple). This resulted in an insertion that caused a frame shift mutation at amino acid 318. GFP-labeled embryos were obtained from Tg (*vsx2*:GFP) (Higashijima et al., 2004b) or Tg(*gata1*:GFP) (Kobayshi et al., 2001) carriers or *scl*;Tg(*vsx1*:GFP) or *scl*;Tg(*gata1*:GFP) carriers created from a cross of identified heterozygous carriers of *scl* and Tg(*vsx2*:GFP) or Tg(*gata1*:GFP) fish. Embryos were staged according to Kimmel et al., 1995 by number of somites, or hours post-fertilization at 28.5 °C.

scl mutants are lethal recessive and not viable after 4 days post fertilization, therefore all *scl* embryos were obtained from an in-cross of *scl* heterozygote fish. *scl* heterozygote fish were identified by random in-crosses. Two *scl* heterozygote fish produce embryos where 25% of the embryos are *scl* homozygous mutants (exhibit heart edema phenotype).

Unfortunately, *gata3* mutant embryos cannot be identified by their morphology; therefore PCR and DNA sequencing were used to identify heterozygous *gata3* fish.

Fixation

Embryos for *in situ* hybridization were fixed at 24 hours post fertilization (hpf), whereas embryos for antibody staining were fixed at 48 hpf. Embryos for fixation were placed in 1 mL of 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight at 4°C. Embryos were then washed four times in PBS. They were washed twice for 5 minutes and twice for 10 minutes. Embryos for *in situ* hybridization were dehydrated with 1 mL of methanol, whereas embryos for antibody staining were left in 1 mL of PBS. Embryos for *in situ* hybridization were stored at -20°C and embryos for antibody staining were stored at +4°C.

Preparation of RNA probes

in situ hybridization probes were prepared using the following templates: *gata2* (Read et al., 1998), *gata3* (Neave et al, 1995); *sox1b* (Okadu et al., 2006); *sox1a* (Okadu et al., 2006); *foxn4* (Li et al., 2010). A mixture of *gad65*, *gad67a, gad67b* was used to create the *gad* mixture (Higashijima et al., 2004a; Higashijima et al., 2004b). An equal mixture of *islet1* and *islet2* was used to create the *islet 1/2* mixture *(*Appel et al., 1995).

RNA *in situ* hybridization probes was made from DNA templates. 10µg of DNA was linearized by digestion using appropriate restriction enzymes. The restriction enzyme EcoRI was used for digestion of *gata2, gata3, gad65, gad67a, gad67b, islet1* and *islet 2,* whereas XhoI was used for *sox1a* and *sox1b* and Hind III for *foxn4.* The linearization of the DNA was confirmed on 1% agarose gels. Linearized DNA was treated with proteinase K (0.05 µg/µl) for 30 minutes at

37°C. The treated DNA was extracted using equal volumes of phenol-chloroform and phenol-chloroform-isoamyl. The DNA was precipitated with 1/10 volume 4M sodium chloride and 2 volumes of ethanol, then placed at -20°C overnight. The next day, the DNA was spun at 13300 rpm for 30 minutes. The supernant was removed and replaced with 1mL of 80% ethanol: 20% distilled water (dH_20) and spun for 10 minutes. The ethanol supernant was removed and the precipitant was air-dried. Once dried, the precipitant was resuspended in 14 µL of dH_20.

To make RNA probes, 40 units of appropriate RNA polymerase, 0.5 µL RNase inhibitor, 2 µL transcription buffer and 2 µL of digoxigenin (dig) NTP mix were added to 1µg of template DNA and water to make a final volume of 20 µL. RNA polymerase T3 was used for *foxn4, gad65*, *gad67a*, *gad67b*, *islet1, islet 2, sox1a* and *sox1b,* whereas T7 was used for *gata2, gata3.* This reaction was incubated for 2 hours at 37°C. The synthesized RNA was treated with 40 units DNase I inhibitor, and then precipitated with 2.5 µL of LiCl and 75 µL pre-chilled ethanol. RNA was resuspended in 100 µL RNase-free water with 40 units RNase Inhibitor. Confirmation of translation was made on 1% agarose gels. The RNA was then mixed with 400 µL of fish hybridization buffer (cheap hybridization buffer; 500 µg/ml yeast RNA; 50 µg/ml heparin; 200 µl of 1 M citric acid for pH of 6.0) and stored at -20°C.

in situ hybridization and immunohistochemistry

Embryos fixed for *in situ* hybridization were rehydrated through a series of 10 minutes 75% methanol: 25% distilled water (dH_20), 50%, methanol: 50%

dH_20 and 25% methanol: 75% dH_20 washes then a phosphate buffered saline + 0.1% tween (PBT) wash. Embryos fixed at 24 hours were incubated with proteinase K at a concentration of 10µg/mL for 24 minutes, then refixed with 4% PFA for 30 minutes, and washed again with PBT. The embryos were then washed for 5 minutes in 50% chloroform: PBT, 100% cheap hybridization buffer (50% formamide; 5X SSC; 0.1% tween), and then fish hybridization buffer. Embryos were placed in a 70°C oven for 2 hours pre-hybridization before they were incubated overnight with 25 µL of RNA probes in 1 mL fish hybridization buffer. After incubation, embryos were washed twice for 5 minutes with cheap hybridization buffer, three times for 20 minutes with 2x SSC (3M NaCl and 0.03M sodium citrate), twice for 20 minutes with 0.2x SSC, once for 20 minutes with 0.1X SSC, and three times with PBT at 70°C. Embryos were then washed one time with PBT at room temperature. 1 mL of block solution (1X PBT; 2 mg/ml BSA; 5% sheep serum; 1% DMSO) was added to the embryos for one hour. RNA hybridization was detected using 1 mL of anti-dig (Roche) antibody diluted in 1/2000 blocking solution. This block solution was removed and eight washes of PBT for 15 minutes each, were done. The embryos were then left in PBT overnight. Then three NTMT (0.1M NaCl, 0.05M MgCl, 0.1M Tris pH 9.5, 0.1% tween) washes for 5 minutes washes were. 20 µL of NBT/BCIP (Roche) in 1 mL of NTMT was used to stain the embryos. Staining was stopped with two quick and three 5 minute NTMT buffer washes. Following the NTMT washes three 15 minute washes of PBT were done.

For immunohistochemistry, fixed embryos were permeabilized for 5

minutes in distilled water, 7 minutes in acetone at -20°C, 5 minutes in distilled water and then PBT. 1% BSA was added to PDT (1x PBS; 1% DMSO; 0.1% Triton) to make antibody blocking solution. The antibody blocking solution was added to embryos and the embryos were left to rock on a shaker for one hour. The antibody blocking solution was replaced with 0.4 µL of Rabbit anti-GFP (1/1000) in 1 mL of blocking solution and left overnight at +4°C. Two quick washes and eight washes of 15 minutes with PDT followed. PDT was then replaced with 0.4 µL of Goat anti-Rabbit Alexa Fluor 488 (1/500) in 1 mL of antibody blocking solution and left overnight at +4°C. After secondary incubation, two quick washes and eight washes of 15 minutes with PDT were performed.

Preparation for photo analysis

Once stained, *in situ* embryos were washed in 30% glycerol: 70% PBS, 50% glycerol: 50% PBS, 70% glycerol: 30% dH_20. Since there is no detectable *scl* or *gata3* mutant morphological phenotype at 24hpf, embryos were sorted by their *in situ* hybridization staining pattern. Approximately 25% of the embryos had a distinct pattern with the exception of *foxn4, islet1/2* and *sox1a.* The best representative embryos of the *in situ* hybridization staining were selected. The embryos were deyolked and placed on glass coverslips.

Antibody stained embryos were placed in DABCO for preservation of antibody staining and stored at +4°C.

Cell counts and row numbers

Cell counts were made in the region of the spinal cord between the 6th and 10th somite. All results are an average of 5 different embryos unless otherwise stated. Error bars show standard deviation. Cell row numbers are assigned ventral to dorsal.

Photographs

Photographs were taken using Zeiss Axio Imager M1 microscope (DIC images) and processed using Adobe Photoshop CS.

Results

gata3 may be downstream of *scl*

Both *scl* and *gata3* are known to be expressed by V2b cells (Batista et al., 2008; Karunaratne et al., 2002; Kimura et al., 2006; Li et al., 2005; Muroyama et al., 2005; Smith et al., 2002). As mentioned in the introduction, in zebrafish, KA cells also express both of these genes. Work from Batista et al., 2008 and Yeng et al., 2010, suggested that *scl* is expressed before *gata3* in the V2b and KA cells. Additionally, preliminary findings from the Lewis Lab suggest that *scl* maybe required for *gata3* expression (Jacobstein, 2008) (**Fig. 6)**.

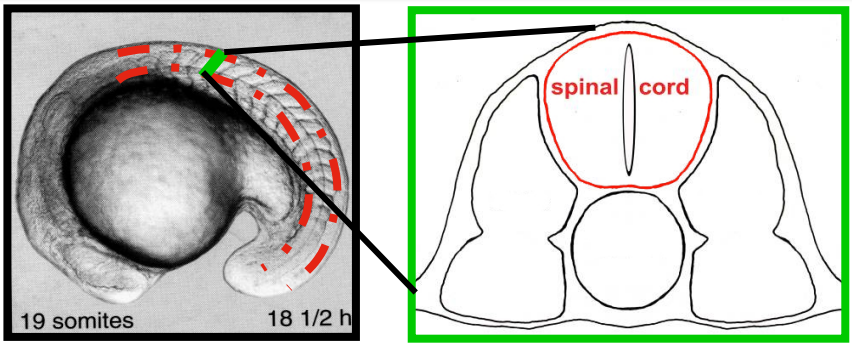


Fig. 5 Depiction of a cross section of the zebrafish spinal cord (green box). Black box shows the lateral view of a zebrafish embryo at 18.5 hpf. Red dashes show the dorsal and ventral boundaries of the spinal cord. All *in situ* hybridization photos are a lateral view of the spinal cord.

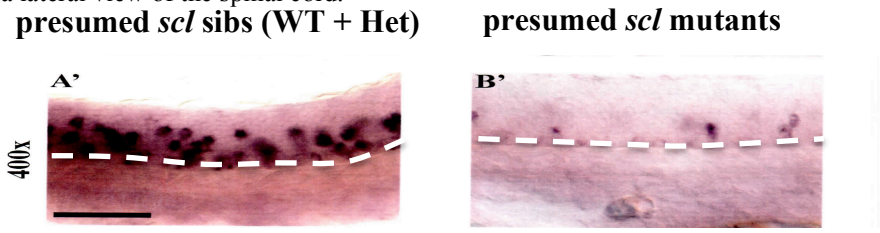


Fig. 6 Expression of *gata3* in presumed *scl* siblings and mutants. Lateral views of trunk showing anterior to the left. Scale bar = 50 µm. White-dashed line marks the most ventral part of the spinal cord. Data was taken from Jeff Jacobstein's thesis (Jacobstein, 2008). Expression of *gata3* is severely reduced in presumed *scl* mutants when compared to *scl* sibs. Demonstrates that *scl* may be required for the expression of *gata3*.

With the use of *in situ* hybridization in *scl* siblings (WT+ Het) and presumed mutants, my results also demonstrated a reduction in the number of *gata3* expressing cells at 24 hours post fertilization (hpf) in presumed *scl* mutants (**Fig. 7** and **Chart 1**). There appears to be a significant reduction of *gata3* expressing cells in the V2 region, but retention of *gata3* expressing cells in the KA region (most ventral domain of spinal cord). This data supports the hypothesis that *scl* is required for proper *gata3* expression in V2b cells.

28 embryos were analyzed from the *gata3 in situ* hybridization experiment. Out of the 28 *scl* embryos, 6 were thought to be *scl* mutants based on their *in situ* hybridization cell staining. The presumed *scl* mutants made up 21% of the 28 embryos, which is close to the expected frequency of *scl* mutants to *scl* siblings (25%). The genotype of the *scl* embryos could not be confirmed phenotypically since *scl* mutants look identical to *scl* siblings at 24 hours post fertilization. To determine if the frequency of presumed *scl* mutants was accurate, I performed a Chi-square test. The null hypothesis was that there would be no significant difference between the observed and expected presumed *scl* siblings and observed and expected presumed *scl* mutants (**Fig. 7**). The p value was close to 0.70 (x^2 =0.19048), which means that there is a 70% probability that the difference between the observed and the expected is just due to chance. Therefore, I accepted my null hypothesis and determined that the deviation between the observed and expected values is small enough that chance alone may account for it.

A Student t-test was performed to determine if the number of cells expressing *gata3* was significantly different between presumed *scl* siblings and mutants. The null hypothesis was that there wouldn't be a statistically significant difference between the number of *gata3* expressing cells in presumed *scl* siblings and mutants. The p value of the Student t-test was 0.00497, which is less than 0.05. This suggests that the number of cells expressing *gata3* is statistically significant in presumed *scl* siblings and mutants.

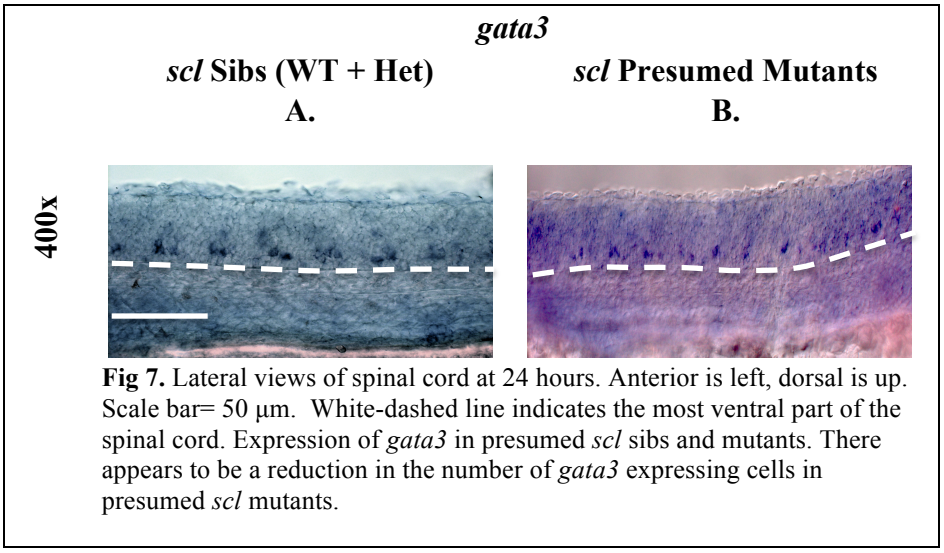


Fig 7. Lateral views of spinal cord at 24 hours. Anterior is left, dorsal is up. Scale bar= 50 µm. White-dashed line indicates the most ventral part of the spinal cord. Expression of *gata3* in presumed *scl* sibs and mutants. There appears to be a reduction in the number of *gata3* expressing cells in presumed *scl* mutants.

If there was more time, I would have extracted DNA from the embryos' heads to PCR and DNA sequence. PCR and DNA sequencing would have allowed me to determine the genotype of each of the presumed *scl* siblings and mutants. By knowing the embryos' genotype, I would have been able to compare the *in situ* hybridization phenotypes and embryos' genotypes. I would have expected to see a strong and clear correlation between the phenotypes and genotypes of the presumed *scl* siblings and mutants.

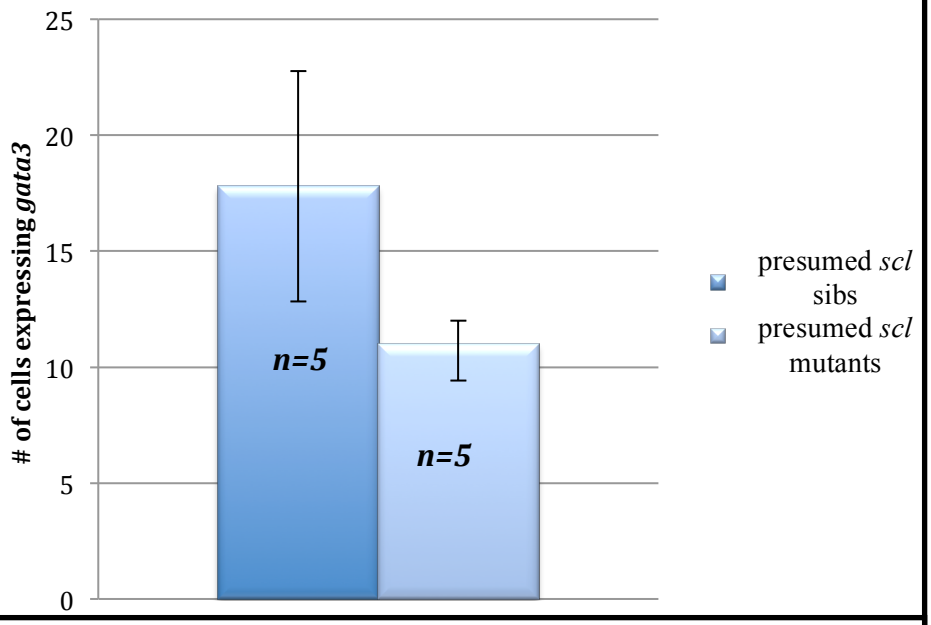


Chart 1. Cell count of *gata3* in presumed *scl* sibs and mutants. All cell counts are from a 5-somite length of spinal cord adjacent to somites 6–10. Values shown are the mean from 5 different embryos from one *in situ* hybridization experiment. Error bar represents the standard deviation. Standard deviation was ± 4.9699 for presumed *scl* sibs and ± 1.5811 for presumed *scl* mutants. Student t-test was performed to determine if the results were significantly different. The p value was 0.00497, which is less than 0.05. This shows that the number of cells expressing *gata3* in presumed *scl* sibling and mutant embryos was significantly different.

sox1a expression is unaffected by loss of function of *scl* or *gata3*

Previous findings from Panayi et al., 2010 determined that in mouse *sox1* was expressed in the V2 domain and was not co-expressed by other V2 markers, such as *chx10* and *gata3.* Furthermore, Panayi et al., 2010 discovered that in absence of *sox1*, V2c cells became V2b cells. This data suggests that *sox1* is necessary for regulating the V2b versus V2c fate choice.

The zebrafish orthologs for mouse *sox1* are *sox1a* and *sox1b.* In this experiment, I wanted to determine if *sox1a* was expressed in the V2 domain. If *sox1a* was expressed in the V2 domain, I would test if the loss of function of *scl*

or *gata3* affected the expression of *sox1a.*

in situ hybridization was used to determine the expression of *sox1a* in the spinal cord of 24 hpf wild-type, *scl* (sibling and mutant) or *gata3* (sibling and mutant) embryos. *sox1a* expression was found in the V2b and KA region of the spinal cord (**Fig 8)**. Although 25% of the *scl* or *gata3* embryos were expected to be mutants, there was no phenotypic difference found. 12 embryos from *scl* or *gata3 in situ* hybridization experiments were analyzed. I wanted to confirm that *sox1a* expression was the same amongst these embryos. There was no phenotypic difference amongst these embryos, suggesting that neither *scl* nor *gata3* is required for *sox1a* expression. These results were consistent with Panayi et al., 2010 findings that *sox1* $^{GFP/+}$ expressing cells did not co-express *gata3*.

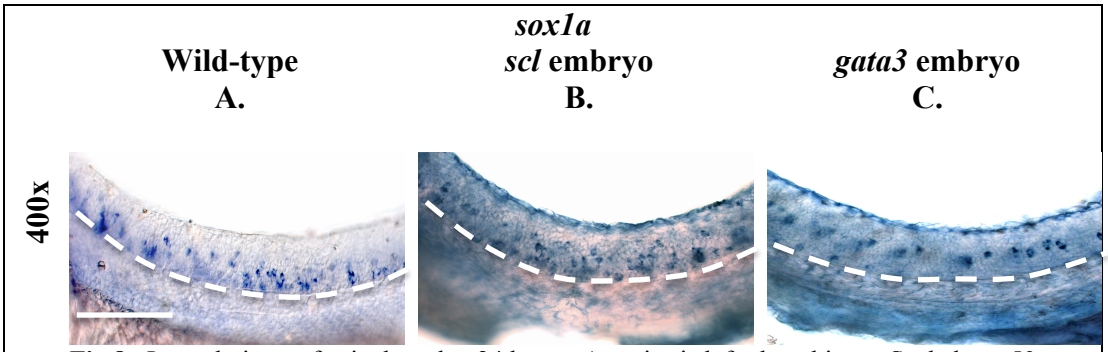


Fig 8. Lateral views of spinal cord at 24 hours. Anterior is left, dorsal is up. Scale bar= 50 µm. White-dashed line indicates the most ventral part of the spinal cord. Photos are representative of *sox1a* expression in the wild-type, *scl* and *gata3* embryos. There was no apparent phenotypic difference amongst the *scl* or *gata3* embryos. This suggests that neither *scl* nor *gata3* is required for proper *sox1a* expression. Two wild-type, one *scl* and one *gata3 in situ* hybridization experiments were analyzed. 12 embryos from the *scl* or *gata3 in situ* hybridization experiments were analyzed.

The next logical step for this project would be PCR and DNA sequencing. PCR and DNA sequencing would determine the genotype of the *scl* or *gata3* sibling and mutant embryos. With the knowledge of embryos' genotype, further analysis of *sox1a* expression in these mutant lines could be done.

sox1b expressing cells are reduced due by the loss of function of *scl*

in situ hybridization and 24 hpf embryos from a cross of *scl* heterozygous carriers were used to determine the expression of *sox1b* in the spinal cord. As expected, *sox1b* was expressed in the V2 and KA region of the spinal cord (**Fig 9**). However, there was a phenotypic difference found amongst the *scl* embryos. There appeared to be a reduction of *sox1b* expressing cells in rows 2 and 3 of the spinal cord (V2 domain) (**Fig 9**).

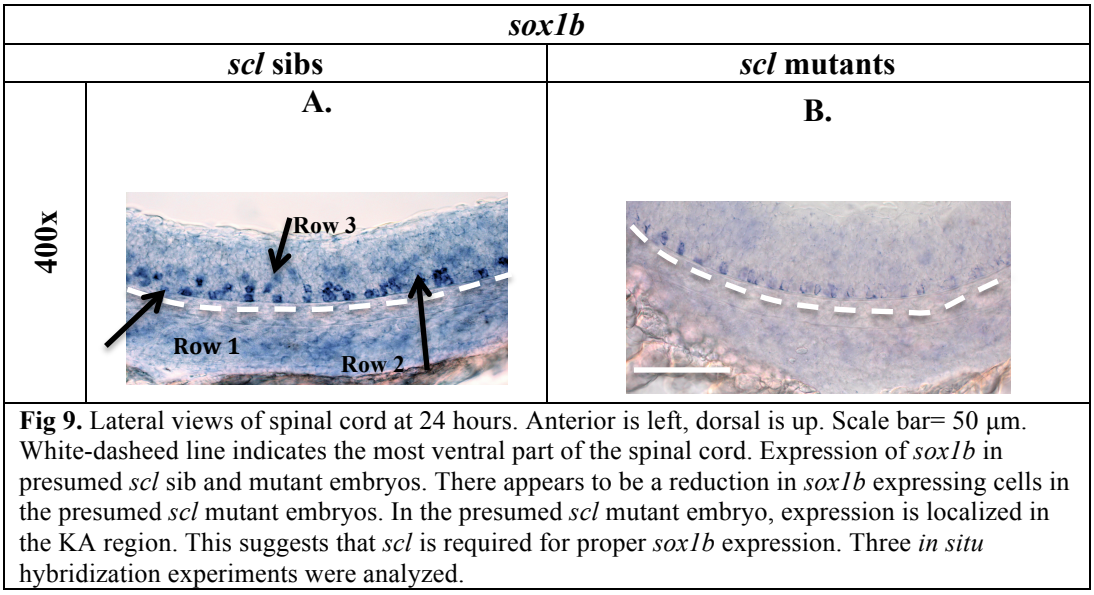


Fig 9. Lateral views of spinal cord at 24 hours. Anterior is left, dorsal is up. Scale bar= 50 µm. White-dasheed line indicates the most ventral part of the spinal cord. Expression of *sox1b* in presumed *scl* sib and mutant embryos. There appears to be a reduction in *sox1b* expressing cells in the presumed *scl* mutant embryos. In the presumed *scl* mutant embryo, expression is localized in the KA region. This suggests that *scl* is required for proper *sox1b* expression. Three *in situ* hybridization experiments were analyzed.

Three *in situ* hybridization experiments were analyzed to determine the expression of *sox1b* in *scl* embryos. 5 out of 32, 7 out of 26, and 7 out of 34 embryos were thought to be *scl* mutants. The ratio of presumed *scl* mutants to the total number of embryos were 16%, 27%, 21%, respectively. Therefore, two out of the three experiments approximately exhibited *scl* mutants at the appropriate frequency of 25%.

A Chi-square test of the three *in situ* hybridization experiments was performed to determine if the frequency of presumed *scl* sibling and mutants was accurate. The null hypothesis was that there would be no significant difference between the observed and expected presumed *scl* siblings or presumed *scl* mutants (**Fig. 9**). The p value was close to 0.30 (x^2= 0.92753), which is greater than the 0.05. Therefore, I accepted my null hypothesis and determined that the deviation between the observed and expected values is small enough that chance alone may account for it.

A Student t-test was used to determine if there was significant difference in the total cell counts of the presumed *scl* sibling and mutant embryos. 15 presumed *scl* siblings and 10 presumed *scl* mutants were counted from the three *in situ* hybridization experiments. My null hypothesis was that there wouldn't be a significant difference between the combined cell counts of the presumed *scl* siblings and mutants. The p value for of the combined cell counts were 0.00055. This p value is less than 0.05, suggesting that there is a significant difference between the total number of *sox1b* expressing cells in the presumed *scl* sibling and mutant embryos (**Chart 3**).

Additionally, a Student t-test of the three *in situ* hybridization experiments was performed. The Student t-test was used to determine if there was a significant difference in the number of *sox1b* expressing cells in *scl* sibling and mutant embryos by cell rows. My null hypothesis was that there wouldn't be a significant difference between the number of *sox1b* expressing cells in the rows of presumed *scl* sibling and mutant embryos. The p value for row 1 and 3 was less than 0.05,

suggesting that there is a significant difference in the number of cells expressing *sox1b* in these cell rows (**Table 1**). However, the p value of row 2 was greater than 0.05, suggesting that there isn't a significant difference in the number of cells expressing *sox1b* in row 2 of the presumed *scl* sibling and mutant*s*.

Student t-test of the presumed *scl* mutants and siblings- Table 1		
Cell counts from Row 1-3	**p value**	**Significance/ No significance**
Cell counts from row 1	0.03131	Significance
Cell counts from row 2	0.0602	No Significance
Cell counts from row 3	5.54954E-06	Significance

Chart 2 shows the cell count of the presumed *scl* sibling and mutant embryos by rows. **Chart 3** shows the total cell counts of the presumed *scl* sibling and mutant embryos.

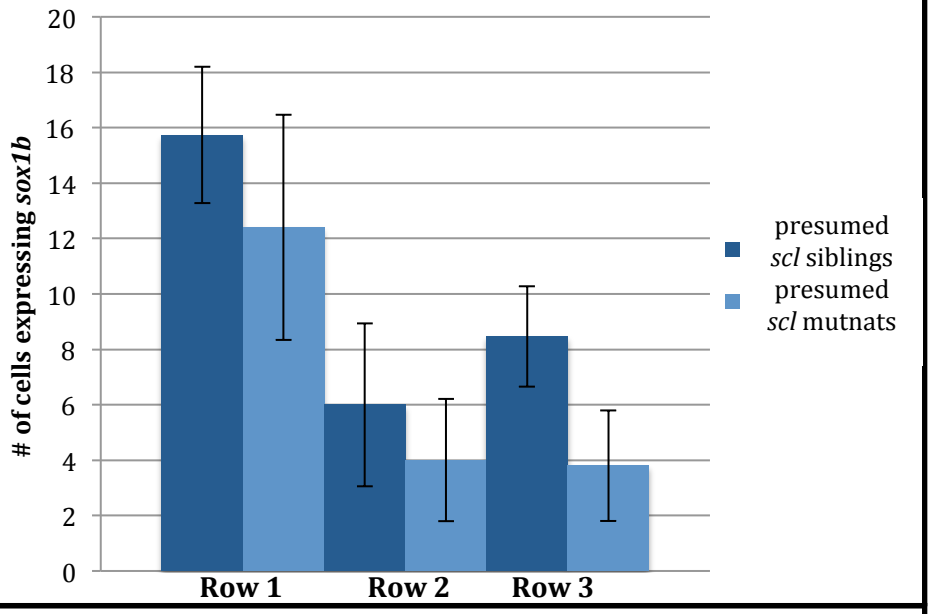


Chart 2. Cell count of *sox1b* in presumed scl sibs and mutants. All cell counts are from a 5-somite length of spinal cord adjacent to somites 6–10. Values shown for *scl* siblings are the mean from 5 embryos from each *in situ* hybridization experiment (n=15). Values shown for *scl* mutants are the mean of 3/4 embryos from three different *in situ* hybridization experiments (n=10). Error bar represents the standard deviation. Standard deviation for the *scl* sibs and mutants for rows 1: ± 2.58751 and 4.06065 ±; row 2: ± 2.46306 and ±2.94392; row 3: ± 1.99523 and ± 1.813529. A Student t-test was performed for each *in situ* hybridization experiment to determine there was a significant difference in the number of *sox1b* expressing cells in the presumed *scl* siblings and mutants. The p values were 0.03131, 0.0602, 5.54954E-06. These p values for row 1 and 2 are less than 0.05, demonstrating significant difference between the number of *sox1b* expressing cells in *scl* sibling and mutant embryos.

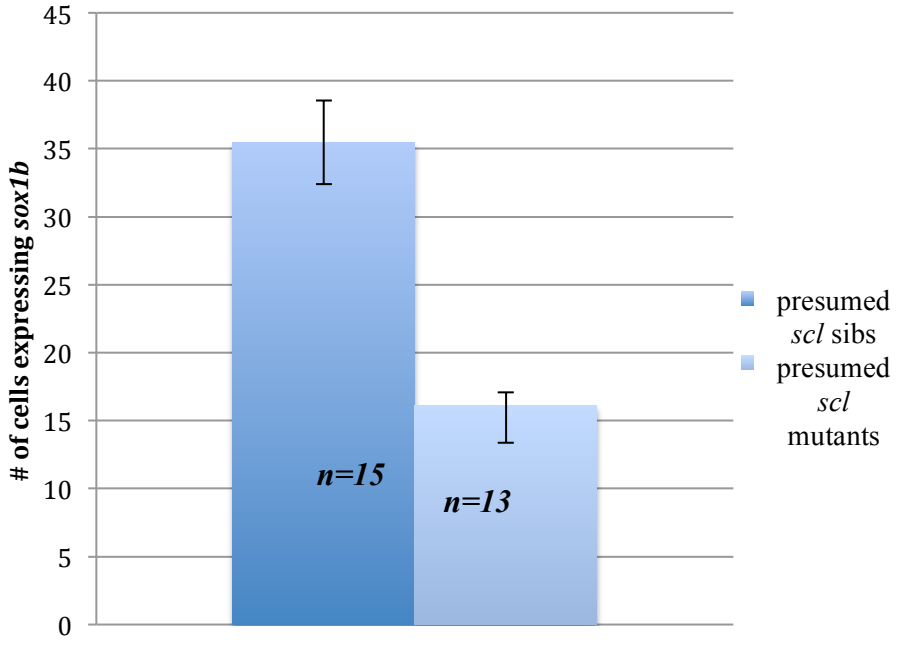


Chart 3. Average cell count of *sox1b* in presumed *scl* sibs and mutants. All cell counts are from a 5-somite length of spinal cord adjacent to somites 6–10. Values shown for *scl* sibling and mutant embryos an average cell count from rows 1-3. Standard deviation was ± 3.067494712for presumed *scl* sibs and ± 2.726414006 for presumed *scl* mutants. A Student t-test was performed to determine if the number of *sox1b* expressing cells between the presumed *scl* siblings and mutants was significantly different. The p value was 0.00055, which was less than 0.05. This shows that the results were significantly different.

To check the reproducibility of my results, I preformed two Student t-tests to show that there was no significant difference in the number of *sox1b* expressing cells in the presumed *scl* sibling or mutants. The p values from these tests were greater than 0.05, demonstrating that that the staining and the counting of cells was reproducible for each *in situ* hybridization experiment (**Table 2** and **Table 3)**.

Student t-test of the presumed *scl* siblings-Table 2		
***in situ* hybridization experiments**	**p value**	**Significance/ No significance**
scl siblings from *in situ* experiments 1 and 2	0.17939	No significance
scl siblings from *in situ* experiments 1 and 3	0.70306	No significance
scl siblings from *in situ* experiments 2 and 3	0.413340	No significance

Student t-test of the presumed *scl* mutants siblings-Table 3		
***in situ* hybridization experiments**	**p value**	**Significance/ No significance**
scl mutants from *in situ* experiments 1 and 2	0.13323	No significance
scl mutants from *in situ* experiments 1 and 3	0.32805	No significance
scl mutants from *in situ* experiments 2 and 3	0.36986	No significance

Data from these results show that there is a reduction of *sox1b* expressing cells in presumed *scl* mutants. If there were more time, I would have extracted DNA from the embryos' heads to PCR and DNA sequence. PCR and DNA sequencing would have allowed me to determine the genotype of the presumed *scl* siblings and mutants. By knowing the embryos' genotype, I would have been able to compare the *in situ* hybridization phenotypes and embryos' genotypes. Hopefully, I should have seen a correlation between the phenotypes and genotypes of the presumed *scl* siblings and mutants.

sox1b expressing cells are reduced by the loss of function of *gata3*

Since the expression of *sox1b* was reduced by the loss of function of *scl* in *scl* mutant embryos, I wanted to determine if the loss of function of *gata3* would provide the same results. *in situ* hybridization and 24 hpf *scl* sibling and mutant

embryos were used to determine the expression of *sox1b* in *gata3* embryos. As anticipated, *sox1b* was expressed in the V2b and KA region of the spinal cord in *gata3* presumed siblings (**Fig 10**).

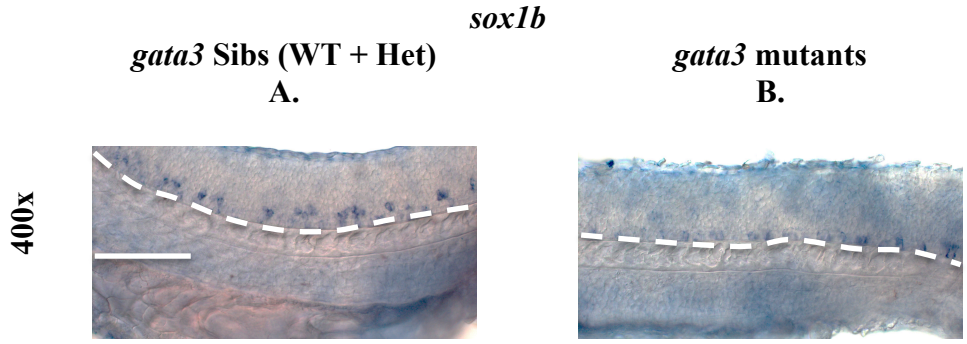


Fig 10. Lateral views of spinal cord at 24 hours. Anterior is left, dorsal is up. Scale bar= 50 µm. White-dotted line indicates the most ventral part of the spinal cord. Expression of *sox1b* in presumed *gata3* sib and mutant embryos. There appears to be a reduction in *sox1b* expressing cells in presumed *gata3* mutant embryos. In the presumed *scl* mutant embryo, expression is localized in the KA region. This suggests that *scl* is required for proper *sox1b* expression. Two *in situ* hybridization experiments were analyzed.

Within the presumed *gata3* mutants, there appeared to be a reduction of *sox1b* expressing cells in rows 2 and 3 (V2 domain) (**Fig 10**). Two *in situ* hybridization experiments were analyzed to determine the expression of *sox1b* in *gata3* embryos. 34 embryos were analyzed from one of the *in situ* hybridization experiments, where 6 of the embryos were thought to be *gata3* mutants. The presumed *gata3* mutants made up 18% of the 34 embryos, which is not close to the expected frequency of *gata3* mutants to *gata3* siblings (25%). In the other *in situ* hybridization experiment, 2 out of 15 embryos were thought to be *gata3* mutants. The presumed *gata3* mutants made up 13% of the 15 embryos, which is not close to the expected frequency of *gata3* mutants to *gata3* siblings (25%). The number of embryos in each *in situ* hybridization experiment may have contributed to the discrepancy between the real and expected frequency of presumed mutants.

If there were more embryos from each *in situ* hybridization experiment, I may have seen frequencies closer to 25%.

A Chi-square test of the two *in situ* hybridization experiments was used to determine if the frequency of observed and expected presumed *gata3* sibling and mutants was accurate. The null hypothesis was that there would be no significant difference between the observed and expected presumed *scl* siblings or presumed *scl* mutants. The p value of the Chi-square test was close to .20, which is greater than 0.05 (x^2=1.96560). This result suggests that the deviation between the observed and expected values is small enough that chance alone may account for it.

A Student t-test was performed to determine if there was a significant difference in the number of *sox1b* expressing cells in the 5 presumed *gata3* sibling and 5 presumed *gata3* mutant embryos. My null hypothesis was that there wouldn't be a significant difference between the number of *sox1b* expressing cells in the presumed *gata3* sibling and mutant embryos. The p value was less than 0.05 (0.00182), suggesting that there is a significant difference in the number of cells expressing *gata3* between the presumed *scl* sibling and mutant embryos. Additionally, a student t-test was performed do determine if there was a significant difference in the number of *sox1b* expressing cells in *gata3* sibling and mutants embryos by cell rows. My null hypothesis was that there wouldn't be a significant difference between the number of *gata3* expressing cells in the rows of presumed *scl* sibling and mutant embryos. The p values for row 1 and 2/3 were

less than 0.05 (0.01578 and 0.01511), suggesting that there is a significant difference in the number of cells expressing *sox1b* in these cell rows.

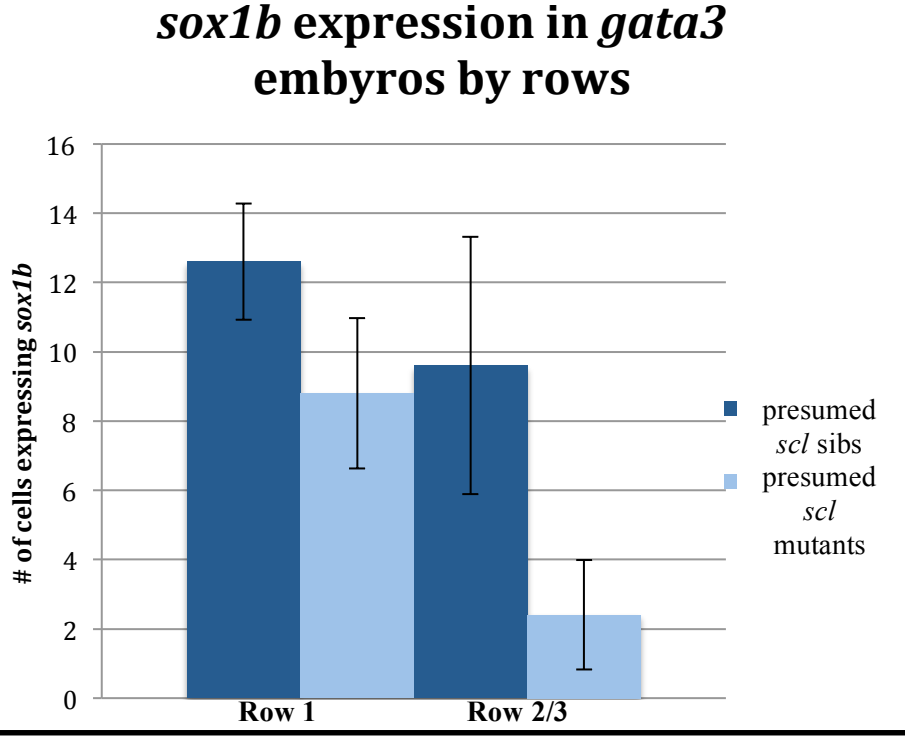


Chart 4. Cell counts of 5 *sox1b* presumed gata3 sibling and mutant embryos. All cell counts are from a 5-somite length of spinal cord adjacent to somites 6–10. Values shown for *scl* siblings are the mean from 5 embryos from each *in situ* hybridization experiment. Values shown for *scl* mutants are the mean of 3-4 embryos from three different *in situ* hybridization experiments. Error bars represent the standard deviations. Standard deviations for the *gata3* sibs and mutants for rows 1: were ± 1.67332, ± 2.16795; row 2/3 were ±4.02492 and ± 0.70711. Student t-test was performed to determine if the results were significantly different. The p value for the average cell counts of row 1 and row 2/3 was 0. 001578 and 0.01511, respectively. These p values are less than 0.05, demonstrating significant difference between the *gata3* sibling and mutant embryos.

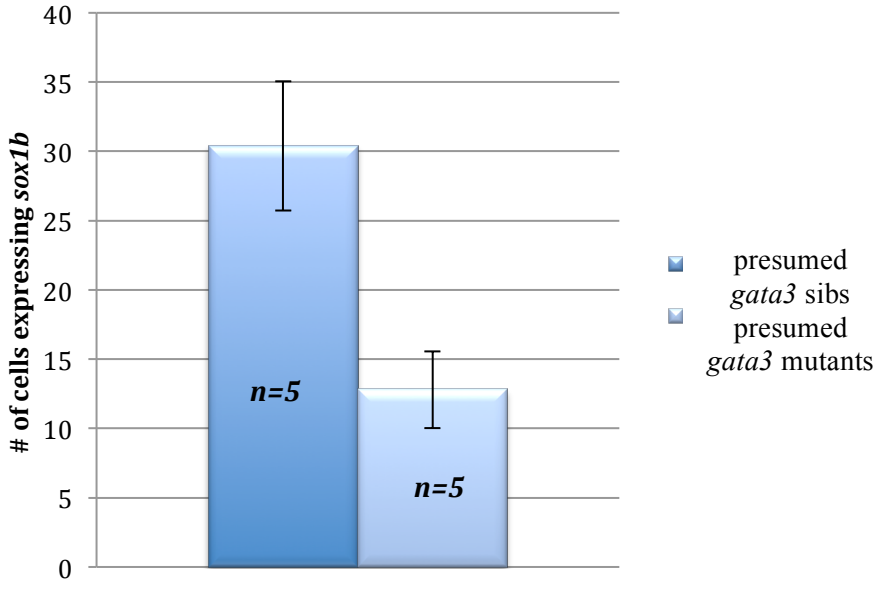


Chart 5. Average cell count of *sox1b* in presumed *gata3* sibs and mutants. All cell counts are from a 5-somite length of spinal cord adjacent to somites 6–10. Values shown are the mean from 5 different embryos from two *in situ* hybridization experiment. Three embryos were from one *in situ* hybridization and two were from another. Standard deviation was ± 4.96991 for presumed *gata3* sibs and ± 1.58114 for presumed *scl* mutants. The p value for the average cell counts was 0.00182, which is less than 0.05, demonstrating significant difference between the *gata3* sibling and mutant embryos.

The data from the results show that there is a reduction of *gata3* expressing cells in presumed *scl* mutants. The Student t-test of the presumed *sox1b* expressing *scl* siblings and mutants from **Chart 4 & 5** confirmed that there was a significant difference.

Like the other *in situ* hybridization experiments PCR and DNA sequencing is needed to, hopefully, confirm a correlation between the phenotypes and genotypes of the presumed *gata3* sibling and mutant embryos. This would also allow us to determine if some *gata3* mutants were considered to be *gata3* siblings because their expression pattern was less severe. This additional

information would allow us to determine the correct frequency of *gata3* mutants in each *in situ* hybridization experiment.

foxn4 expression is localized solely in the V2 region

Work in mouse has suggested that *a* distinct class of V2 cells may express *foxn4*, V2c cells (Li et al., 2010). In my experiment, I first determined if *foxn4* is expressed in the spinal cord of the zebrafish. Secondly, I determined if the loss of function of *scl* or *gata3* affected the expression of *foxn4.*

in situ hybridization was used to determine the expression of *foxn4* in the spinal cord. Interestingly, *foxn4* was expressed solely in the V2 region of the spinal cord of the wild-type, *scl* and *gata3* embryos (**Fig 11**). Additionally, there was no apparent reduction of *foxn4* expressing cells in *scl* or *gata3* embryos. Lack of *foxn4* phenotypic variance suggests that *foxn4* is not affected the by the loss of function of *scl* or *gata3*. This suggests that neither *scl n*or *gata3* is required for *foxn4* expression.

The wild-type expression pattern of *foxn4* is exciting because this is the first gene we have found in zebrafish that specifically labels V2b cells. In the future, *foxn4* can be used for studies that solely want to look at V2bs without labeling KAs. If I were to have more time, I would have PCR and DNA sequenced the embryo heads of the *scl* or *gata3* embryos to determine their genotypes and see if there is a subtle variance in the number of *foxn4* expressing cells.

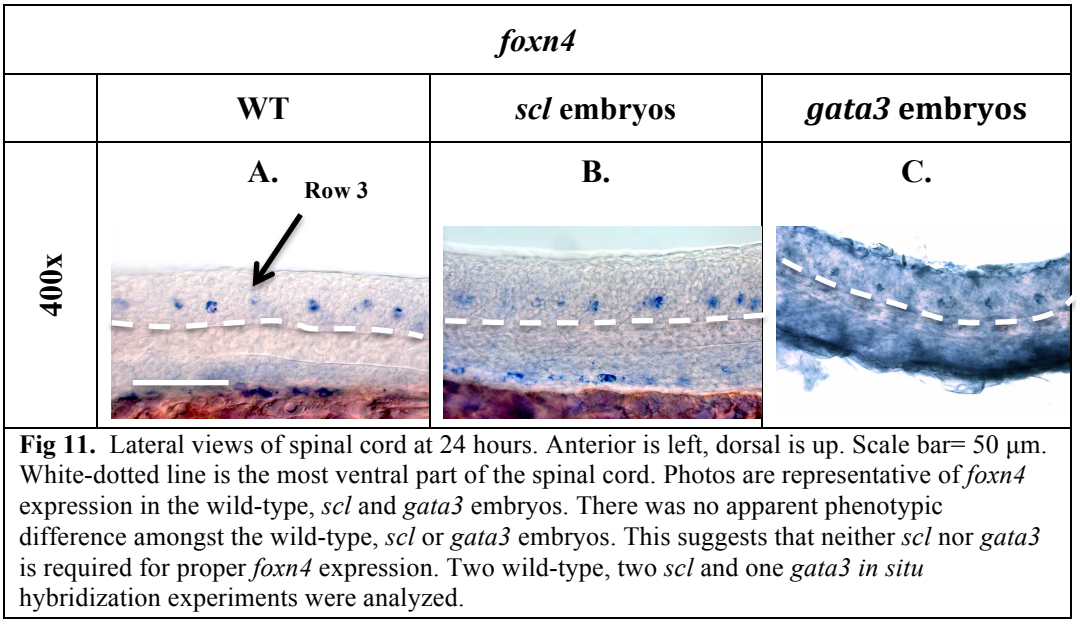


Fig 11. Lateral views of spinal cord at 24 hours. Anterior is left, dorsal is up. Scale bar= 50 µm. White-dotted line is the most ventral part of the spinal cord. Photos are representative of *foxn4* expression in the wild-type, *scl* and *gata3* embryos. There was no apparent phenotypic difference amongst the wild-type, *scl* or *gata3* embryos. This suggests that neither *scl* nor *gata3* is required for proper *foxn4* expression. Two wild-type, two *scl* and one *gata3 in situ* hybridization experiments were analyzed.

Reduction of *gad* expressing cells in *scl* and *gata3* mutant embryos

gad65, *gad67a*, and *gad67b* are GABAergic (inhibitory) markers that label GABAergic cells in the zebrafish spinal cord. Previous work has demonstrated that *gads* (*gad65*, *gad67a*, and *gad67b*) are expressed by V2b interneurons (Batista et al., 2008; Higashijima et al., 2004). Given that *gads* are expressed by V2b cells and V2b cells co-express *scl* and *gata3*, I wondered if there would be a reduction of *gad* expressing cells in *scl* or *gata3* mutant embryos. To answer this question, I used in *situ* hybridization and a mixture of RNA probes *gad65*, *gad67a*, and *gad67b* to look for changes in the expression of these genes in *scl* or *gata3* embryos.

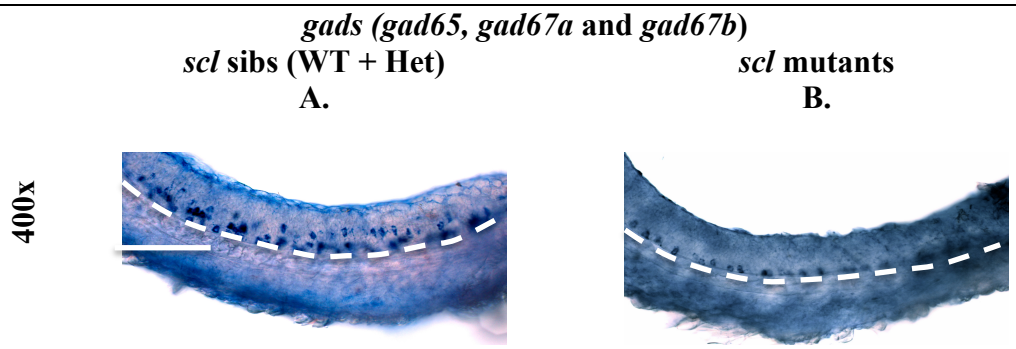


Fig 12. Lateral views of spinal cord at 24 hours. Anterior is left, dorsal is up. Scale bar= 50 µm. White-dotted line is the most ventral part of the spinal cord. Expression of *gad* in presumed *scl* sib and mutant embryos. There appears to be a reduction in *gad* expressing cells in *scl* mutant embryos. In the *scl* mutant embryo, expression is localized in the KA region. This suggests that *scl* is required for proper *gad* expression. Two *in situ* hybridization experiments were analyzed.

To determine if the loss of function of *scl* or *gata3* affected the number of GABAergic cells, *in situ* hybridization experiments were used. Two *scl* and two *gata3 in situ* hybridization experiments were analyzed for this paper. As expected, cells expressing *gads* were found in the V2b and KA regions as both of these cell types are GABAergic (**Fig 12** and **Chart 7**) (Batista et al., 2008; Bernhardt et al., 1992; Higashijima et al., 2004a). However in the presumed *scl* or *gata3* mutant embryos, *gad* expression was almost exclusive to the KA region. This data suggests that in the absence of *scl* or *gata3,* V2b cells expressing *gads* are lost, reduced or are no longer GABAergic. This data is consistent with the Yang et al., 2006 order of expression schematic (**Fig 3**) that speculates that *gads* may be expressed downstream of *scl* and *gata3.*

To confirm that there was a reduction of *gad* expressing cells in the presumed *scl* or *gata3* mutant embryos cell counts of rows 1-3 were done (**Chart 6** and **Chart 7)**. 5 presumed *scl* siblings and mutant embryos were counted from the two *in situ* hybridizations experiment. Two embryos were counted from one *in situ* hybridization experiment and three embryos were counted from the other. 5

presumed *gata3* siblings and mutant embryos were counted from the one *in situ* hybridization experiment.

The frequency of presumed *scl* mutants was approximately 23% (8 out of 36 and 6 out of 27 embryos were thought to be *scl* mutants). A Chi-square test of the total cell counts was performed to determine if the frequency of presumed *scl* mutant embryos was accurate. The null hypothesis was that there would be no significant difference in the number of observed and expected presumed *scl* sibling or mutant embryos and *gata3* sibling or mutant embryos. The p value for the *scl* embryos were close to 0.70, which is greater than 0.05 (x^2= 0.22683). This data suggests that the deviation between the observed and expected values is small enough that chance may account for it (**Chart 6**).

The frequency of presumed *gata3* mutants was approximately 20% for each *in situ* hybridization (3 out of 15 and 4 out of 22 embryos were thought to be *gata3* mutants). This frequency was a bit low in comparison to the 25% of *gata3* mutants expected. A Chi-square test was performed to determine if the frequency of *gata3* mutant embryos was accurate. The null hypothesis was that there would be no significant difference in the number of observed and expected presumed *gata3* sibling or mutant embryos. The p value for the *gata3* embryos were close to 0.50, which is greater than 0.05 (x^2= 0.72973). This data suggests that the deviation between the observed and expected values is small enough that chance may account for it (**Chart 7**).

A Student t-test was performed to determine if there was a significant difference between the total cell counts of the presumed *scl* or *gata3* siblings and

mutants. My null hypothesis was that there wouldn't be a significant difference in the number of *gad* expressing cells in the presumed *scl* or *gata3* sibling and mutant embryos. The p values for comparing the *scl* or *gata3* siblings and mutants were 2.9073E-05 and 0.00066, respectively. These p values were less than 0.05, suggesting that there is likely a significant difference between the total cell counts of the presumed *scl* siblings and mutants or presumed *gata3* siblings and mutants (**Chart 6** and **Chart 7**).

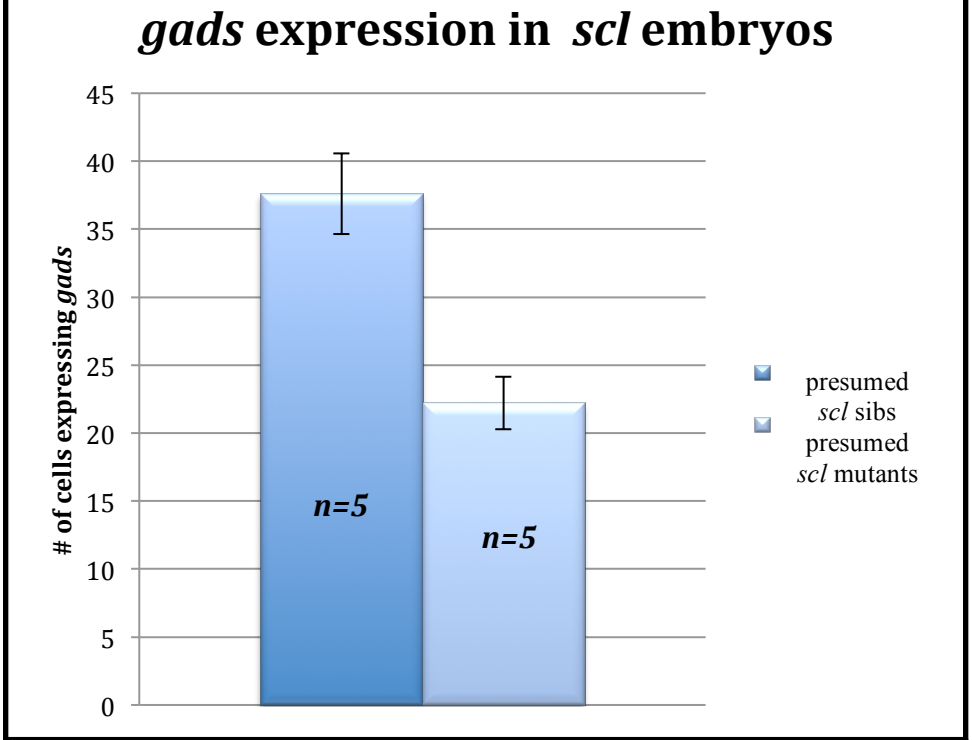


Chart 6. Average cell count of *gad* in presumed *gata3* sibs and mutants. All cell counts are from a 5-somite length of spinal cord adjacent to somites 6–10. Values shown are the mean from 5 different embryos from two *in situ* hybridization experiment. Three embryos were from one *in situ* hybridization and two were from another. Standard deviation was ± 2.966479395 for presumed gata3 sibs and ± 1.923538406 for presumed *gata3* mutants. A Student t-test was performed to determine if the results were significantly different. The p value was 2.9073E-05, which was less than 0.05. This shows that the results were significantly different.

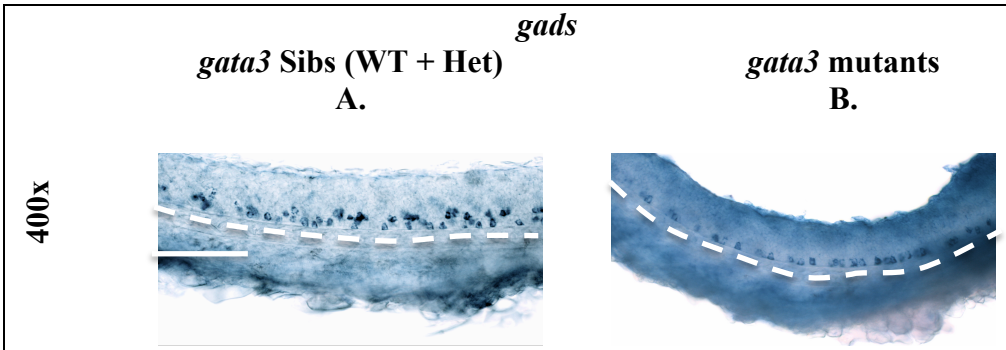


Fig 13. Lateral views of spinal cord at 24 hours. Anterior is left, dorsal is up. Scale bar= 50 µm. White-dotted line indicates the most ventral part of the spinal cord. Expression of *gad* in presumed *gata3* sib and mutant embryos. There appears to be a reduction in *gad* expressing cells in *gata3* mutant embryos. In the *gata3* mutant embryo, expression is localized in the KA region. This suggests that *gata3* is required for proper *gad* expression in V2b cells. Two *in situ* hybridization experiments were analyzed.

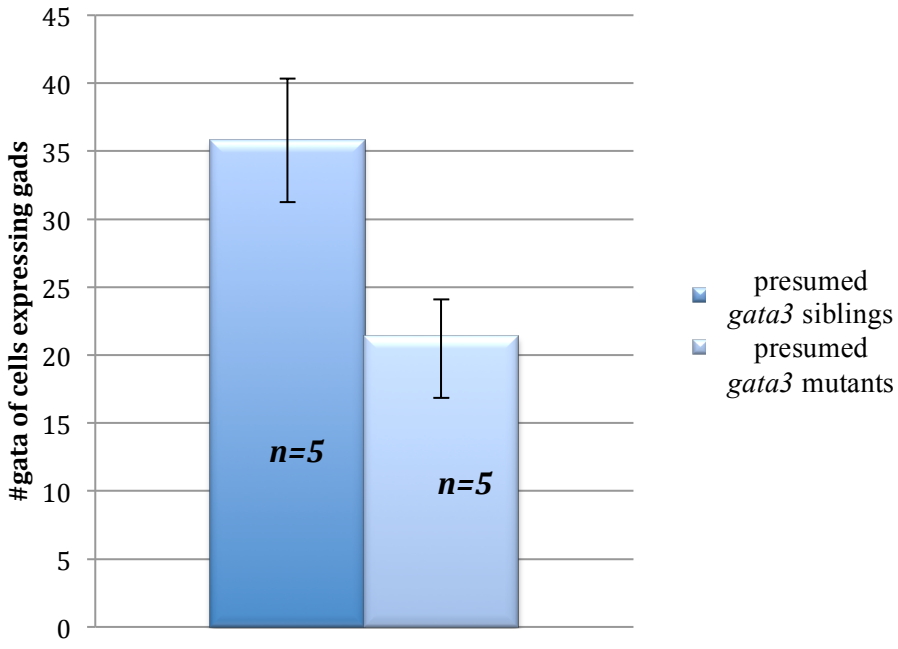


Chart 7. Average cell count of *gad* in presumed *gata3* sibs and mutants. All cell counts are from a 5-somite length of spinal cord adjacent to somites 6–10. Values shown are the mean from 5 different embryos from two *in situ* hybridization experiment. Three embryos were analyzed from one *in situ* hybridization and two embryos were analyzed from the other. Standard deviation was ± 4.549725266 for presumed *gata3* sibs and ± 2.701851217 for presumed *gata3* mutants. A Student t-test was performed to determine if the results were significantly different. The p value was 0.0006 , which was less than 0.05. This shows that the results were

Expression of *islet 1/2* is unaffected by the loss of function of *scl* and *gata3*

Due to the close proximity of V2 cells and motor neurons, I thought that it would be interesting to test if the loss of function of *scl* or *gata3* led to an expansion of motor neurons. In this experiment, I used *in situ* hybridization and the RNA probes *islet 1/2* (genes expressed by motor neurons) to test this question (Appel et al., 2005). The result of the experiment was that there were no phenotypic difference of cells expressing *islet 1/2* in the zebrafish spinal cord of the *scl* or *gata3* embryos. There were 38 embryos from the *scl in situ*

hybridization experiment and 27 from the *gata3 in situ* hybridization experiment. 12 embryos were analyzed from each experiment. This data suggests that the loss of function of *scl* or *gata3* does not affect motor neuron cell fate or cause V2 cells to transfate into motor neurons. To confirm these results, cell counts of *islet 1/2* cells are needed.

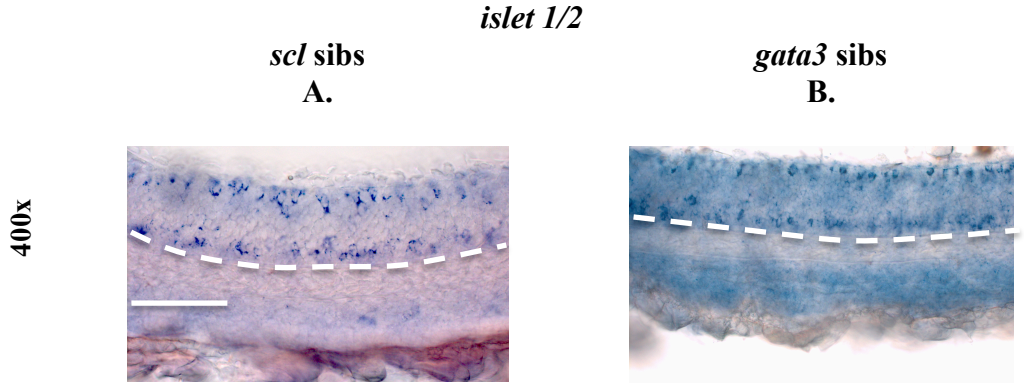
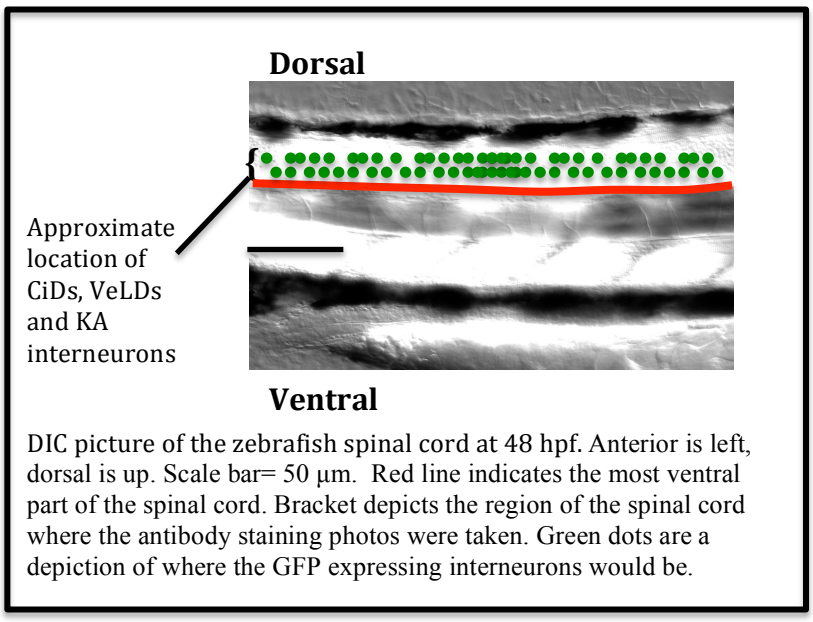


Fig 14. Lateral views of spinal cord at 24 hours. Anterior is left, dorsal is up. Scale bar= 50 µm. White-dotted line indicates the most ventral part of the spinal cord. There appeared to be no reduction of *islet 1/2* expressing cells in the *scl* and *gata3* embryos. Two *in situ* hybridization experiments were analyzed.

scl loss of function affects the presence of V2b interneurons

The *gata3, sox1b* and *gad in situ* hybridization experiments suggested that *scl* may be required for the expression of these genes. Specifically, the *gata3 in situ* hybridization experiment suggested that *scl* is required for *gata3* expression in V2b cells. To determine if *scl* is required for the proper morphology of VeLDs (V2b interneurons), I used antibody staining and *scl*;Tg(*gata1*:GFP), to determine if the loss of function of *scl* affected the morphology of V2b interneurons in the spinal cord. Tg(*gata1*:GFP) is a transgenic line that is driven by a *gata1* promoter and it has been shown to label *gata3* expressing cells in the spinal cord (Batista et al., 2008). Instead of seeing a difference in axon morphology in *scl* $^{-/-}$;Tg(*gata1*:GFP) embryos, my results show a reduction or loss of GFP-labeled

V2b interneurons in the spinal cord. This suggests that *scl* is required for proper V2b development or maintenance. Alternatively, it could be the that *scl* is required for the proper expression of the transgene. Additionally, *scl* $^{-/-}$ *;*Tg(*vsx2*:GFP) embryos were used to determine if the loss of function of *scl* may drive the V2b cell fate to V2a cells. Tg(*vsx2*:GFP) is a transgenic line that labels V2a cells. The data from my results showed that there was no obvious expansion in Tg(*vsx2:*GFP) expressing cells. This data suggests that loss of function of *scl* does not result in V2b cells becoming V2a cells.



DIC picture of the zebrafish spinal cord at 48 hpf. Anterior is left, dorsal is up. Scale bar= 50 µm. Red line indicates the most ventral part of the spinal cord. Bracket depicts the region of the spinal cord where the antibody staining photos were taken. Green dots are a depiction of where the GFP expressing interneurons would be.

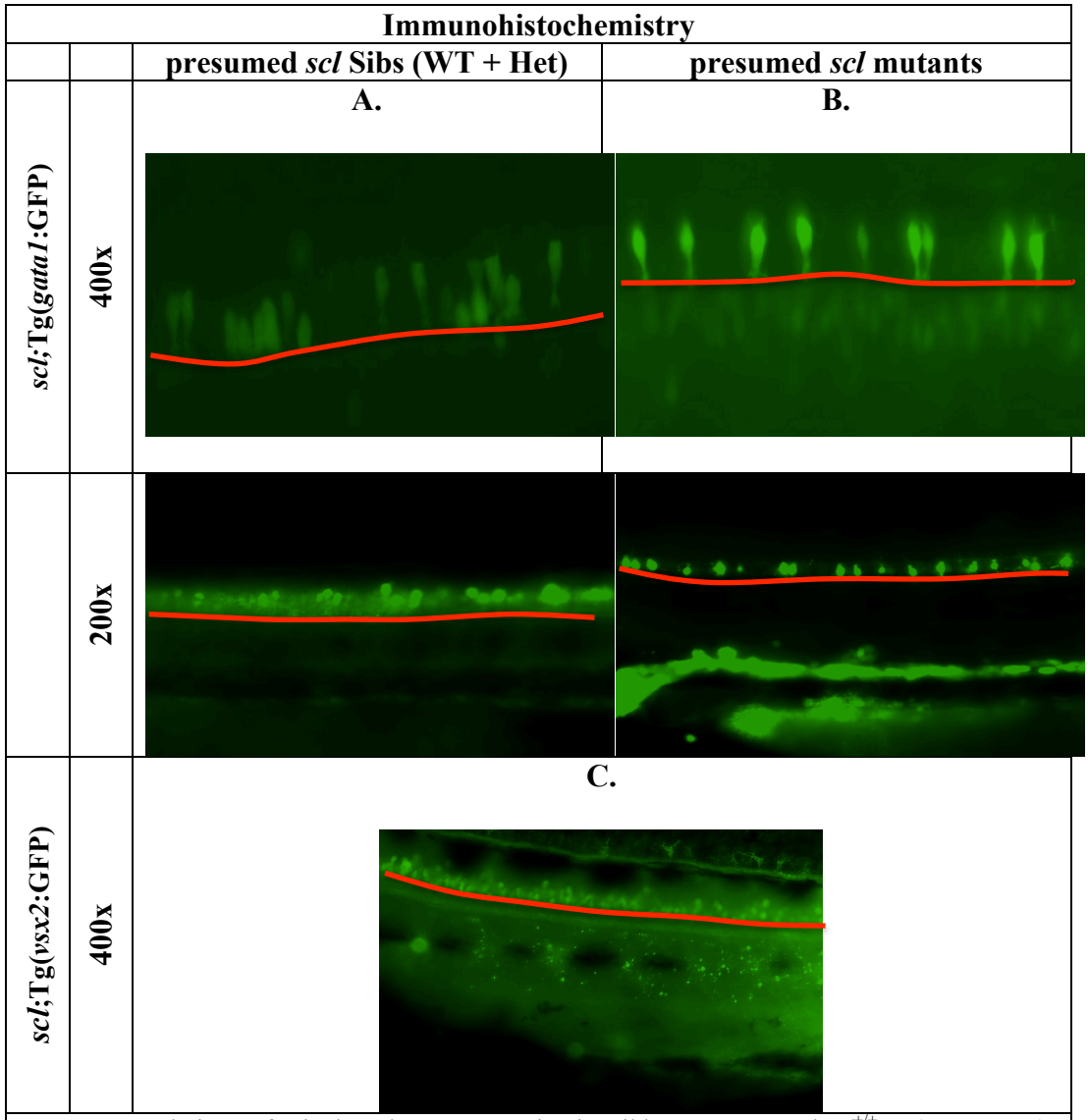


Fig 15. Lateral views of spinal cord gene expression in wild-type, presumed $scl^{+/+}$;Tg(*gata1:*GFP), $scl^{-/-}$;Tg(*gata1:*GFP) and $scl^{+/+}$;Tg(*vsx2:*GFP) embryos at 48 hours. Anterior is left, dorsal is up. Red line is an assumption of the most ventral position of the spinal cord. *scl*; Tg(*gata1:*GFP) photos are taken from two antibody staining experiments. Magnification of scl; Tg(*gata1:*GFP) is at 200x and 400x whereas scl; Tg(*vsx2:*GFP) is at 200x. Cells express either gata1:GFP or *vsx2:*GFP. There was no expansion or reduction of GFP transgene expressing cells in the $scl^{+/+}$;Tg(*vsx2:*GFP) embryos, whereas there was a reduction of *gata1* GFP expressing cells in the presumed $scl^{-/-}$;Tg(*gata1:*GFP) embryos. Better photos would have included *scl*;Tg(*vsx2:*GFP) at 400x.

Discussion

scl may be required for *gata3* expression in V2b cells

One of the objectives for this project was to determine if the loss of function of *scl* affected the expression of other genes. It is already known that *scl* and *gata3* are co-expressed in the V2b cells and *scl* is expressed before *gata3* (Batista et al., 2008; Karunaratne et al., 2002; Kimura et al., 2006; Li et al., 2005; Muroyama et al., 2005; Smith et al., 2002). However, it was uncertain whether *scl* is required for the proper expression of *gata3.* In this study I was able to recapitulate preliminary data from the Lewis lab that suggested that *scl* is required for proper *gata3* expression (Jacobstein, 2008). Approximately 25% of the presumed *scl* mutants had a severe reduction of *gata3* expressing cells in the V2 region. Yet, expression of *gata3* in the KA region remained the same. The result of this experiment was not only consistent with preliminary data, but it expanded it. Not only was I able to suggest that there was a severe reduction of *gata3* expressing cells in the spinal cord, but I was able to obtain quantitative values.

Unfortunately, there was not enough time to PCR and DNA sequence the heads of the presumed *scl* sibling and mutant embryos. This information would have allowed me to confirm that the *gata3* phenotypic variance was a result of the embryo's genotype.

Future work for this experiment should include *in situ* hybridization using the RNA probe *gata2, gata3* and *scl.* Since *gata2* is thought to be expressed before *scl* and *gata3,* it would be interesting to see the expression of *gata2* in the *scl* or *gata3* mutant embryos (Batista et al., 2008). Also, *in situ* hybridization

experiments using the RNA probe *scl* in *scl* mutants and the RNA probe *gata3* in *gata3* mutants should be done. This will help us to determine if these genes regulate their own expression. Lastly, an *in situ* hybridization of *scl* in *gata3* embryos will help us determine if *gata3* is needed for the proper expression of *scl*.

sox1a expression is unaffected the by the loss of *scl* or *gata3*

The *in situ* hybridization result for *sox1a* in *scl* or *gata3* embryos was consistent with the data from previous findings in mouse (Panayi et al., 2010). In my work, the expression of *sox1a* seemed to be unaffected by the loss of function of *scl* or *gata3.* This data suggest that neither *sox1b* nor *gata3* is required for the expression of *sox1a.* Since *scl* or *gata3* mutant embryos could not be determined in this experiment, future work should include PCR and DNA sequencing. PCR and DNA sequencing would identify the genotypes of the *scl* or *gata3* embryos. With this information, precise analysis of *sox1a* expression in *scl* or *gata3* embryos could be made. If this analysis is consistent with mine, future studies using, *in situ* hybridization, can test if *sox1a* labels a distinct class of V2 cells. Hopefully, this testing would confirm the presence of V2c cells in the zebrafish spinal cord.

Loss of function of *scl* and *gata3* reduced *sox1b* expressing cells

The notion to test *sox1b* expression in the zebrafish spinal cord arose from the Li et al., 2010 paper, that referenced *sox1*as a marker for V2c cells in the mouse spinal cord. Prior to my *in situ* hybridization experiment, there was only one paper that referenced *sox1b* expression in the zebrafish (Okadu, et al., 2006).

However, Okadu, et al., 2006 focused primarily on the expression of *sox1b* expression in the zebrafish's forebrain. Based on the Panayi et al., 2010 data, I expected *sox1a* and *sox1b* expressing cells to be positioned in the V2 domain. However, I was unsure if the *sox1a* and *sox1b* would be affected by the loss of function of *scl* or *gata3*.

The results of my *in situ* hybridization experiments showed *sox1a* and *sox1b* expression in the V2 domain of the zebrafish spinal cord. However, *sox1a* and *sox1b* expression differed because *sox1b* expressing cells were reduced by loss of function in presumed *scl* or *gata3* mutant embryos. *sox1a* appeared to be expressed similarly to *sox1* in mouse, in the sense that it was unaffected by the loss of function of *scl* or *gata3*. However, this cannot be determined definitely until double *in situ* hybridization experiments are performed. These *in situ* hybridization experiments should include *vsx1*, *vsx2*, *gata2, gata3* and *scl* markers to determine if *sox1a* cells are V2a or V2b cells. If *sox1a* expressing cells do not co-express the V2a and V2b cell markers, it can be proposed that *sox1a* must label another class of V2 cells, V2c cells.

scl and *gata3* expressed before *gads*

The reduction of *gad* (*gad65, gad67a, gad67b*) expressing cells in the *scl* or *gata3* mutants was consistent with the data from Peng et al., 2007. In Peng et al., 2007, they showed that *gad67* was downstream of *scl* and *gata3*. Since *gad67* is in the family of *gads* and *gad67* is downstream from *scl* and *gata3,* I expected *gad* expression to be affected by the loss of function of *scl* or *gata3*. My hypothesis

was proven correct by the reduction of *gad* expressing cells in the presumed *scl* and *gata3* mutants. However, I noticed a reduction of *gads* mainly in the V2 region as opposed to the KA region. One explanation for this result is that proper expression of *scl* or *gata3* is not required in KAs in order for *gads* to be expressed.

The reduction of *gata1* GFP labeled cells in *scl* $^{-/-}$;Tg(*gata1*:GFP), suggested that *scl* is required for proper V2b development or maintenance. Alternatively, it could be the that *scl* is required for the proper expression of the transgene.

The *in situ* hybridization experiments of *sox1b* and *gads,* suggest that *scl* and *gata3* are necessary for the expression of these genes. Additionally, antibody staining of *scl* $^{-/-}$; Tg(*gata1*:GFP) suggest that *scl* is required for proper maintenance or development of VeLDs or expression of the transgene. Further analysis is needed to determine the role of *scl* in VeLD development or maintenance. However, it appears that proper *scl* function might be required for the presence of V2b interneurons in the zebrafish spinal cord.

islet 1/2 is unaffected by the loss of function of *scl* and *gata3*

The study of *scl* or *gata3's* effect on motor neurons was due to the close proximity of the V2 and the motor neuron domain. I wanted to test if the loss of function of *scl* or *gata3* resulted in V2b cells expressing *islet 1/2* (motor neuron markers). The result of this experiment was that there was no significant difference in *islet 1/2* expression in *scl* or *gata3* embryos. The lack of significant difference in the *in situ* hybridization experiments, suggests that the *scl* or *gata3*

siblings and mutants express *islet 1/2* similarly. From these results it can be inferred that the loss of function of *scl* or *gata3* does not increase the number of *islet 1/2* expressing cells in the spinal cord. Therefore, loss of function of *scl* or *gata3* does not drive V2b cells to become motor neurons.

foxn4 only labels V2 cells in the spinal cord

The expression of *foxn4* solely in the V2 domain was a very important discovery since we don't know of any other genes that are specifically expressed in the V2 region and not KAs. This probe now gives us the opportunity to study V2 cells without the additional complications of KA interference. Future studies will determine what cell-type *foxn4* expressing cells are. With the use of double *in situ* hybridization and the markers for V2a and V2b cells, this question can be answered.

Conclusion

The purpose of this experiment was to analyze the roles of *scl* and *gata3* in the zebrafish spinal cord interneuron specification and function. With the use of presumed *scl* and *gata3* sibling and mutant embryos, I was able to determine that these genes do affect the gene expression of other genes. Additionally, I was able to propose the hypothesis that *scl* may affect the presence of V2b cells in the spinal cord. This experiment has allowed us to gain a better understanding of how interneurons are specified. Hopefully, future interneuron studies will help us to gain a better understanding of how the neural circuitry of the spinal cord specifies and functions.

Future work

PCR and DNA Sequencing

Throughout this project, the genotype of the embryos was hypothesized based on the staining pattern of the cells. However, we can determine the real genotypes of the embryos by PCR and DNA sequencing. Hopefully, a correlation can be made between the *in situ* hybridization staining pattern and the genotype of the embryos. Additionally, this experiment will allow us to determine the *scl* and *gata3* mutants of the *sox1a, foxn4* and *islet 1/2 in situ* hybridization experiments. With this knowledge, we can determine if there might be slight differences between the mutants and siblings and if my preliminary conclusions are supported.

Future *in situ* hybridization experiments

in situ hybridization experiments using markers for the V2a cells (*vsx1* and *vsx2*) and excitatory markers (*vglut1, vglut2a,* and *vglut2b*), should also be another step. This experiment would help us determine if the loss of function of *scl* or *gata3* causes V2b cells to become V2a cells or switch their neurotransmitter phenotype to Glutamatergic (excitatory).

Future work with *foxn4* and *sox1a*

foxn4 and *sox1a* appeared to be unaffected by the loss of function of *scl* or *gata3.* Interestingly, I was able to discover that *foxn4* was not expressed in the KA domain. Future work with these RNA probes would determine if *foxn4* or *sox1a* expressing cells are V2c cells. A double *in situ* hybridization using V2a, V2b and

motor neurons markers should be used. Hopefully, there will be a lack of co-expression with these markers, suggesting that *foxn4* and *sox1a* label V2c cells in zebrafish.

Future Antibody Staining

The transgenic line *scl*$^{+/-}$*;gata3*$^{+/-}$*;*Tg(*vsx2*:GFP) was created from an in-cross of *scl*$^{+/-}$*;*Tg(*vsx2*:GFP) and *gata3*$^{+/-}$. With the use of antibody staining, this line could be used to determine how the loss of function of *scl* and *gata3* affects the number of CiDs in the spinal cord. Hopefully, data collected from this antibody staining will be consistent with the *scl*$^{+/-}$*;*Tg(*vsx2*:GFP) results, where there was no apparent expansion of V2a cells. Therefore, it could be proposed that the loss of function of *scl* and *gata3* does not drive V2b cells to become V2a cells. *gata3*$^{+/-}$*;*Tg(*gata1*:GFP) and *gata3*$^{+/-}$*;*Tg(*vsx2*:GFP) fish lines should also be created. These transgenic lines will help determine if the loss of function of *gata3* affects the morphology or presence of VeLDs.

Analyzing the Swim Pattern of *scl* mutant embryos

Given that CiDs arise from V2a cells and assist in the escape movement of zebrafish, it would be plausible to hypothesize that VeLDs (V2b cells) may also assist in movement (Ritter et al., 2001). My preliminary observation of startled *scl* mutant embryos at 4 days post fertilization is that they move in a circle motion rather than in a straight line. This restriction of movement is most likely due to the weight of the heart edema or the lack of a swim bladder. However, this may also

suggest that *scl* may play a role in altering VeLD's morphology. Unfortunately adult *scl* mutants cannot be analyzed since they are lethal recessive. Only *scl* embryos younger than 5 days post fertilization can be analyzed. Differences in the swim pattern/movement of these embryos would suggest that VeLDs contribute to the movement of zebrafish. It would also suggest that *scl* severely alters the morphology of VeLDs or causes the reduction/loss of VeLDs in the spinal cord.

Appendix

gata3 may be downstream of *scl*

***gata3* expressed in presumed *scl* siblings**					
	Embryo 1	Embryo 2	Embryo 3	Embryo 4	Embryo 5
count	20	17	15	12	25
avg	17.8				
std	4.9699				

***gata3* expressed in presumed *scl* mutants**					
	Embryo 1	Embryo 2	Embryo 3	Embryo 4	Embryo 5
count	12	10	13	9	11
avg	11				
std	1.58114				
Student t-test	0.00497				

Embryos analyzed for *gata3 in situ* hybridization experiments			
	exp 1		
	scl sibs	scl mu	total number
real	22	6	28
expected	21	7	28
chi square total	0.19048		

sox1a expression is unaffected by the loss of function of *scl* or *gata3*

sox1a		
	scl embryos	*gata3* embryos
Analyzed	12	12

sox1b expressing cells are reduced by the loss of function of *scl*

***sox1b* expressed in presumed *scl* siblings**					
		Row1	Row2	Row3	Total
6-10 som					
Embryo 1		14	7	6	27
Embryo 2		16	8	11	35
Embryo 3		14	6	9	29
Embryo 4		11	5	7	23
Embryo 5		14	7	8	29
Embryo 1		16	5	9	30
Embryo 2		17	4	8	29
Embryo 3		17	8	12	37
Embryo 4		20	3	8	31
Embryo 5		18	6	10	34
Embryo 1		17	9	8	34
Embryo 2		19	10	6	35
Embryo 3		15	2	5	22
Embryo 4		16	5	9	30
Embryo 5		12	5	11	28
average	15.73333333	6	8.466666667	30.2	
standard dev	2.46306	2.20389	1.99523		

***sox1b* expressed in presumed *scl* mutants**					
		Row1	Row2	Row3	Total
6-10 som					
Embryo 1		10	5	3	18
Embryo 2		11	5	4	20
Embryo 3		7	8	5	20
Embryo 1		11	6	5	22
Embryo 2		10	4	6	20
Embryo 3		9	8	4	21
Embryo 1		13	0	6	19
Embryo 2		20	0	1	21
Embryo 3		16	2	3	21
Embyro 4		17	2	1	20
average		12.4	4	3.8	20.2
standard dev		4.06065	2.94392	1.813529	4.90845

Embryos analyzed for *sox1b in situ* hybridization experiments									
	exp 1			exp 2			exp3		
	scl sibs	scl mu	total	scl sibs	scl mu	total	scl sibs	scl mu	total
real	27	5	32	19	7	26	27	7	34
expected	24	8	32	19.5	6.5	26	25.5	8.5	34
Chi square of t	0.92753								
Student t-test	.00055								

sox1b expressing cells are reduced by the loss of function of *gata3*

***sox1b* expressed in *gata*3 siblings**					
	Embryo 1	Embryo 2	Embryo 3	Embryo 4	Embryo 5
	14	14	12	13	10
	6	11	15	5	9
Total	22	25	27	18	19
avg	17.8				
std	4.96991				

***sox1b* expressed in *gata*3 mutants**					
	Embryo 1	Embryo 2	Embryo 3	Embryo 4	Embryo 5
	10	7	12	7	8
	2	3	1	2	4
Total	12	10	13	9	12
avg	11				
std	1.58114				
Student t-test for all	.000182				
Student t-test for row 1	0.01578				
Student t-test for 2/3	0.01511				

Embryos analyzed for sox1b *in situ* hybridization experiments

	scl sibs	scl mu	total	scl sibs	scl mu	total
real	28	6	34	13	2	15
expected	25.5	8.5	34	11.25	3.75	15
chi square total	0.22683					

foxn4 expression is unaffected by the loss of function of *scl* or *gata3*

foxn4		
	scl embryos	*gata3* embryos
Analyzed	12	12

Reduction of *gad* expressing cells in *scl* and *gata3* mutant embryos

***gads* expressed in presumed *scl* siblings**					
	Embryo 1	Embryo 2	Embryo 3	Embryo 4	Embryo 5
count	35	37	35	42	39
avg t	37.6				
std	2.96648				
Student t-test	0.00003				

***scl* embryos analyzed for *gads in situ* hybridization experiments**			
	scl sibs	*scl* mu	total
real	12	3	15
expected	11.25	3.75	15
chi-square	0.05	0.15	
chi square total	0.2		

***gads expressed in* presumed *gata3* mutants**					
	Embryo 1	Embryo 2	Embryo 3	Embryo 4	Embryo 5
count	21	18	25	20	23
avg	21.4				
std	2.70185				
Student t-test	0.00066				

***gata3* embryos analyzed for *gads in situ* hybridization experiments**			
	gata3 sibs	*gata3* mu	total
real	18	4	22
expected	16.5	5.5	22
chi-square	0.13636	0.40909	
chi square total	0.72973		

Expression of *islet 1/2* is unaffected by the loss of function of *scl* and *gata3*

islet 1/2		
	scl embryos	*gata3* embryos
Analyzed	12	12

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Capstone Summary

Introduction

The neuronal circuitry of the spinal cord is an integral component of the vertebrate central nervous system, because it coordinates sensory inputs as well as the movement of an organism. Proper formation and communication of the spinal cord neurons is vital because regeneration of the neurons is almost impossible. However, little is known about the processes that regulate the formation and proper connections of spinal cord neuronal circuitry.

My project specifically focuses on the V2 cells of the zebrafish spinal cord. The V2 cells originate from identical p2 progenitor cells in the spinal cord. As the p2 progenitor cells stop dividing and differentiate (specialize), subsets of these cells start to express different transcription factor genes. The expression of these genes allows the p2 progenitor cells to initially develop into at two sets of molecularly distinct cells, V2a and V2b cells. In zebrafish (*Danio rerio*), V2a and V2b cells differentiate into two functionally distinct classes of vertebrate spinal interneurons. These interneurons are excitatory Circumferential Descending interneurons (V2a) and inhibitory Ventral Lateral Descending interneurons (V2b) (Batista et al., 2008; Bernhardt et al. 1990; Kimura et al., 2006).

Little is known about how the particular functional properties of V2a and V2b interneurons are specified. However, the transcription factors that these cells express as they start to differentiate are thought to specify at least some of the neuronal characteristics that they acquire as they differentiate into functional neurons (Batista et al, 2008). V2a cells are known to express *chx10* and *vsx2*,

whereas V2bs express *gata2, gata3,* and *scl* (Batista et al., 2008; Kimura et al., 2006; Neave et al.,1995; Passini et al., 1998). It is important to note that *gata2, gata3,* and *scl* are also expressed in Kolmer-Agduhr (KA) interneurons (Batista et al., 2008). KAs are a type of interneuron that is located in the most ventral (lowest) part of the zebrafish spinal cord and derive from the p3 domain. Additionally, work in mouse suggests that another class of cells may be present in the V2 region, V2c cells. In mouse, *foxn4 and sox1* have been shown to mark V2c cells (Li et al., 2005; Panayi et al., 2010).

In this study, I determine if *scl* and/or *gata3* are necessary for proper V2b cell development. With the use of *scl* and *gata3* mutants and GFP transgenic lines, I determine that the loss of function of *scl* or *gata3* effects:

- The expression of genes co-expressed in V2b cells
- The presence of V2b cells in the spinal

Furthermore, I use *sox1a* and *sox1b* (comparable to *sox1* in mouse) or *foxn4* RNA probes to determine if *sox1a, sox1b* or *foxn4* are:

- Expressed in the V2 domain
- Affected by the loss of function of *scl* and/or *gata3*

For my research, zebrafish embryos were used for their unique qualities. Unlike mouse, whose embryos must be extracted from the mother and the mother euthanized, zebrafish embryos are easily accessible because eggs are laid outside of the mother's body. Additionally, the zebrafish has a functional spinal cord as early as 24 hours post fertilization. This fast development of the zebrafish spinal cord is one of the main reasons why the zebrafish is an ideal model organism to

study interneuron specification and function.

Methods and Materials

Various materials were used for my experiment. The most important were the zebrafish lines, *scl* and *gata3*. With the use of these fish, I was able to determine how loss of function *scl* or *gata3* affected gene expression or VeLD morphology. One method that I used was *in situ* hybridization. For this project, *in situ* hybridization is an experiment that labels specific cells with a RNA probe. Once these cells are labelled with the specific RNA probe, a staining solution is added. The staining solution allows the cells labelled by the RNA probe to be visualized. Below is a list of the different RNA probes used in this experiment and what zebrafish spinal cord cell they are expressed in.

Spinal Cord Cell	RNA Probe
V2b	*gata3;scl*
V2c (unknown)	*foxn4;sox1a;sox1b*
Motor neurons	*islet 2; islet 2*
GABAergic (inhibitory neurotransmitter)	*gad65;gad67a;gad67b*

Another procedure used in this study was antibody staining. Antibody staining is an experiment that exploits the antibody-antigen relationship. A primary antibody detects a targeted antigen on the cell surface and binds to it. A secondary antibody, with a fluroscent tag, detects the first antibody. UV light is shown on the embryo and the cells that are bound by the antibodies flurosece. This experiment was used to determine if the loss of function of *scl* affected the

morphology of VeLDs.

Results/Discussion

My *in situ* hybridization experiments suggested a severe reduction of cells expressing *gata3,* or *gads* (*gad65, gad67a,* and *gad67b*) in presumed *scl* mutants (embryos were considered presumed *scl* or *gata3* siblings or mutants because their genotype was unknown). This reduction demonstrates that proper *scl* function is required for the proper expression of these genes. Likewise, there was a severe reduction of *sox1b* and *gad* (*gad65, gad67a,* and *gad67b*) expressing cells in presumed *gata3* mutants. This also suggests that *gata3* may be required for the proper expression of *sox1b* and *gad.* The results of my experiment are consistent with previous findings from Yang et al., 2010 that suggested that *scl* and *gata3* were upstream of *gad67b.* Ultimately, this experiment showed that loss of function *scl* or *gata3* affects the expression of other genes.

Future work should determine the genotype of the presumed *scl* and *gata3* siblings and mutants by PCR and DNA sequencing. Also, *in situ* hybridization experiments using markers for V2a, V2b, and motor neurons can give us a better understanding of the roles of *scl* and *gata3* in the spinal cord.

Interestingly, there appeared to be no reduction of *sox1a, foxn4* or *islet 1/2* expressing cells in *scl* or *gata3* embryos, whereas there there was a reduction of *sox1b* expressing cells in presumed *scl* or *gata3* mutants. This suggests that neither *scl* or *gata3* is required for *sox1a, foxn4* or *islet 1/2* expression but they are needed for *sox1b* expression. The results for *sox1a* were expected since

Panayi et al., 2010 suggest that the majority of *sox1* expressing cells do not co-express *gata3* in mouse. However, the reduction of *sox1b* in presumed *scl* and *gata3* mutants suggest that *sox1a* and *sox1b* differ in the zebrafish.

Additionally, *foxn4* expressing cells were only found in the V2 region. This was fascinating, because before this we had yet to find a RNA probe that labels V2b cells and not KAs. My data is also conistent with Li et al., 2010 that suggests that *foxn4* expressing cells co-express with *sox1* in mouse (don't co-express with *gata3* or *scl*). Therefore if this translated to zebrafish, *foxn4* expressing cells shouldn't have been affected by the loss of function of *scl* or *gata3*.

Future work in zebrafish would determine what cells *foxn4* and *sox1a* are expressed in by double *in situ* hybridization (use two RNA probes). This procedure will allow us to determine if they are any other genes that are co-express *foxn4* or *sox1a*. If *foxn4* or *sox1a* cells are shown to not be co-expressed by V2a, V2b or motor neuron markers, it can be propsed that these cells are V2 cells.

in situ hybridization experiments were done using the RNA probes *islet 1* and *islet 2* because V2 cells are immediately dorsal to motor neurons. I wanted to test if the loss of function of *scl* or *gata3* would increase the number of motor neurons (given that it looks like the number of V2b cells may be reduced). The result of this experiment was that there was no significant difference in *islet 1/2* expression in *scl* or *gata3* embryos. This suggests that the loss of function of *scl* or *gata3* does not push V2b cells to become motor neurons or affect the motor neuron cell fate.

Lastly, an antibody staining in *scl*;Tg(*gata1*:GFP) and *scl*;Tg(*vsx2*:GFP) was performed to determine if the loss of function of *scl* affected the morphology of VeLDs. Tg(*gata1*:GFP) is a transgenic line that is driven by a *gata1* promoter and it has been shown to label *gata3* expressing cells in the spinal cord (Batista et al., 2008). Whereas, Tg(*vsx1*:GFP) is a transgenic line that labels V2a cells. Instead of seeing a variance in axon morphology in *scl* $^{-/-}$*;*Tg(*gata1*:GFP) embryos, my results showed a reduction or loss of gata1:GFP-labeled V2b interneurons in the spinal cord. This suggests that *scl* is required for proper V2b development or maintenance. Or this suggests that *scl* is required for the proper expression of the transgene. Additionally, my results from the *scl* $^{-/-}$ *;*Tg(*vsx1*:GFP) embryos showed that there was no obvious expansion in *vsx1:*GFP expressing cells. This data suggests that *scl* must not play a role in allowing p2 cells to become V2a or V2b cells. If *scl* did play a role, loss of function of *scl* should have resulted in the expansion of V2a cells. Future work will look at the loss of function of *gata3* in Tg(*gata1*:GFP) and Tg(*vsx*:GFP) determine if it affects the morphology of VeLDS or increases the number of V2a cells in the spinal cord.

Conclusion

The purpose of this study was to analyze the roles of *scl* and *gata3* in the zebrafish spinal cord interneuron specification and function. With the use of presumed *scl* and *gata3* sibling and mutant embryos, I was able to determine that these genes do affect the gene expression of other several genes. Additionally, I was able to determine that *scl* affects the number of V2b cells in the spinal cord,

suggesting a role in specification and/or maintenance of these cell types. This experiment has allowed us to gain a better understanding of how interneurons are specified. Hopefully, future interneuron studies will help us to gain a better understanding of how the neural circuitry of the spinal cord works.