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Rushay Amarath-Madav December 21, 2018

The development of a temporally controllable lineage system for labeling enteric neural crest precursors in D. rerio and assessment of the possible role of ETV1 as a transcriptional regulator of ENS specification

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An abstract of
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Abstract

The development of a temporally controllable lineage system for labeling enteric neural crest precursors in D. rerio and assessment of the possible role of ETV1 as a transcriptional regulator of ENS specification

By Rushay Amarath-Madav

The enteric nervous system (ENS) makes up the largest and most complex portion of the peripheral nervous system. It is the intrinsic nervous system of gut musculature and is responsible for maintaining gut homeostasis and motility. Complications involving the specification and differentiation of enteric neural crest cells (ENCCs) can lead to the development of Hirschsprung's Disease, and Gastric Esophageal Reflux Disease (GERD), and a number of other diseases related to gut immobility. Here, we aimed to further analyze the lineage of ENCCs during their proliferation and migration from the vagal neural crest to the gut to populate the ENS with up to 17 different neuronal subtypes. Our primary aim was to develop a direct system for labeling enteric neural precursors for temporal analysis of ENS specification. Additionally, we also assessed the role of ETS transcription factor ETV1 in the development of the ENS. Labeling utilizing the Brainbow (PriZm) transgenic line was utilized in labeling studies. A β-actin2 driven multicolor construct was induced to recombine when exposed to Tol2phox2b::Cre-ER constructs treated with (Z) 4-hydroxytamoxifen (4-OHT). Limited recombination has been observed; only some expression of green fluorescence protein (GFP) in a mosaic patterning was seen after 4-OHT treatment. Regarding ETV1, mutants obtained from TILLING were sequenced, and heterozygote mutants were crossed to generate homozygous mutants of ETV1. A distinct no-swim bladder phenotype was seen in the one trial performed thus far at a frequency of 17%. Antibody staining with pan-neuronal markers for Hu and 5-HT were performed, and stained embryos were imaged with a confocal microscope. Images will be used to quantify enteric neurons and assess depletion in the ENS within mutants compared to controls. More trials are needed to further assess the significance of the observed morphant phenotype, as well as a method for genetically proving that suspected mutants are heterozygous.

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Chapter 1: Background

The enteric nervous system (ENS) is an intricate network of neurons that is part of the autonomic nervous system. It is composed of up to 17 different neuronal subtypes responsible for gut function. The ENS functions independently of the central nervous system (CNS), maintaining and regulating intestinal homeostasis and motility[1] [2]. All classes of enteric neurons are distributed equally along the gut and together form an overlapping set of neural networks. The cooperative nature of these interacting networks is what drives motor patterns seen in the gut more generally known as peristalsis[3]. Problems with the specification and differentiation of enteric neurons has been seen to compromise gut motility and digestion. Some congenital disorders that cause peristaltic inhibition (e.g. Hirschsprung's Disease) result from abnormal development of the ENS network[4]. Further understanding lineage patterning and transcriptional regulation within ENS specification can help further define the causes of less understood disorders that involve improper ENS development or function.

Additional findings have brought to attention a group of mesenchymal derived cells known as the Interstitial Cells of Cajal (ICC). These cells are believed to help facilitate smooth and rhythmic depolarizations that establish peristalsis via smooth muscle contraction along the gut in conjunction with more intricate contractile patterns derived from functional ENS signaling.[5] The complex interconnected circuitry within the ENS ranges through a number of mucosal and muscular layers within the gut architecture (Figure 1).

The Shepherd lab is primarily interested in neural development, and more specifically, the specialization and differentiation of various enteric nervous system (ENS) neurons and glia, using *Danio rerio* (*D. rerio*), more commonly known as the zebrafish. In the past two and a half decades *D. rerio* has emerged as an important biomedical model system for vertebrate species

studies. Numerous factors give the zebrafish a distinct advantage for research. *D. rerio* are not only genetically tractable, but they are more economical for both breeding and keeping than other vertebrate model systems. Furthermore, they are relatively easy to use in large-scale genetic screening studies and can be easily genetically manipulated permitting increased efficiency in experimental procedures[6]

Regarding ENS architecture, the neural network structure within the zebrafish gut is simpler in than that of their mammalian counterparts.[7] [8] Examples of differences include the lack of a of submucosal layer and associated neuronal plexus within the gut. There is also a notable lack of a ganglionated myenteric plexus within the teleost model as compared to that of the mammalian one. Additionally, differences include a simpler mucosal surface structure with the teleost surface consisting of large folds instead of the characteristic villi, which are seen in mammals (Figure 1).

FIGURES PENDING APPROVAL

Figure 1. ENS circuitry and gut architecture in the mammalian and teleost model

(A) A simplified view of the ENS circuitry gives insight into the complex interactions between various enteric neuronal classes within the mammalian model. These neurons innervate multiple layers within the gut, including the longitudinal muscle (LM), myenteric plexus (MP), circular muscle (CM), and submucosal plexus (SMP). (B) Mammalian gut architecture is more complex than that of the teleost.

Image source: Bertram G. Katzung. Anthony J. Trevor: Basic and Clinical Pharmacology, 13th Ed.

Submucosal and myenteric ganglia exist as clusters of innervating neurons within the gut. (B) Villi are present on the luminal surface of the mammalian gut. (C) Teleost gut architecture lacks a muscularis layer as well as a ganglionated MP. Instead, the gut is singly innervated and has a thin layer of connective tissue connecting mucosa to circular smooth muscle. The luminal surface in the teleost model consists of large folds in epithelial tissue as opposed to villi.

Image source: Shepherd, I. & Eisen, J., 2011; Adapted from: Wallace, K.N. et. al, 2005

The entire ENS in all vertebrate species including zebrafish is derived from a transient embryonic stem cell population known as the neural crest. Specifically, the ENS primarily arises from a discrete axial population of cells from the vagal region (Figure 2). Cells that are derived from the neural crest that give rise to the ENS are known as enteric neural crest cells (ENCC's). Substantial proliferation and differentiation of these ENCCs will form the interconnected nervous network along the gut.[9]

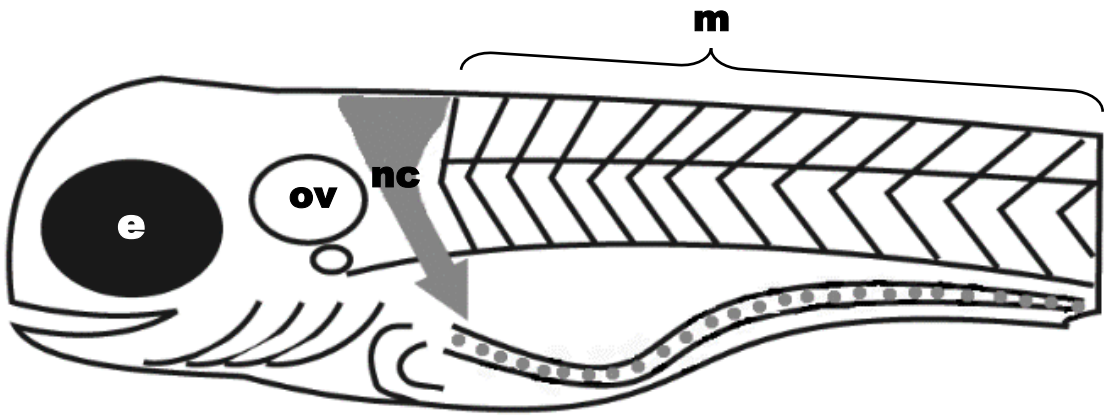

Figure 2. Zebrafish neural crest proliferation and migration visualization

(A) The vagal neural crest region (nc) is defined just posterior to the otic vesicle (ov). From here, neural crest cells that have gone through an epithelial to mesenchymal transformation become ENCCs that migrate along the entire length of the developing embryonic gut to populate and innervate the gut (punctate patterning in gut tube). The eye (e) is identified anterior to the crest region. Mesodermal cells (m) that further develop and constitute the sclerotome and derma myotome are just ventral to the crest region.

Graphic adapted from the Shepherd Lab

While research efforts to better understand ENCC specification are afoot, a clear understanding of how these ENCC's are specified and determined to give rise to the many different subtypes of the ENS does not exist. Two plausible models of fate specification of ENS neuron subtypes have been postulated: the stochastic model and the fated model (Figure 3) (Harrison, C. & Shepherd, I.T., 2013). Recent studies in mice have brought a better understanding of ENCC lineage patterns (Lasrado, R. et. al, 2017). Furthermore, variable expression patterns of key developmental genes are seen to drive ENS specification. While there is an increasing understanding of how the onset specification in the ENCC specification occurs, more work needs to be done to further define the mechanisms that regulate and determine how

these neural precursors are specified into their various subtypes (Figure 4). Possible intermediate checkpoints may exist that further divide the 17 ENS subtypes into smaller groups with similar lineage patterns. Establishing an effective labeling system for temporal analysis of ENCC proliferation and differentiation will help bring a greater understanding to the developmental steps between neural precursors and ENS neurons[10].

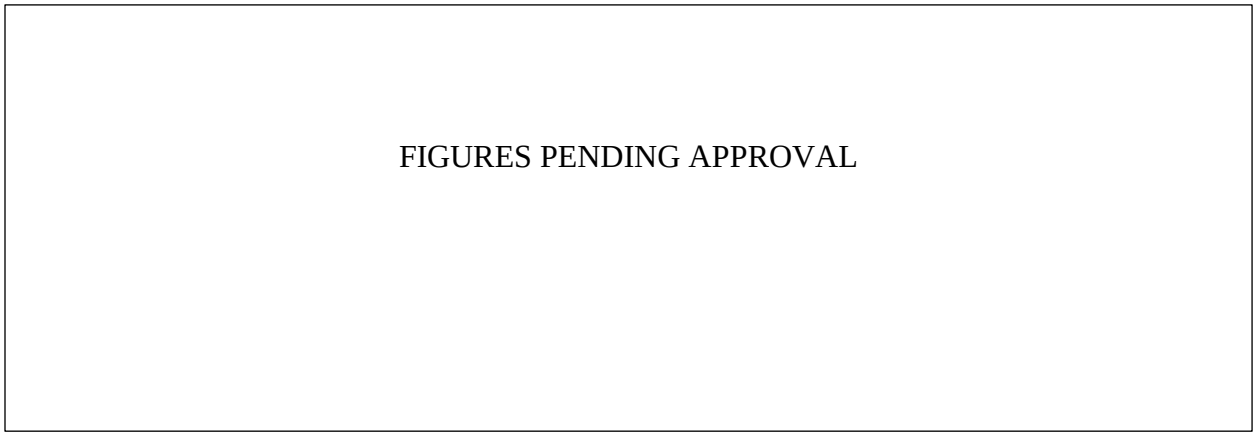

Figure 3. Proposed models for ENS neuronal specification within the gut

Two mechanistic models for ENS neuronal subtype specification have been proposed. The different shapes represent different ENS subtypes, and different colors represent an individual clone of cells that is derived from a single precursor cell. The stochastic model (left) infers that ENCC specification is not predetermined within the neural crest. Thus, ENCCs arising from a specific region within the neural crest can give rise to multiple neuronal subtypes. The fated model (right) describes the mechanisms of specification where different ENs neuronal cell fates are predetermined in the vagal neural crest prior to its migration to and along the gut. According to the fated model, a single labeled neural crest in the pre-migratory vagal neural crest will be already specified to give rise to a specific ENS subtype, so the cells descendent of said cluster are only able to develop into one specific neuronal subtype.

Image source: Harrison, C. & Shepherd, I.T., 2013

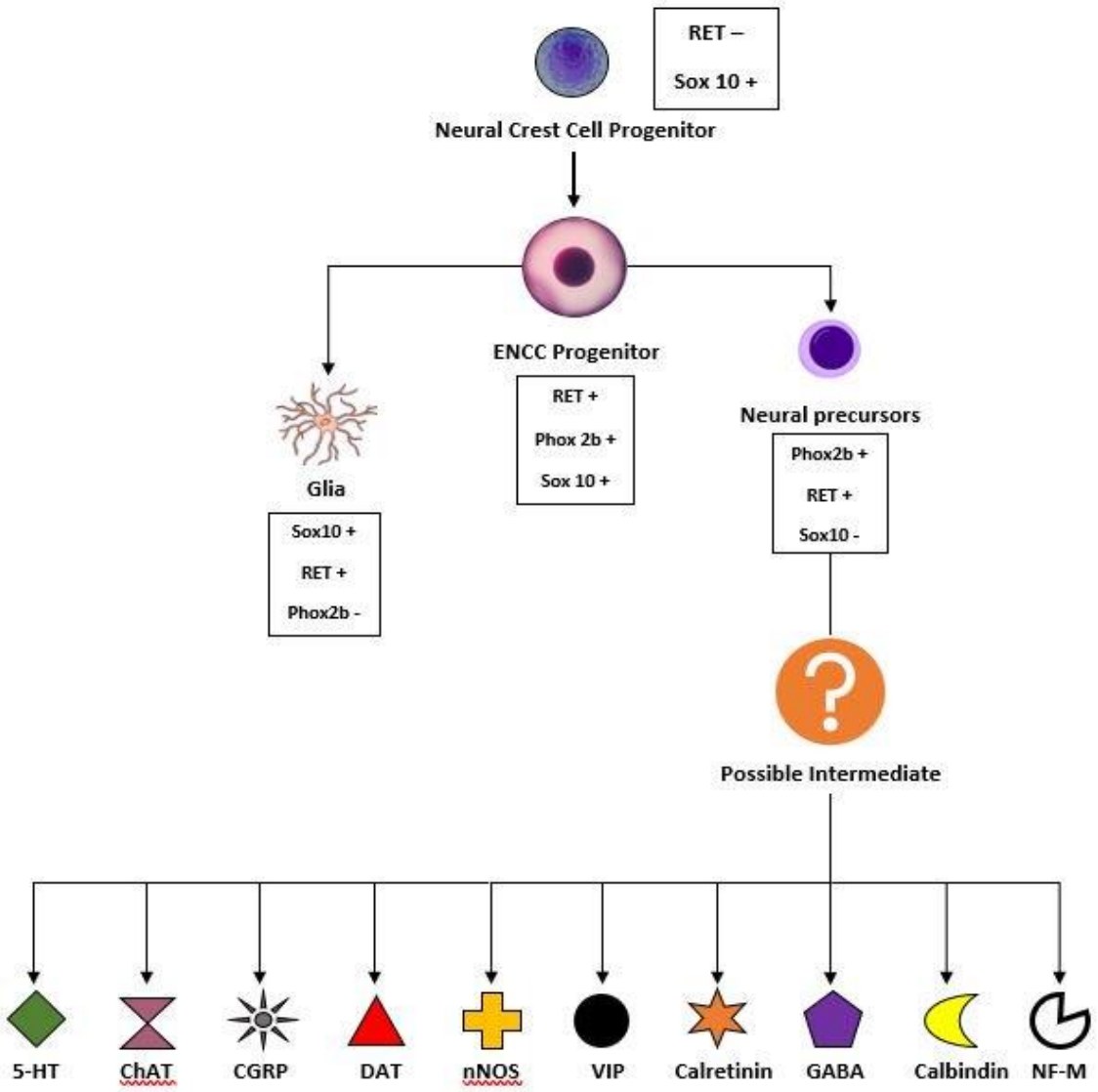

Figure 4. Variable expression pattern lineage map of ENCC differentiation

The variable expression patterns of key developmental genes drive the specification of neural crest precursors into various enteric neurons and glia. *Ret* and *Sox10* expression specifies glial cells. *Ret* and *Phox2b* expression specifies neural precursors. Currently, little is known about the possible intermediate checkpoints between the neural precursor level and the various neuronal subtypes. An intermediate may further separate neuronal subtypes into various subclasses based on their lineage history.

Adapted from Lasrado, R. et. al, 2017

The primary aim of this thesis is to establish a fish line that will permit a temporal study of ENCC specification using the colorful array of cell types established within the PriZm line. Evidence supporting either of the proposed models for ENS neuronal subtype specification should be easily determined from these experiments. A second aim of this project was to exploit recently published data that used an alternative method to gain insights into the neuronal differentiation process in the ENS. This second approach involves undertaking an expression analysis of ENCCs at various time points during ENS development. This approach potentially can reveal characteristic signatures that correspond to specific neuronal subtypes. While a complete systematic analysis of ENCCs at the single cells level at different embryonic ages during ENS development has not, to date, been undertaken a recent study analyzing the transcriptional profile of groups of ENCCs at different ages has provided some evidence of potential new transcriptional regulators that are involved in ENS development. These potentially may help determine specific subsets of ENS neuronal subtypes. For example, the transcription factor Sox6 is thought to be vital in the specification of gastric dopaminergic neurons.[11] Previous studies have outlined transcription factors FoxD3, Pax3, Phox2b, Sox10, and Zeb2 as crucial to ENS development (Table 1). Mutations to these key genes have resulted in an array of deficiencies from total aganglionosis of the gut to neuronal deficiency throughout the length of the GI tract in mice.[12] Furthermore, the Memic et al. study specifically revealed a potential role of another transcriptional factor of the ETS family, ETV1, as an early neuronal marker of the ENS neurons. Based on this new data, we decided to further investigate the role of ETV1 in zebrafish ENS neuron subtype specification**.**

Gene	Developmental Effects on ENS	Effect on Mature ENS
FoxD3	Affects the maintenance of progenitors and their proliferation. Involved in neural patterning and glial differentiation	Lack of neurons in the entire gastrointestinal track.
Pax3	ENCCs fail to enter fetal intestine.	Total intestinal aganglionosis in homozygotes. Perinatal lethality with colonic aganglionosis.
Phox2b	Impaired neuronal differentiation and decrease proliferation of the enteric ganglion progenitors	Perinatal lethality in heterozygotes. Total intestinal aganglionosis.
Sox10	Depletion of ENCCs. Delayed migration in heterozygotes. vagal cell death in homozygotes.	Variable aganglionosis in heterozygotes. Complete aganglionosis in homozygotes.
Zeb2	Vagal neural crest cells do not develop or delaminate from the neural tube.	Homozygote lethality

Table 1. Single gene mutations in genes for various transcriptional factors and their effects on ENS development in mice

Chapter 2: Development of a temporally controllable lineage system for labeling enteric neural crest precursors in D. rerio

Introduction

D.rerio is an excellent model system for studying vertebrate development on a cellular and molecular level due to embryo transparency and accessibility. The use of fluorescent dyes to trace cell lineage within zebrafish was pivotal in establishing the fate maps that have been used in numerous zebrafish development studies.[13] The later discovery of fluorescent proteins – accompanied by more advanced imaging technology and other methodologies— has been revolutionary and allowed researchers to further visualize various cellular subtypes and developmental processes temporally in living embryos. One advancement included the discovery of the Tol2 transposon that permitted a more efficient establishment of transgenic zebrafish lines. Using this technique, one can quickly generate lines of zebrafish with tissue-specific fluorescent reporters. These lines are extremely useful in visualizing specific cell types.[14] Initially these lines only expressed green fluorescent protein, making their use in lineage analysis limited. To overcome difficulties associated with having only a single fluorochrome, multi-colored reporter lines have been generated.

The transgenic approach generated by Livet, J. et. al to visualize various neuronal subtypes within the zebrafish ENS was published in 2007. Their work gave the field the ability to establish combinatorial expression patterns of various fluorescent proteins by utilizing the Cre/lox recombination system.[15] Gupta and Poss applied this approach to analyze cardiomyocyte lineage specification within zebrafish.[16] [17] The PriZm transgenic lines used by Gupta & Poss contain a *β-actin2* driven multicolor construct that can be induced to recombine when crossed with a strain of fish that express a tissue specific tamoxifen-inducible Cre

recombinase (Cre-ER) (Figure 5). Additionally, studies have also worked to optimize Cre induction by utilizing the zebrafish *ubiquitin B* (*ubi*) driver.[18] These transgenics, known as Zebrabow lines, showed higher expression levels after induction.[19] In this study PriZm lines are used to visualize and quantify the behaviors of small clones of labelled ENCCs during migration, proliferation and differentiation stages of ENS development.

Depending on the developmental stage at which recombination of the Brainbow cassette is induced, experimentation should result in ENCCs expressing a range of fluorescent proteins. It should then be possible to determine whether ENS subtype specification in zebrafish is stochastic or a fated. Temporal analysis henceforth will potentially give new insights into physiological abnormalities and disorders that involve compromised ENS development and function.

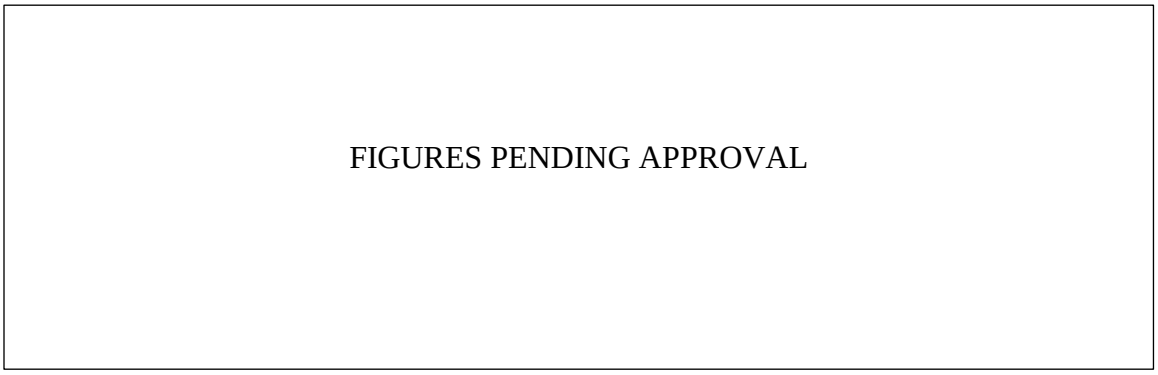

Figure 5. Brainbow fluorescence cassette and accompanying expression

(A) Specific arrangement of fluorescent protein precursors and their Lox active sites. Lox sites pictured are recognized by Cre recombinase and can result in varying degrees of fluorescent protein expression in a mosaic fashion. (B) Default fluorochrome expression is red (RFP); Cre induction leads to the expression of other colors like blue (CFP), and yellow (YFP) in subsequent generations of cells. The creation of an array of fluorescent reporters can help distinguish cellular subtypes within studies.

Image source: Gupta, V. & Poss, K.D., 2012

Methods

Zebrafish

PriZm fish were crossed to generate large clutches of progeny that express the Brainbow fluorochrome cassette under control of the β-actin promoter. The PriZm line was generously provided by the Poss Lab at Duke University.[20] Embryos derived from crosses of the PriZm^{pd49} allele were injected. sorted at 24hpf to identify RFP expressing embryos from non-RFP using an Olympus U-HGLGPS UV light burner in conjunction with an Olympus SZX12 dissecting microscope. PriZm zebrafish have default red fluorochrome expression and were sorted accordingly to include only embryos expressing RFP. Injected and uninjected control PriZm embryos were stored in a 28℃ incubator to further develop. Non-RFP expressing embryos were euthanized following standard protocols.

DNA Preparation and Microinjection of Embryos

A *Tol2phox2b::Cre-ER* construct was generated and amplified within *E. coli.* This construct along with synthetic RNA encoding the Tol2 transposase was injected into PriZm embryos at 1-4 cell stage. Both the DNA reporter construct and the Tol2 transposase RNA were injected at a concentration of 12.5 ng/µl. The dosage used within this experiment was a half dosage of an experimentally determined concentration of 25 ng/µl used in previous Tol2 studies (Kawakami, K., 2004). To ensure Cre-ER incorporation into host genome was successful, embryonic DNA extraction was performed on embryos after 48hpf following standard protocol. Polymerase chain reactions (PCR) were run with the following primers obtained from Integrated DNA Technologies (IDT): Cre ORF— Forward: 5'—CAT TTG GGC CAG CTA AAC AT—3';

Reverse: 5'—TGC ATG ATC TCC GGT ATT GA—3'. DYAD Engine PTC 220 thermal cyclers by MJ Research Inc. were run with an annealing temperature of 53℃ and an extension time of 1 minute for 50 cycles. As a second confirmation for incorporation, reverse transcription polymerase chain reaction (RT-PCR) will be performed in the future at 48hpf to test for CRE mRNA presence. Conditions for RT PCR will be 60 cycles at an annealing temperature of 53℃ with an extension time of 3 minutes. Cre ORF primers will be utilized here as well.

Tamoxifen treatment

Brainbow embryos were treated with (Z)4-hydroxytamoxifen(4-OHT) (Sigma Aldrich) dissolved in 70% ethanol. A final concentration of 10 – 15 µM of 4-OHT solution in 3 ml of embryo medium with 1-phenyl 2-thiourea (PTU) was prepared. 4-OHT in the Z form is light sensitive and easily converted to its less active E isomer; therefore, all work was performed in the dark and with time considerations. Aliquots of 4-OHT in ethanol were made the day of treatment, as the instability of (Z)4-OHT permits only short-term storage. Caution was taken to make ethanol and 4-OHT aliquot concentrations strong enough to avoid using too much of the solution in the embryo medium mix during treatment. Volumes of ethanol and 4-OHT over 30 µl in 3 ml of embryo medium proved to be lethal dosages. Embryos were treated with 4-OHT for 15 minutes, then rinsed at least twice and incubated in embryo medium with PTU at 28℃ until imaging. Too long of a 4-OHT exposure time has been shown to result in the reversal of desired effects and failed recombination.[21]

Imaging: Dissecting and confocal microscopy

4-OHT treated embryos at E7 were anesthetized with tricaine and observed with the aforementioned Olympus dissecting microscope and various UV filters to assess recombination success. For further imaging, an Olympus IX2-UCB confocal microscope with a PRIOR Lumen200 lamp box and accompanying Hamamatsu C10600 camera controller was used to capture high resolution images with depth correction using the SlideBookPro software for Windows. Gut dissections were performed in attempt to isolate illuminated enteric neurons and avoid interference of fluorescent cells in surrounding tissue (results shown in this paper were of guts that were not dissected from embryos).

Results/Discussion

Cre-ER incorporation

Cre-ER incorporation into host genome was successful. This suggests that unsuccessful recombination results are not due to failure of construct microinjection and integration (Figure 6).

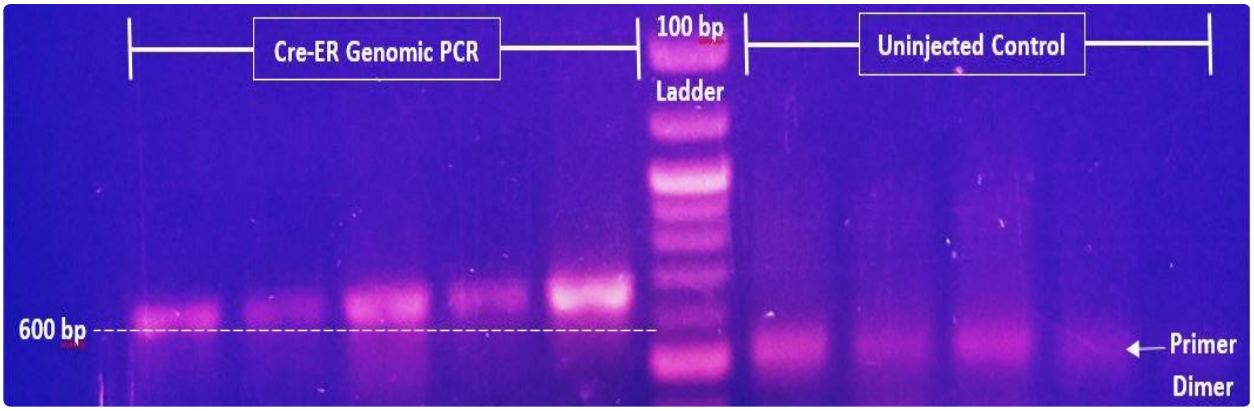

Figure 6. Gel results show successful incorporation of Cre-ER into host genome

Recombination

Currently, we have observed GFP expression in clones of cells in the gut from 7dpf. Additional expression in the hindbrain was observed (Figure 7). Both locations are where *phox2b* is normally expressed and are consistent with recombination being induced in a tissue specific manner. Successful recombination would generate a mosaic expression pattern of an array of colors (Figure 4), thus observing GFP suggests that other fluorochromes are being expressed. Further imaging under varying wavelengths using a confocal microscope should show variable fluorochrome expression patterns in a mosaic fashion and will be carried out with future experiments.

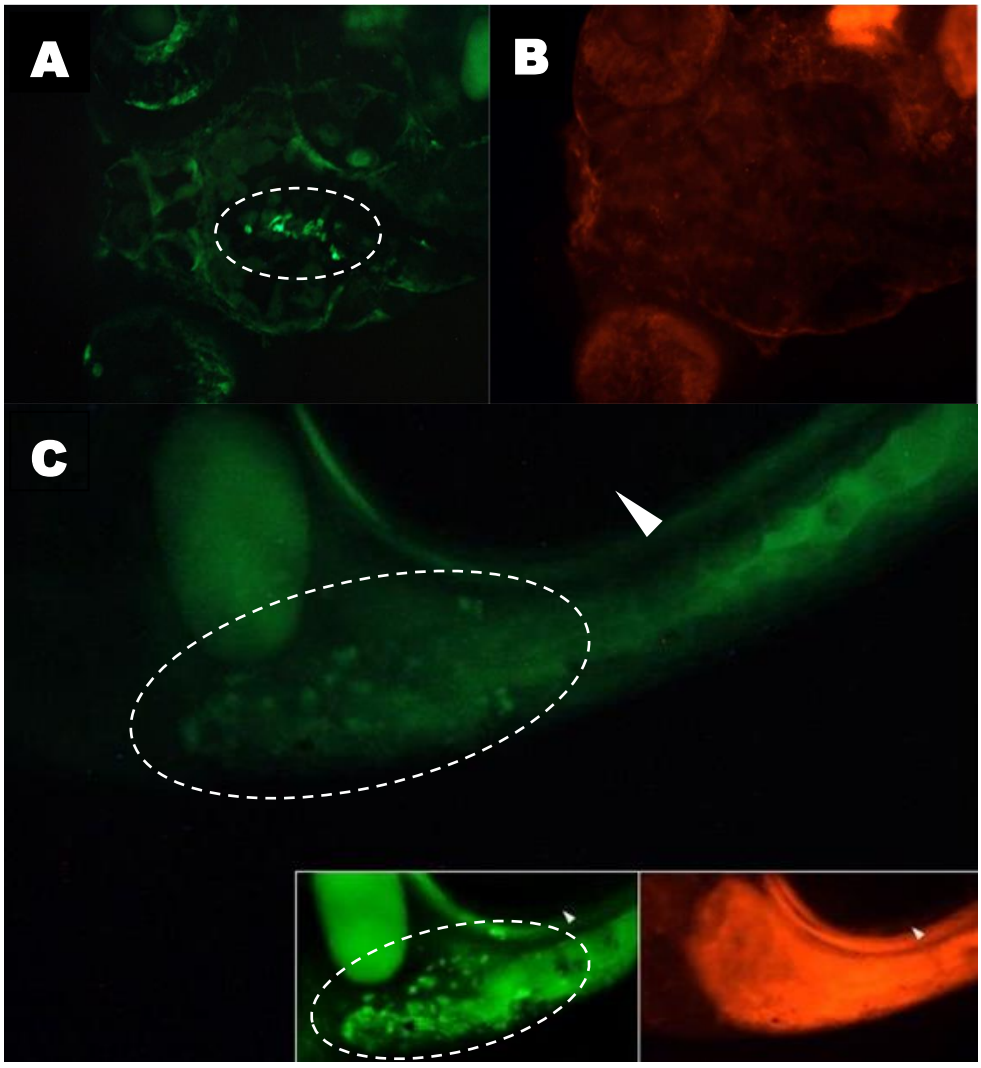

Figure 7. Recombination results in treated embryos at 6dpf

(A) Dorsal view of embryonic head with anterior to the left. Observed GFP expression corresponds to endogenous *phox2b* expression patterns in the hind brain, suggesting the *Tol2phox2b::Cre-ER* construct was successfully induced in a targeted tissue specific manner. (B) Hindbrain RFP expression is not observed. (C) Lateral view of gut with anterior to the left. (Upper) A clone of GFP expressing cells (dashed circle) in a punctate patterning is observed in the anterior of the gut directly below the swim bladder (arrow) and posterior to the liver. (Lower left) Combined GFP and RFP expression. (Lower right) Default RFP expression observed.

Inducing recombination via 4-OHT treatment at varying times will help further analyze the nature of ENCC specification and establish if it follows a fated or stochastic model. Induction at 48, 64, 72, and 96 hpf will be performed and recombination results imaged. If as we predict ENS neuronal subpopulations are specified by the fated model, later induction times should yield

smaller and less diversified clones, as adding 4-OHT at later stages in development would induce recombination in progenitor cells that undergo less division and will give rise to smaller clones with fewer neuronal subtypes (Figure 8). If recombination does not follow the fated model there should be smaller clones at later ages of 4-OHT treatment, but these clones should always contain a diverse range of neuronal subtypes. This would suggest that the ENCC remain unspecified throughout the developmental period investigated and suggests that ENS neuronal subtype specification follows a stochastic model of specification.

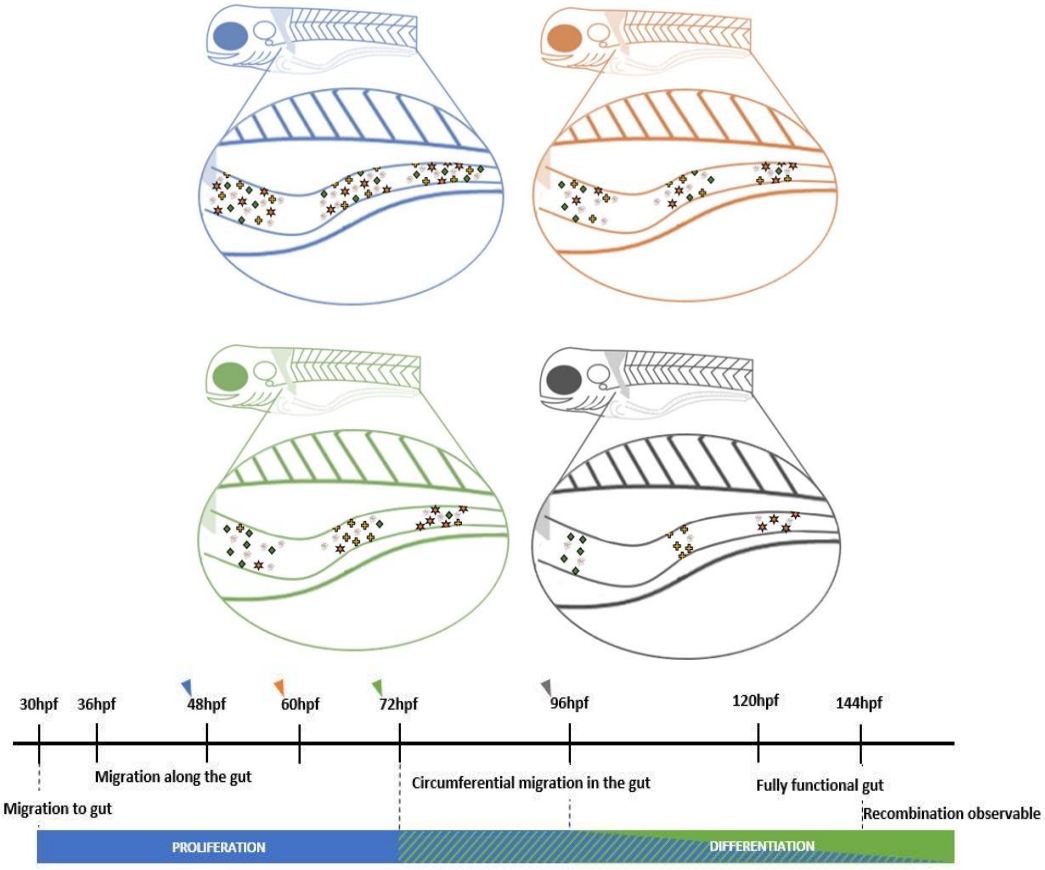

Figure 8. Expected recombination results following the fated model at time-dependent 4-OHT treatments

Induction at 48hpf (blue) affects earlier progenitor cells and is expected to result in large, non-specific clones throughout the gut; whereas, at 60hpf (orange), smaller and more specific clones are predicted. As per the fated model, this trend would continue through treatment at 72hpf (green) and result in the smallest clones of primarily one neuronal subtype when induction occurs at 96hpf (gray).

Chapter 3: Possible role of ETV1 as a transcriptional regulator of ENS specification

Introduction

ETV1 is a transcription factor of the ETS family. Previous studies in the Shepherd lab have shown that ETV1 is expressed in a punctate manner in the zebrafish gut from 72hpf through 120hpf (Figure 9). This expression is promising, as it does appear to be similar to the punctate *phox2b* expression patterning in the ENS observed in the Shepherd lab. Furthermore, ETV1 has recently been shown to serve as an early onset neuronal marker for the ENS.[22] ETV1 has previously been linked to intestinal motility as studies have suggested that the overexpression ETV1 in coordination with a KIT mutation, drives the formation of gastrointestinal stromal tumors (GIST). Interstitial Cells of Cajal (ICCs) are mesenchymal-derived cells that serve as pacemakers for gut musculature and are crucial to establishing proper peristalsis; ICCs are thought to give rise to GIST.[23] Past studies have shown that ETV1 expression is high in ICCs, suggesting a potential role of the transcription factor as a regulator of ICC development.[24] Coordination with the ICCs to maintain gut motility and function[25]. Previous studies in the Shepherd Lab aimed to analyze ETV1's role in ICC formation. As part of these studies, an ETV1 splice-blocking morpholino oligonucleotide (SBMO) was made (Gene Tools, LLC). *Tg(Phox2b::kaede)* fish were in crossed and their embryos were microinjected with the morpholino to knock-down ETV1 expression. These injections resulted in a clear reproducible morphant phenotype (Figure 10); however, due to the inherent problems with the off-site effects of morpholinos and the phenotype observed in the ETV1 morphant embryos, further studies are needed to definitively elucidate the potential role of ETV1 in ENS neuronal subtype specification.[26] Previous work in the Shepherd lab has not fully revealed ICC depletion within ETV1 knock-downs, and with mounting evidence suggesting that enteric neurons have ETV1

expression along the gut, further steps were taken to assess the possible role of ETV1 in the development of the ENS. The aforementioned complications associated with SBMO knock-downs drove the Shepherd Lab to make use of acquired ETV1 mutants as a worthwhile avenue of observing morphologies linked to ETV1 dysfunction. By directly analyzing homozygous mutants (null mutation) for ETV1, the complete loss of function phenotype could be definitively analyzed, and conclusions regarding the transcription factor's role in ENS specification could be determined. Comparisons between mutants and controls could potentially show a reduction in the total number of enteric neurons in the gut tube of mutants or may have more subtle phenotypic effects by changing number and proportion of specific ENS neuronal subtypes.

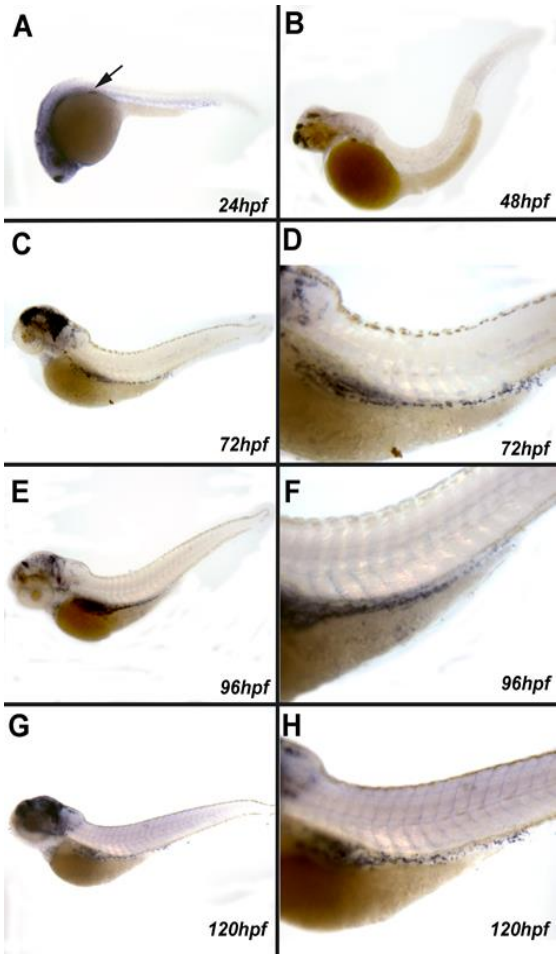

Figure 9. In situ shows ETV1 expression patterns using antisense riboprobes from 24hpf to 120hpf

(A) At 24hpf, ETV1expression appears to be localized to the anterior of the embryo (arrow). (B) 48hpf—when ENS precursors are proliferating and migrating to the gut. (C and D) By 72hpf, punctate expression patterns are observed along the gut around the same developmental stage where differentiation begins for various ENS precursors. (E – H) Punctate expression patterns of ETV1 remain consistent through 120hpf within the gut.

Image source: Shepherd Lab. Unpublished work by Schapmann, N. & DeGuzman, K.

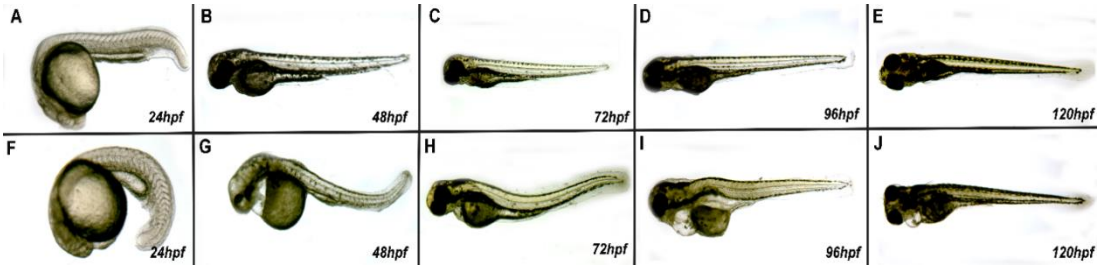

Figure 10. ETV1 SBMO knockdown phenotype

ETV1morphant phenotype in knockdowns (F-J) is apparent when compared to control (A-E). Key phenotypes include cardiac edema small eyes and some hindbrain death in morphants.

Image source: Shepherd Lab. Unpublished work by Schapmann, N. & DeGuzman, K.

Methods

Sequencing for heterozygotes

Zebrafish with target induced local lesions (TILLING) in alleles of interest (genomic region sa14953) were obtained from the Zebrafish International Resource Center (ZIRC). After raising IVF derived ETV1 embryos to adulthood, fin clip DNA was extracted under standard protocol and genomic PCR was performed using the Taq DNA Polymerase Kit (Qiagen). Primers used: Forward: 5'—CCT GTC ATT CGT TCC CCT CT –3' ; Reverse: 5'—TGT ACA GAC TGC CAT GGT GA—3'. Thermal cycler conditions were set to annealing temperatures of 58.4℃ and an extension time of 1.5 minutes with 60 cycles. Purification using the PCR Purification Kit and accompanying protocol (Qiagen) was performed following the included protocol. 30µl purified product was isolated and analyzed for purity and concentration with Nanodrop 2000 (Thermo Scientific). Gel electrophoresis was run to further analyze sample

concentrations. Purified DNA was then sent for sequencing to Macrogen. Results were analyzed with Sequencher software to identify heterozygotes mutants.

Screening

Identified heterozygotes were in crossed to generate homozygous mutants, and subsequent progeny were screened for phenotypic abnormalities. Hereafter, isolated mutants and controls were analyzed further under an Olympus SZX12 dissecting microscope and imaged.

Enteric neuron quantification

Immunostaining was performed at 7dpf. Primaries used were Anti-Hu (1:100) and 5-HT (1:100). Secondary antibodies used were Anti-Mouse (1:500) and Anti-Rabbit (1:500). Antibody staining protocols were performed following standard procedures outlined in precious studies.[27] Stained embryos were analyzed for successful staining using an Olympus U-HGLGPS UV light burner in conjunction with an Olympus SZX12 dissecting microscope. Thereafter, Olympus IX2-UCB confocal microscope with a PRIOR Lumen200 lamp box and accompanying Hamamatsu C10600 camera controller was used to capture high resolution images with depth correction using the SlideBookPro software for Windows. Confocal microscope magnification was standardized at 10x to take lateral images of the gut over a roughly 10 dorsal root ganglion length. Images taken with the SlideBookPro software will be converted to black and white to generate increased contrast and then analyzed with Fuji Image J software to quantify cells along the gut tract.

Results/Discussion

Sequencing results

Sequencing results from Macrogen identified a subset of heterozygous mutants when analyzed in Sequencher (Figure 11).

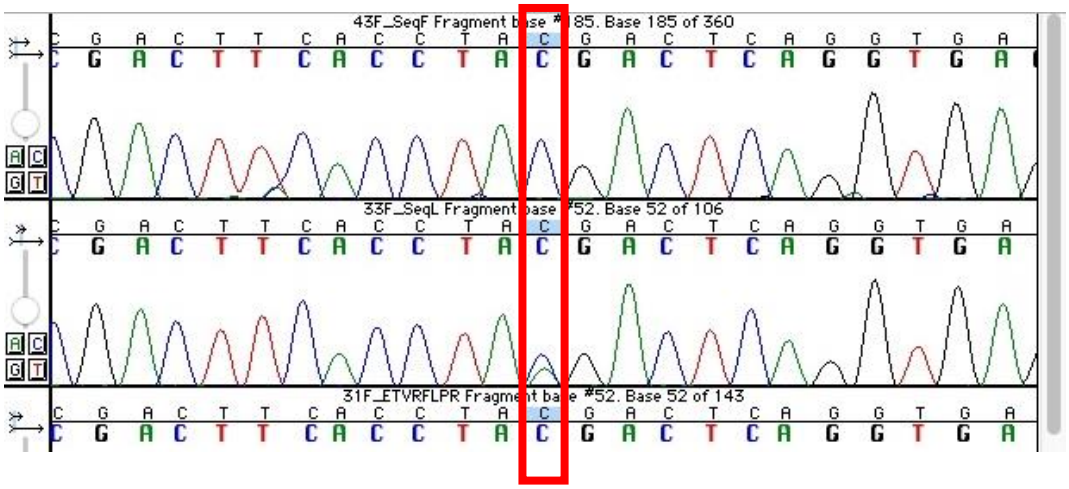

Figure 11. Chromatogram analysis of mutations in ETV1 mutants revealed a subset of heterozygous mutants

Sequencher software analysis reveals a doublet in chromatogram results within base 52 of 143 within the genomic region of interest (red box). Heterozygous mutants have doublets in their chromatogram results. Those fish with doublets at base 52 of 143 were isolated and used for further crosses to

Screening for abnormal morphology

A distinct no-swim bladder phenotype was observed in approximately 17% of progeny (Figure 12), but only one trial has been run thus far. Previous research has shown a link between swim bladder and ENS development, and ENS mutants are found consistently show a depleted swim bladder phenotype. Several neuronal subtypes present within the ENS are thought to innervate the musculature of the swim bladder.[28] Improper swim bladder development is therefore a promising observation and would be considered consistent with a potential ENS phenotype, but more trials are needed to further prove that this observed abnormality is genetically linked to the homozygous mutants we are aiming to generate.

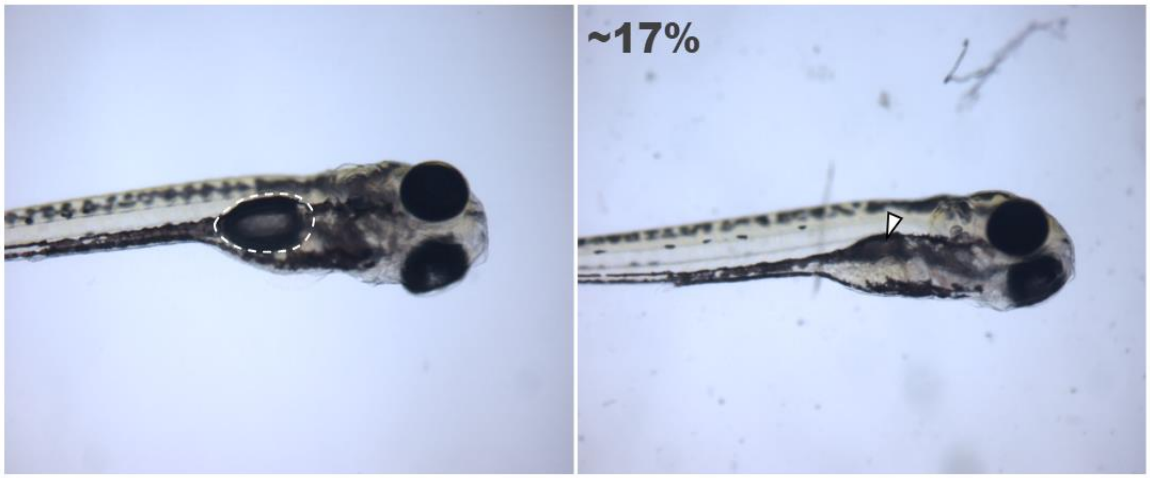

Figure 12. WT (left) and ETV1 mutant (right) morphologies at 6dpf

WT embryos presented with a functional swim bladder (dashed boundary); whereas 17 % of the clutch exhibited a no swim-bladder phenotype. The missing swim bladder is fairly obvious under the dissecting scope (arrow).

A clutch of E7 embryos obtained from an in cross of ETV1 heterozygotes was stained with pan-neuronal antibodies Anti-Hu and 5-HT. These different markers would aid in quantification of two different neuronal subtypes within the ENS, potentially giving the lab the ability to observe discrepancies in neuronal subtype proportions within the ENS. It is possible;

however, that affected neurons within the ENS do not follow developmental pathways that produce the neuronal subtypes being marked, so more immune-staining will be carried out in the future with other markers to further analyze a number of other subtypes via quantification. Embryos were successfully stained with Anti-Hu markers, but not with 5-HT. Future trials will be conducted to successfully stain with 5-HT to analyze subtype proportions between mutants and controls (Figure 13). While no dramatic difference is apparent in neuronal numbers between mutant and WT embryo confocal images, more information will regarding neuronal cell number will be derived from image analysis of the guts using Fuji Image J software to complete unbiased neuronal quantification. This analysis may reveal a statistically significant difference in the number of ENS neurons in ETV1 mutants as compared to WT. If no differences are seen, it is still a possibility that ENS depletion may be present, and staining embryos with other pan neuronal markers may reveal this. Additionally, future trials will generate data that may reveal differences between mutants and WT embryos in the number and proportion of specific ENS neuronal subtypes.

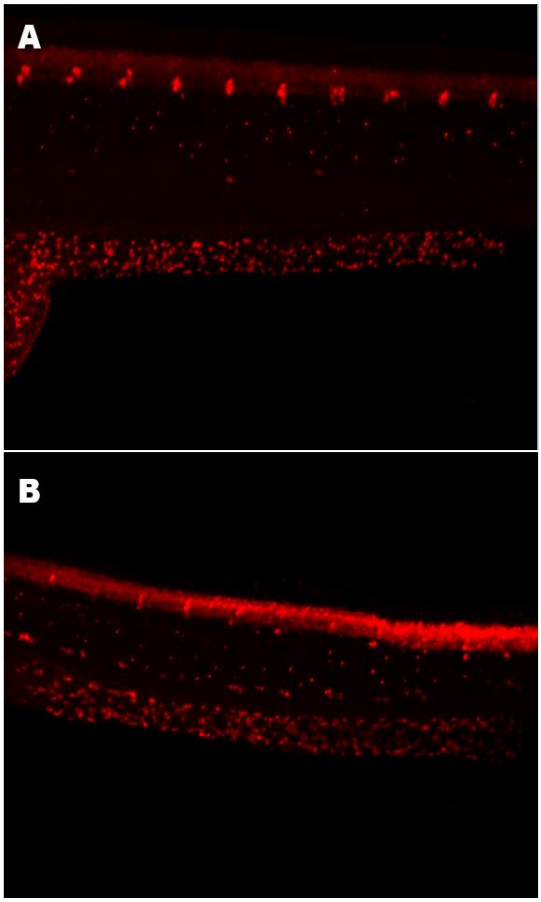

Figure 13. Anti-Hu antibody staining of embryos at 10x magnification

WT (A) and mutant (B) embryos that were isolated with previous screening showed no obvious difference in neuronal cell body count for neurons marked with Anti-Hu.

Chapter 4: Conclusions

The aim of this study was to study ENS lineage specification in zebrafish by taking two different lines of research. The primary aim was to establish a direct labelling system for enteric neural crest precursors to enable temporal analysis of ENS specification. A second aim was to further explore the transcriptional regulation of ENS differentiation and specification potentially mediate by the transcription factor ETV1. Assessing the role of ETV1 in ENS development could elucidate a previously unrecognized role for ETV1 as a regulator of specific neuronal subtypes within the ENS, an idea suggested in previous studies with mice.[29]

With limited recombination success in direct labelling studies, more work will need to be done to further optimize 4-OHT activation and develop a strict protocol for Cre induction. Currently, results show that recombination occurs in a tissue specific manner in injected and tamoxifen treated embryos which is promising, however the lack of consistency in these experiments needs to be resolved in order to be able to undertake more meaningful lineage analysis studies. More work is required to optimize our tamoxifen treatment which should bring more consistent recombination results. Literature has suggested that final concentrations of 4-OHT ranging from 8 µM to 10 µM in 3 ml of embryo medium is sufficient for successful Cre-ER induction. Concentrations within this range and as high as 15 µM were tested, but recombination results remained inconsistent. The difficulties of working with such a small mass of 4-OHT when making ethanol solutions for treatment may contribute to the inconsistent recombination results observed, as it can be increasingly difficult to manage concentrations due to human error in material transfer. Finding a way to more accurately measure and aliquot solutions of 4-OHT in ethanol could greatly increase recombination rates observed in the future. As a control, PCR and RT-PCR experiments should be performed with every future trial to

ensure that Cre-ER integration remains consistent between clutches. Obtaining consistent recombination results will take this study further in the future and help allow for temporal analysis of ENCC migration and differentiation. This analysis is of clinical relevance, as it can bring about more information regarding ENS specification that can help understand the lesser understood disorders linked to improper ENS function and formation.

Regarding the study's second aim, a preliminary observed ETV1 mutant phenotype of no swim bladder is promising, as a relationship between its development and that of the ENS exists. More trials are needed to analyze the frequency of mutant morphologies within clutches including confirmation of the mutant genotype in swim bladderless embryos. This includes optimizing antibody staining protocols for 5-HT markers to allow for accurate imaging and quantification of another neuronal subset within the ENS, as only Anti-Hu immunocytochemistry was successful from the first staining of a clutch of ETV1 mutants, limiting our ability to fully assess more subtle changes in subtype proportions. Furthermore, more neuronal markers will need to be used to label more ENS subtypes, as it may be possible that ETV1 plays a regulatory role in the development of neurons not directly labeled by either Hu or 5-HT neuronal markers. Using quantification tools within the Fuji Image J software, ETV1's role in ENCC specification could be further analyzed by looking at a range of ENS abnormalities in neuronal count. Further analysis of mutants may show enteric neuron depletion or ENS subtype proportion discrepancies when compared to WT embryos. Looking for the possible results within homozygous ETV1 mutants can help the Shepherd Lab further describe a phenotype and potentially lead to the ultimate goal of more fully describing the transcription factor's role in the specification and differentiation of ENCCs in the developing zebrafish ENS. Further defining said role can also

provide ETV1 as a useful early neuronal marker for ENS development in lineage studies moving forward. Ultimately, clinical significance is apparent in this line of research because work to assess ENCC proliferation and differentiation has the potential to bring clarity to less understood physiological conditions linked to improper ENS formation.

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