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Swet Patel April 14, 2020

The development of a temporally controlled lineage system for visualizing enteric neural precursor specification in *D. rerio*

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Abstract

The development of a temporally controlled lineage system for visualizing enteric neural precursor specification in *D. rerio*

By Swet Patel

Composed of at least 17 different neuronal subtypes, the enteric nervous system (ENS) is the largest part of the peripheral nervous system and responsible for regulating gut motility and homeostasis. Proper differentiation of enteric neuron subtypes is vital for digestive functioning, with improper differentiation of enteric neural crest cells (ENCCs) leading to a number of human gastrointestinal motility disorders. Despite recent advancements, the exact lineage and specification of ENCCs remains unclear. This study aims to develop a system for labeling enteric neural precursors to permit the temporal analysis of ENCCs differentiation during their migration and proliferation from the vagal neural crest to and along the gut. This study utilizes the *ubi:Zebrabow* transgenic line of *D. rerio*, which expresses the Brainbow fluorescent cassette under control of the *ubiquitin-B* promoter. From this line, we created a double transgenic with a *Tol2phox2b::CreER* construct to allow recombination within the Brainbow multicolor constructed specifically in *phox2b* expressing cells when embryos are treated with (Z) 4-hydroxytamoxifen (4-OHT). Successful incorporation and expression of *CreER* via Tol2 transposition was verified by genomic polymerase chain reaction (PCR) and reverse-transcription polymerase chain reaction (RT-PCR). We observed sparse recombination in enteric neurons of embryos treated with 4-OHT, with the majority of recombination occurring ectopically in myocytes. Cells that had undergone recombination expressed a combination of red, yellow, and cyan fluorescence proteins in a mosaic pattern. Future experiments should aim to achieve consistent recombination within enteric neural precursors and alter the timing of tamoxifen treatments to in order to analyze ENCC specification.

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Introduction

Composed of at least 17 different neuronal subtypes, the enteric nervous system (ENS) is the largest and most diverse group of neurons outside of the brain and spinal cord.[1] Uniquely the ENS is the only part of the peripheral nervous system (PNS) that is able to function without input from the central nervous system and regulates gastrointestinal motility and homeostasis.[2, 3] The ENS neurons and glia, in all vertebrates, are predominantly derived from the vagal neural crest cells and form a complex neuronal network primarily in close association with the intestinal smooth muscle tissue in the gut that regulates peristalsis.[4, 5] Congenital human disorders (e.g. Hirschsprung's Disease) that stem from abnormal differentiation of the ENS network lead to improper peristaltic contractions along the gut and often require immediate surgical interventions.[6] While it is understood that proper positioning of each enteric neuron subtype is important for digestive functioning, the exact lineage and specification of the enteric neuronal crest cells remains unclear.[7] A better understanding of ENS lineage and the differentiation of developing neurons can better allow us to understand the specific cause of many currently undetermined gastrointestinal motility disorders that likely results from enteric neurons differentiation defects. Additionally, identifying transcriptional regulators could potentially allow us to artificially induce neuronal differentiation and specification of ENS precursors to replace missing subtypes in disease related states.[7] This project aims to develop a system that allows better study of the lineage relationship and differentiation patterns of enteric neurons and their precursors.

While the ENS plays a central role in coordinating gastrointestinal motility, the Interstitial Cells of Cajal (ICC), which are a group of mesenchymal cells found in the muscle

layers of the alimentary tract, actually initiate peristalsis. These cells facilitate communication between the autonomic nervous system and smooth muscle tissue in the gut to result in peristalsis via smooth and rhythmic depolarizations.[8] The ICC work in conjunction with other ENS neurons to create a complex system that is able to create complicated contractile patterns and responses to stimuli. This web of neurons and synapses are spread across many mucosal and muscular layers within the gut (Figure 1).

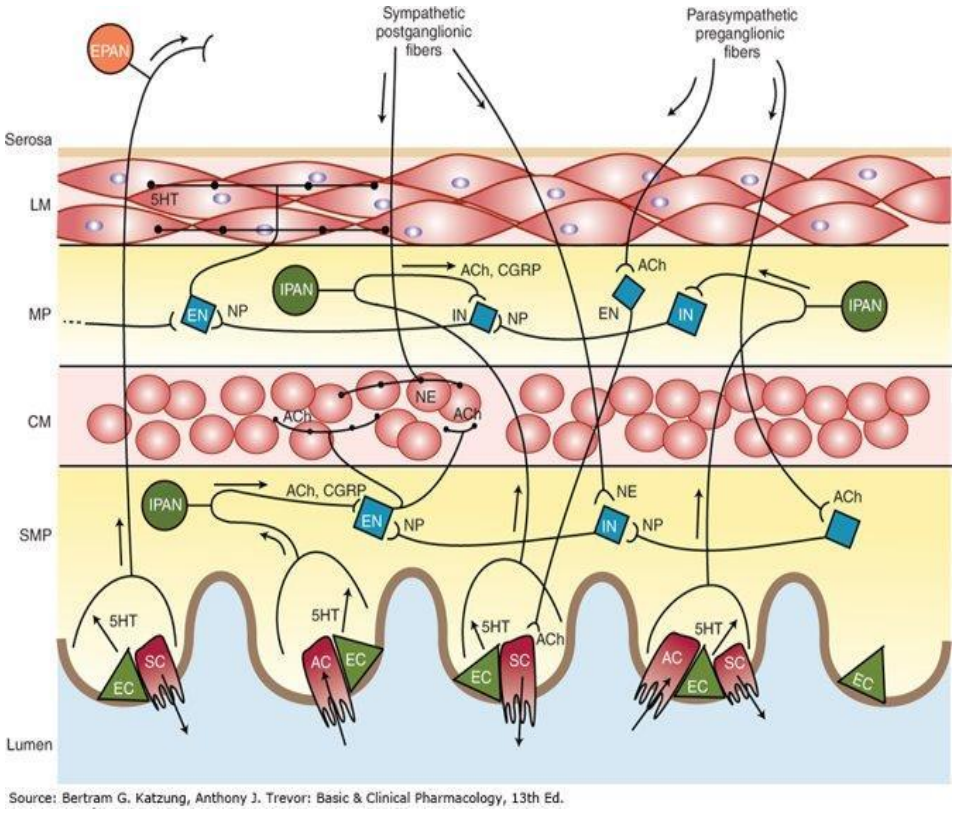

Figure 1. ENS circuitry and gut architecture in a mammalian model.

A simplified diagram of the ENS circuitry between various enteric neuronal classes in mammals. These ENS neurons innervate multiple layers, including the circular muscle (CM), myenteric plexus (MP), longitudinal muscle (LM), and submucosal plexus (SMP).

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

The Shepherd Lab is interested in vertebrate neural development focusing on the mechanisms that regulate the normal development of the neurons and glia within the ENS

using the *Danio rerio* (*D. rerio*) model system, commonly referred to as zebrafish. Since the late 20th century, *D. rerio* has been increasingly used as a model system for biomedical research studies due to the ease to which they can be kept in large numbers and bred.[9] Additionally, zebrafish are genetically tractable and relatively easy to genetically manipulate. This permits comparatively cheap and efficient experimental studies to be undertaken to investigate vertebrate ENS development.[10] The enteric neural network is simpler in zebrafish than that of mammalian models, with the zebrafish gut having no submucosal layer, a less complex myenteric plexus, and having a simpler mucosal structure (Figure 2).[11, 12] Importantly, zebrafish lack a ganglionated submucosal and myenteric plexus, leading to the enteric neurons being more dispersed than mammalian counterparts. This allows easier visualization of individual neuronal subtypes and their developmental path. Despite these differences, there are many similarities that still exist within the development of the ENS of zebrafish compared to that in mammals. All vertebrate species have the ENS derived from a transient embryonic stem cell population, the neural crest. Specifically an axial population of vagal neural crest cells gives rise to the majority of the ENS in all vertebrate species (Figure 3). The proliferation and differentiation of these cells, which are called the enteric neural crest cells (ENCCs), will cause the development of the neuronal network in the gut.[13]

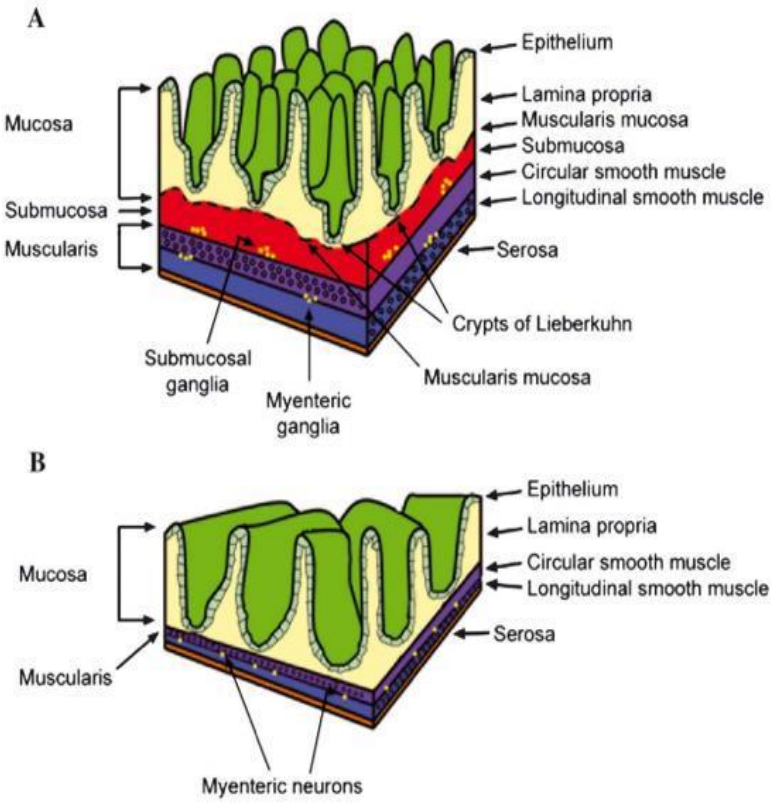

Figure 2. The distribution of the enteric nervous system in (A) mammals and (B) fish.
Submucosal and myenteric ganglia exist as clusters of innervating neurons within the gut. (A) Villi are present on the luminal surface of the mammalian gut. (B) The teleost gut lacks a submucosal layer and does not have a ganglionated myenteric plexus. By contrast myenteric neurons in teleost remain as individual neurons innervating the circular and longitudinal smooth muscle layers of the muscularis as well as innervating the mucosa. The luminal surface in the teleost model is made of large folds of epithelial tissue instead of the villi found in the mammalian gut.
Image source: Shepherd, I. & Eisen, J., 2011; Adapted from: Wallace, K.N. et. al, 2005

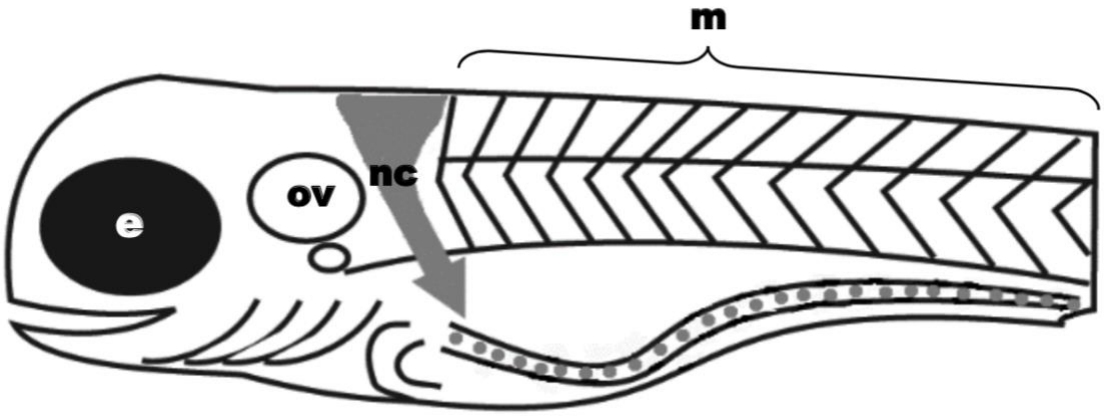

Figure 3. Zebrafish neural crest proliferation and migration visualization.
(A) The vagal neural crest region (nc) is located posterior to the otic vesicle (ov). Neural crest cells that have gone through an epithelial to mesenchymal transformation become ENCCs that migrate along the entire length of the developing embryonic gut to populate and innervate the gut (punctate patterning in gut tube).
Adapted from: the Shepherd Lab

Currently, two plausible models of specification of ENS neuronal subtypes have been proposed: a stochastic model and a fated model (Figure 4).[3] The stochastic model suggests the ENCC specification is not predetermined within the neural crest. Therefore, ENCCs within a specific region of the neural crest and differentiate into a variety of subtypes based on environmental factors. Alternatively, the fated model suggests the path of differentiation of enteric neurons is predetermined within the vagal neural crest prior to their migration to the gut. According to the fated model, an ENCC labeled before migration would only give rise to a specific subtype or subtypes of enteric neurons in the mature gut. Recent studies have shed some light on the lineage patterns of ENCCs by showing there is a variable expression of key developmental genes are driving ENS neuronal specification.[14] While the onset of ENCC specification is becoming more understood, the specific mechanisms that regulate and determine which precursors develop in to which specific ENS neuronal subtype remain

unclear.[7] For instance, there could be various intermediate checkpoints and branching points that divide mature ENS subtypes into groups with similar lineage patterns (Figure 5). These groups could be characterized by the expression of different genes or transcription factors, and knowledge of this could be used to induce ENCCs to differentiate into a specific subtype. Developing an effective labeling system to analyze ENS neuronal subtype differentiation and specification will help determine the developmental mechanisms that regulate how neural precursors become mature ENS neurons and provide methods for further study or treatment of currently poorly understood gastrointestinal motility/function disorders.

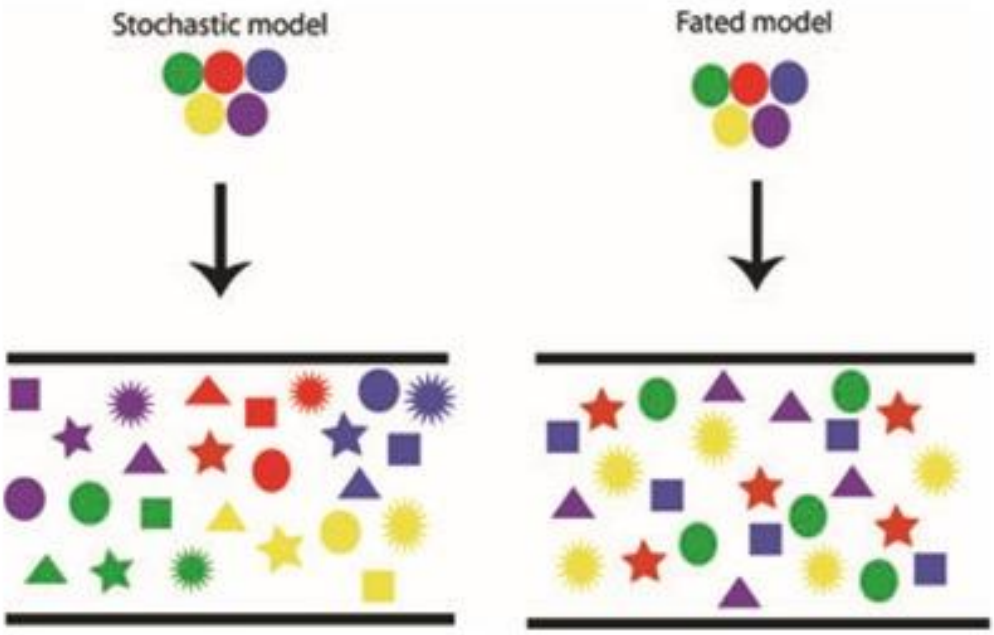

Figure 4. Proposed models for ENS neuronal specification within the gut.
The two proposed mechanistic models for ENS neuronal subtype specification. Different shapes represent different ENS subtypes, and different colors are used to represent an individual clone of cells that is derived from a single precursor cell.
Image source: Harrison, C. & Shepherd, I.T., 2013

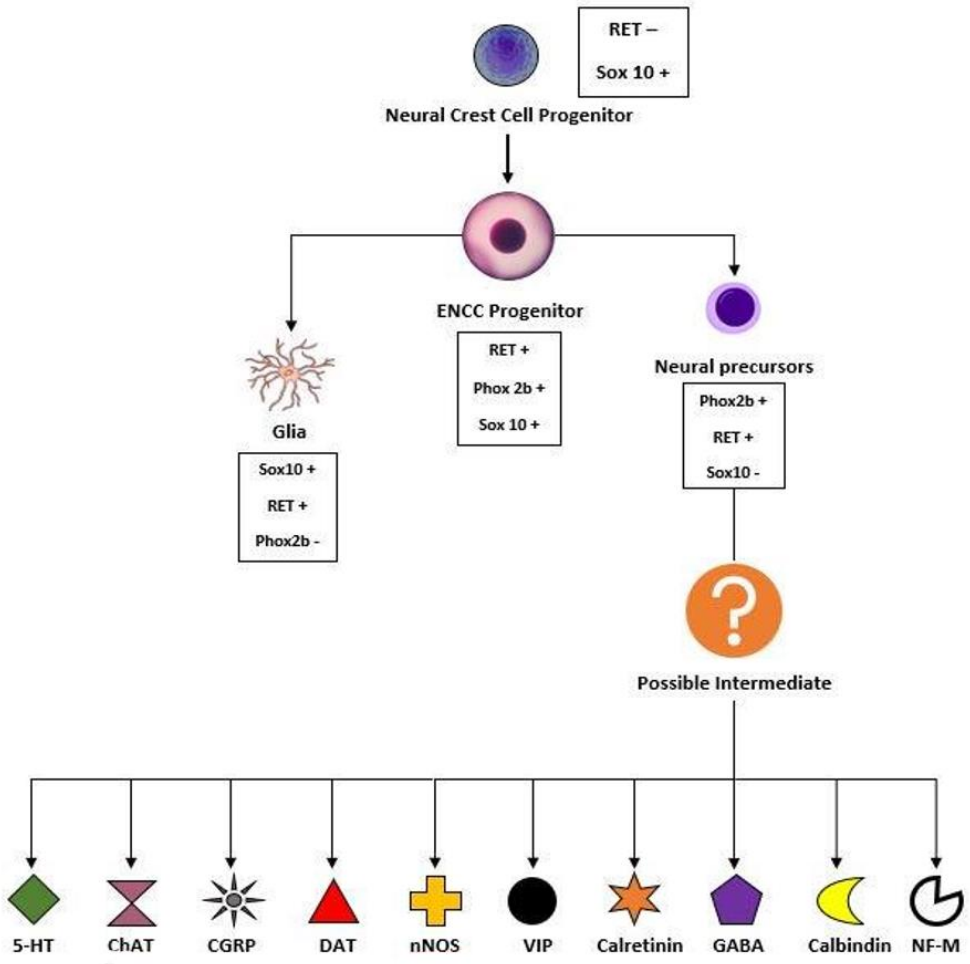

Figure 5. Variable expression pattern lineage map of ENCC differentiation.
Key developmental genes are expressed to drive the specification of ENCC progenitors into enteric neurons and glia. *Sox10* expression specifies glial cells while *phox2b* expression specifies neural precursors. Possible intermediates between neural precursors and the neuronal subtypes could exist that are driven by a unique set of developmental genes.
Adapted from Lasrado, R. et. al, 2017

In order to investigate which of these two models is potentially correct we used the zebrafish model system. Due to external fertilization, *D. rerio* embryos are easy to obtain and can be readily imaged throughout development. Additionally, they can be raised in embryo medium with 1-phenyl 2-thiourea (PTU) to remain transparent throughout development.[15] This has allowed many developmental studies to use fluorescent dyes and imaging to trace cell

lineage within zebrafish embryos.[16, 17, 18] This utility of the model system was further advanced with the discovery of the Tol2 transposon which allowed for the rapid and easy generation of transgenic lines of zebrafish (Figure 6). Using this transposition method, target sequences that are wanted to be expressed in zebrafish, are cloned into plasmids with two Tol2 transposition sites flanking a sequence of DNA to be incorporated. This Tol2 construct is then injected along with Tol2 transposase mRNA into one-cell embryos, resulting in the incorporation of the target DNA sequence into the host's genome.[19] As these injected embryos develop, a mosaic expression pattern of inheritance of the injected construct will be seen. If the injection construct is incorporated into the germ line, raising the offspring from crosses of the F0 injected fish can result in a stable transgenic line that contains the construct in every cell. Generation of this stable line eliminates the need for microinjections in future studies.

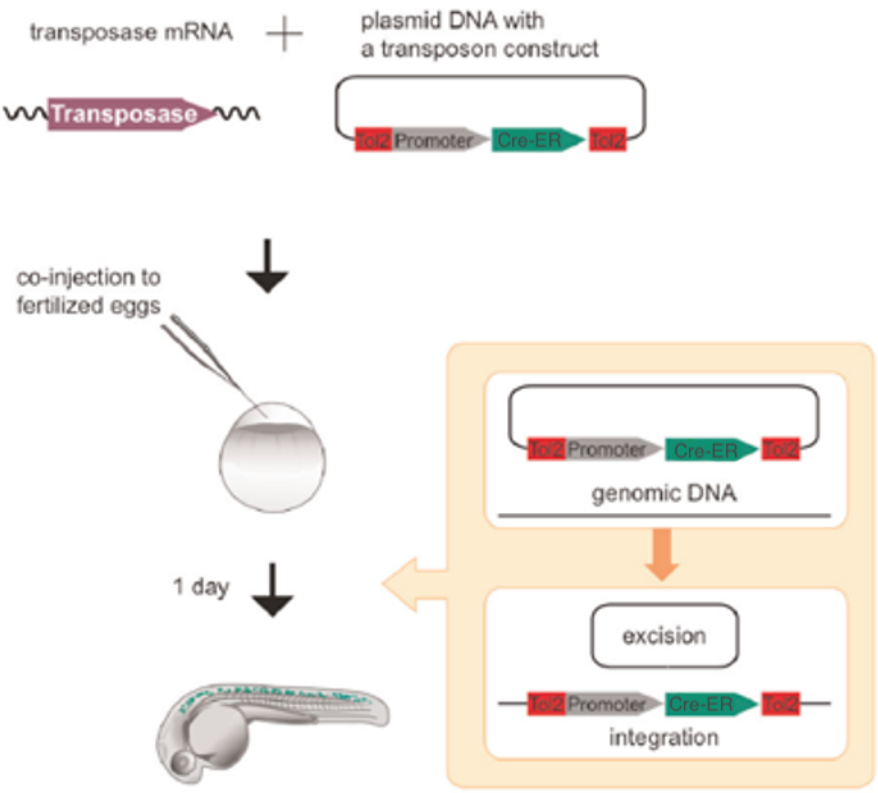

Figure 6. Genetic incorporation of *CreER* using Tol2 transposase.
A plasmid encoding Cre recombinase controlled by a promoter is flanked by Tol2 regions. This is injected alongside mRNA encoding the Tol2 transposase. As the embryo matures, the *CreER* is incorporated into the host genome.
Adapted from: Kawakimi, 2007

To be able to study the lineage relationship of ENS neurons derived from ENCC precursors, we have used two different transgenic lines of zebrafish that nominally ubiquitously express the Brainbow fluorescent cassette.[16, 20] We propose to use these lines in order to distinguish the origins of the different ENS neuronal subtypes in a series of lineage studies. The Brainbow system allows cell lineage study in many model organisms via the variable expression of various fluorochromes. The Brainbow cassette is found within the genome and contains three fluorescent proteins (RFP, YFP and CFP) driven by a promoter (Figure 7). RFP is expressed

by default, but recombination can occur in the presence of Cre recombinase to allow for the expression of YFP and CFP. Lox sites are recognized by Cre recombinase and used to ensure expression of only one type of fluorescent protein per copy. With three copies of the Brainbow cassette in each genome that recombine independently, there can be a large variety of colors due to stochastic recombination and expression of fluorescent proteins in each cell. The combination of colors resulting from recombination results in clones marked by different colors. Since the recombination events in each progenitor cell are inherited by its progeny, Brainbow lines can serve as multi-lineage markers. [17, 20, 21] The *PriZm* line utilizes a *β-actin2* driven Brainbow construct that is induced to recombine when crossed with fish expressing a tamoxifen-inducible Cre recombinase (*CreER*).[20] The *ubi:Zebrabow* line uses the *ubiquitin B (ubi)* promoter to express the Brainbow construct.[16] The *ubi* promoter allows ubiquitous transgenic expression of the Brainbow construct and is able to label a larger variety of cell types than the *β-actin-2* promoter found in the *PriZm* line.[16, 22] This system is further modified by Tol2 transgenesis to allow the incorporation of *CreER* under control of a specific promoter to narrow the range of cells expressing recombination.[18, 19]

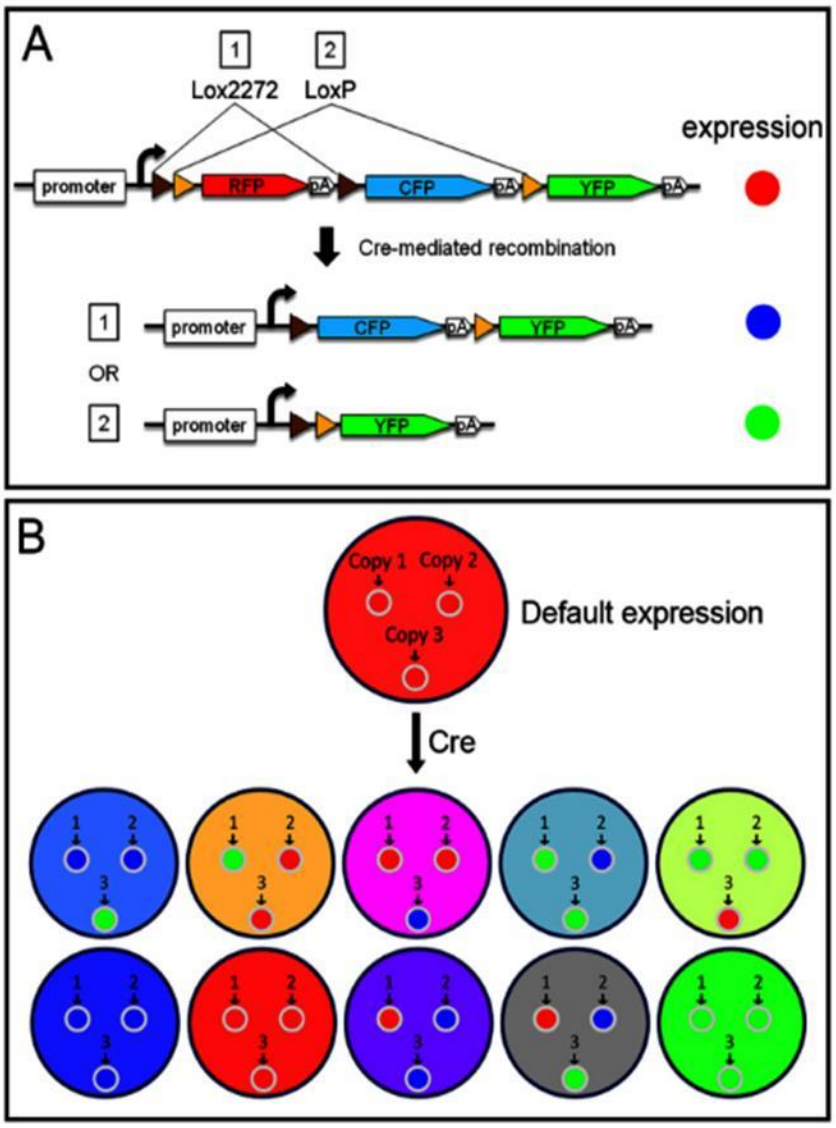

Figure 7. Brainbow fluorescence cassette and accompanying expression.
(A) The arrangement of fluorescent protein genes and their Lox sites. Cre recombinase recognizes the Lox sites resulting in a mosaic pattern of fluorescent protein expression. (B) The default fluorochrome expression is red (RFP). Cre recombinase leads to like blue (CFP), and yellow (YFP) being expressed in cells that were induced to recombine.
Image source: Gupta, V. & Poss, K.D., 2012

This thesis project primarily aims to develop novel transgenic zebrafish lines to allow the temporal study of ENCC differentiation using a Cre/Lox recombination system of the Brainbow fluorescent cassette. This study attempts to derive these new fish lines from the *PriZm* and *ubi:zebrabow* lines in order to visualize and quantify clones of labeled ENCCs during the

migration, proliferation, and differentiation of ENS neurons at different stages of ENS development in the zebrafish. Based upon the developmental stage when recombination is induced, ENCCs should express a range of fluorescent proteins in different sized clones. This information will then be used to determine whether ENS neuronal subtype specification in *D. rerio* follows a stochastic or fated model. This analysis will potentially give new insights into human disorders that involved abnormal ENS development, and possibly better lay the groundwork for their treatment.

Methods

Zebrafish

Initially *PriZm* zebrafish were incrossed to generate progeny that expressed the Brainbow fluorescence cassette controlled by the *β-actin2* promoter.[20] Later, the *ubi:Zebrabow* line was used instead of *PriZm* to create progeny that also expressed the Brainbow fluorescence cassette driven by the *ubiquitin B (ubi)* driver.[16] The u*bi:Zebrabow* line was outcrossed to the *Casper* line since the *Casper* fish's translucence allow for easier visualization of recombination.[23] Most embryos from these crosses were microinjected with a *Tol2phox2b8.3::CreER* construct within 1hpf, with approximately 20-40% serving as water-injected uninjected controls. Injected and uninjected embryos were stored in a 28°C incubator to develop for the remainder of the experiment. *PriZm* and *ubi:Zebrabow* homozygous or heterozygous embryos should ubiquitously express the red fluorochrome of the Brainbow cassette by default (Figure 7) and were sorted at 24hpf using an Olympus SZX12 dissecting microscope with an Olympus U-HGLGPS UV light source. Embryos that did not express RFP were removed. Some RFP expressing *ubi:Zebrabow* and *Casper* heterozygous progeny were selected to be raised to adulthood. Others were euthanized following standard protocol at the conclusion of the experiment.

Embryo microinjection

Experimental embryos from both the *PriZm* and *ubi:Zebrabow* lines were microinjected at the 1-4 cell stage in an injection tray made of 1.0% agarose gel using standard procedure. The injection construct consisted of a *Tol2phox2b8.3::CreER* construct, synthetic mRNA encoding Tol2 transposase, and KCl and RNase-free water to remain isotonic with embryo yolk.

The *Tol2phox2b8.3::CreER* construct was previously made by Kelsey Mayer in the Shepherd Lab. The *CreER* construct and Tol2 transposase mRNA were both injected at a concentration of 12.5 ng/µL. This dosage was within the range experimentally determined concentrations used in previous Tol2 studies.[19, 24] Embryos were promptly removed from injection trays after injection and checked for signs of infection or severe damage. Post-injection survival rates were drastically improved by immediately removing embryos from injection trays and washing with embryo medium. Embryos were kept in embryo medium prepared with 0.2mM 1-phenyl 2-thiourea (PTU) to prevent pigmentation to permit better imaging as the embryos developed.

Polymerase chain reaction

Genomic polymerase chain reaction (PCR) was used 72-96hpf to determine if the *CreER* construct successfully incorporated within the host genome. DNA isolation and genomic PCR were performed using reagents from the Invitrogen Platinum™ Direct PCR Universal Master Mix following standard protocol. PCRs 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'. Genomic PCR thermal cycling occurred in Bio-Rad C1000 thermocycler at an annealing temperature of 60°C for 15 seconds and an extension phase at 68°C for 30 seconds for 40 total cycles.

To ensure the Cre recombinase was being expressed, reverse transcription polymerase chain reaction (RT-PCR) was performed using RNA extracted from injected 48 and 72hpf embryos. RNA was isolated and the *Cre* mRNA was amplified using a Qiagen RTPCR kit following the standard protocol. The same *Cre ORF* primers from the genomic PCR were used. RT-PCR

thermal cycling occurred at an annealing temperature of 53°C for 30 seconds and an extension phase at 72°C for 45 seconds for 40 total cycles. All PCR results were visualized using gel electrophoresis with a 1.0-1.2% agarose gel at 130V for 30min.

Tamoxifen treatment

To induce recombination, embryos were treated with (Z)4-hydroxytamoxifen (4-OHT) (Sigma Aldrich) at 72hpf. The biologically active Z isomer of 4-OHT is light sensitive and easily converted to its less active E isomer, so all work was performed quickly in the dark. Other studies have shown 4-OHT can be kept stable in solution a few weeks and still induce recombination via *CreER*.[25, 26] Due to this, solid 4-OHT was dissolved in 100% ethanol to create 26mM aliquots which were stored at -20°C in the dark. A final working concentration of 100µM 4-OHT was created by bathing approximately 50 embryos in 3ml of embryo medium with PTU and adding 12µL of the 4-OHT in ethanol aliquot. Embryos were treated for 15 minutes and then rinsed and incubated again in embryo medium with PTU at 28°C until imaging.

Imaging

At 5-10 dpf, 4-OHT treated embryos at were anesthetized with tricaine and observed with the aforementioned Olympus dissecting microscope and RFP/YFP/CFP UV filters to assess the presence of recombination. For more detailed imaging and rendering, an Olympus IX2-UCB spinning disc confocal microscope with a PRIOR Lumen200 lamp box and accompanying Hamamatsu C10600 camera controller was utilized to record high resolution images with depth correction using the SlideBookPro software for Windows.

Results

Comparison of RFP expression in the two zebrafish Brainbow transgenic lines

To determine which of the two previously generated Brainbow lines would potentially be better for our lineage studies, we compared the default RFP expression in the *PriZm* line, which we had originally obtained from the Poss Lab to that in the *ubi:Zebrabow* line which we obtained from the Moens Lab.[16, 20] In the *PriZm* line, we observed the strongest default RFP expression in the heart and notochord, with little in the gut. By comparison, the RFP expression in the *ubi:Zebrabow* line is much more uniform throughout the embryo including the enteric nervous system (Figure 8).

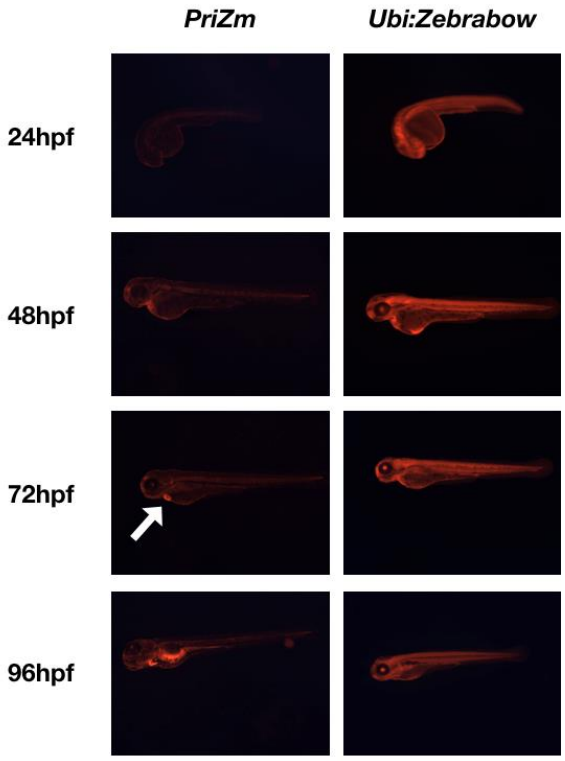

Figure 8. *PriZm* and *ubi:Zebrabow* RFP expression throughout development.
The *PriZm* and *ubi:Zebrabow* lines show significant differences in overall RFP expression. RFP expression is used to infer expression of the Brainbow construct. *ubi:Zebrabow* RFP expression is more intense and appears ubiquitous at all examined ages. By contrast, *PriZm* fluorescence is less intense and appears to be only strongly expressed in the heart (arrow) and notochord. *PriZm* embryos were imaged using 15 second exposure time while *ubi:Zebrabow* embryos were imaged using a 5 second exposure.

Phox2b8.3::CreER construct is transposed into the genomic DNA of injected zebrafish

To determine if the *phox2b:CreER* construct was successfully transposed into the genome of injected embryos, we used PCR to amplify the *CreER* sequence from genomic DNA isolated at 96hpf from injected embryos.

Our initial results using the standard Qiagen *Taq* polymerase were inconsistent (Figure 9). We hypothesize this was due to problems in our standard genomic DNA isolation protocol. We switched to the Invitrogen Platinum™ Direct PCR kit, and this resulted in strong consistent bands for *CreER* within embryos injected with the *Tol2phox2b8.3::CreER* construct and Tol2

transposase mRNA (Figure 9). We amplified a 700bp band representing the expected *Cre* amplicon in PCR of genomic DNA isolated from only *phox2b::CreER* injected embryos and not in water-injected or uninjected embryos (Figure 10). As a positive control for all the isolated genomic DNA, we amplified a 500bp band using primers for the Cerulean ORF. Unlike the *phox2b::CreER* band, the Cerulean band was apparent in all of the experimental and control genomic DNA preps indicating the success of our DNA isolation (Figure 10).

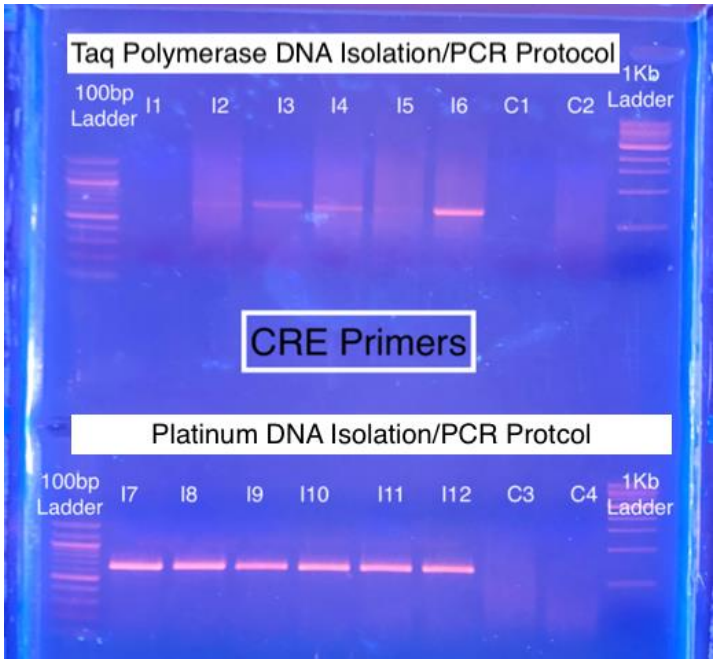

Figure 9. PCR results for *CreER* incorporation using standard *Taq* PCR and DNA isolation protocol vs. PCR using genomic DNA isolated using the Invitrogen Platinum Kit from 72hpf embryos.

Genomic PCR results for *CreER* were improved with the utilization of the Invitrogen Platinum kit. Since we did not change the injection construct or methods, we believe earlier inconsistencies in our experiment was due to inconsistencies within our genomic DNA isolation protocol. I1-I12 designates embryos injected with *Tol2phox2b8.3::CreER*, and C1-C4 designates non-injected control embryos.

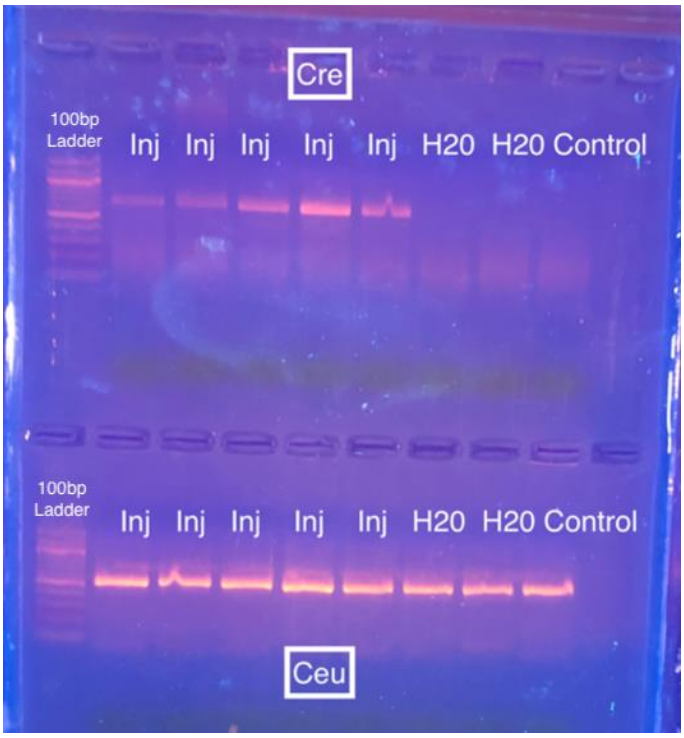

Figure 10. PCR using primers to amplify *CreER* using genomic DNA isolated from 96hpf injected experimental and control embryos and uninjected embryos.
(Top) A *CreER* band can be amplified from genomic DNA isolated from embryos injected (Inj) with Tol2 and *CreER* construct. No *CreER* band is amplified from genomic DNA isolated from embryos injected with water (H_2O) or non-injected embryos (Control). (Bottom) A *cerulean* band can be amplified from genomic DNA from all experimental and control embryos.

To further confirm that the transposed *phox2b8.3::CreER* construct is transcriptionally active in injected experimental embryos, we used RT-PCR to amplify *CreER* from RNA isolated from injected embryos (Figure 11). RT-PCR on RNA isolated from 48hpf injected embryos resulted in a *Cre* band being amplified in a subset of injected embryos. No band was seen in RNA isolated from control embryos.

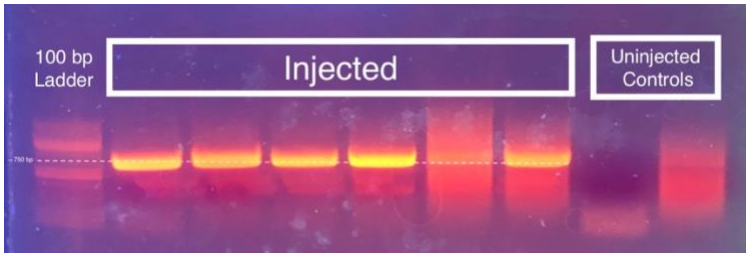

Figure 11. RT-PCR for *Cre* mRNA in RNA isolated from 48hpf injected and control embryos.
Five out of six injected embryos show a clear *Cre* band at 700bp. There are no *CreER* bands amplified in the uninjected controls.

Treatment of injected embryos with tamoxifen results in recombination of the Brainbow cassette

To determine if the *CreER* being expressed in the injected embryos is biologically active and specific to the ENS, we treated *Tol2phox2b8.3::CreER* injected embryos with tamoxifen (4-OHT). Initial experiments in the Shepherd Lab that treated embryos with 4-OHT reconstituted from a small amount of solid powder immediately prior to treatment resulted in little to no observable recombination in treated embryos.[27] Subsequently, we created stock liquid aliquots of 4-OHT in ethanol, and treatment of injected embryos using this resulted in recombination. Afterward, we made a 10-fold increase in the concentration of tamoxifen from 10µM to 100µM which yielded increased recombination. Most of the recombination that we observed appeared to be due to ectopic expression of the *phox2b8.3::CreER* construct in myocytes with one embryo showing recombination in enteric neurons (Figure 12, 13, 14). Under UV filters, we observed YFP and to a lesser extent CFP recombination across multiple myocytes in the majority of injected embryos treated with 100µM tamoxifen. Some myocytes expressed combinations of RFP, YFP, and CFP expression, which indicate different degrees of

recombination are possible in a single cell from the three Brainbow fluorescent cassette copies in each genome (Figure 7, 13, 14). There was no recombination observable in any control embryos which were either non-injected and treated with tamoxifen or injected with the *Tol2phox2b8.3::CreER* construct but not treated with tamoxifen (data not shown).

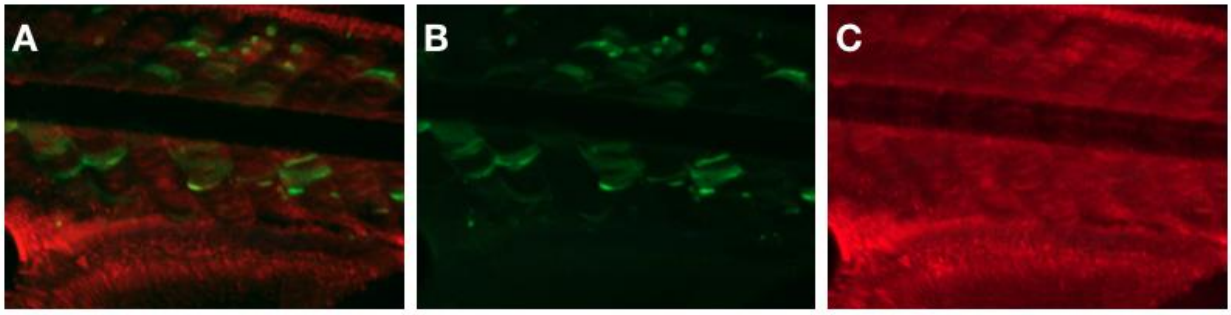

Figure 12. YFP recombination in myocytes of a tamoxifen treated *Tol2phox2b8.3::CreER* injected *ubi:Zebrabow* embryo at 7dpf.

Tamoxifen induced recombination in myocytes. (A) A combined RFP and YFP overlay. (B) Some myocytes express YFP indicating that recombination occurred in these cells. (C) RFP is expressed as in all cells as default from the Brainbow cassette; this does not indicate recombination. Note: the anterior of the fish is to the left, and dorsal towards the top.

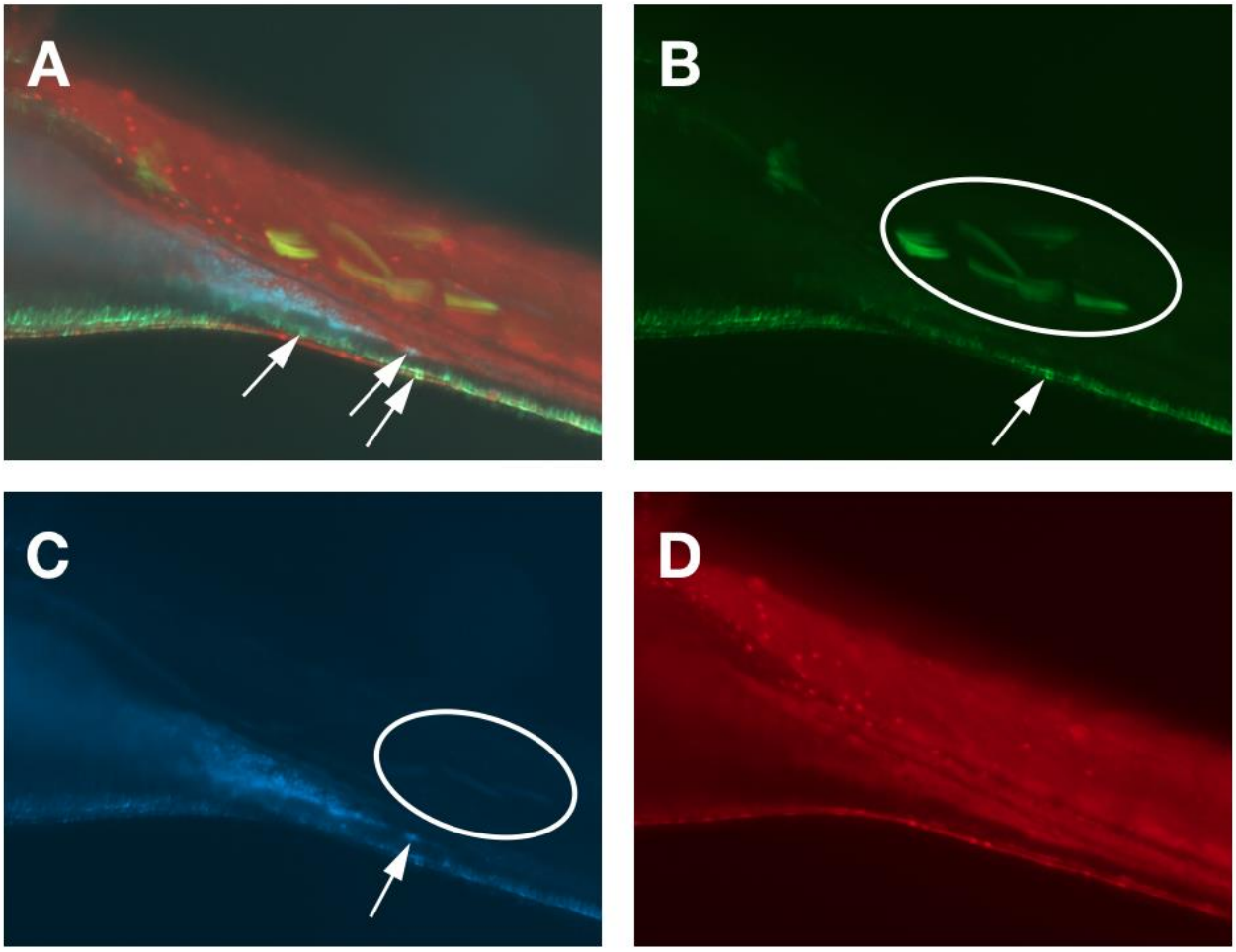

Figure 13. Observed recombination in enteric neurons and myocytes of a tamoxifen treated *Tol2phox2b8.3::CreER* injected *ubi:Zebrabow* embryo at 7dpf.

Recombination of the Brainbow cassette resulting in expression of multiple fluorescent protein can be seen at 7dpf in myocytes of the injected embryo after a tamoxifen treatment. (A) A combined overlay of RFP, YFP, and CFP filters show enteric neurons (arrows) and other cells expressing more than one type of fluorescent protein; this is due to stochastic recombination in each of the three Brainbow cassette copies per cell. (B) YFP expression in myocytes (circled) and enteric neurons (arrows) indicating recombination. (C) Recombination resulting in CFP expression is observed in the myocytes (circled) and enteric neurons (arrows). (D) RFP expression from the unrecombined Brainbow construct. Note: the anterior of the fish is to the left, and dorsal towards the top.

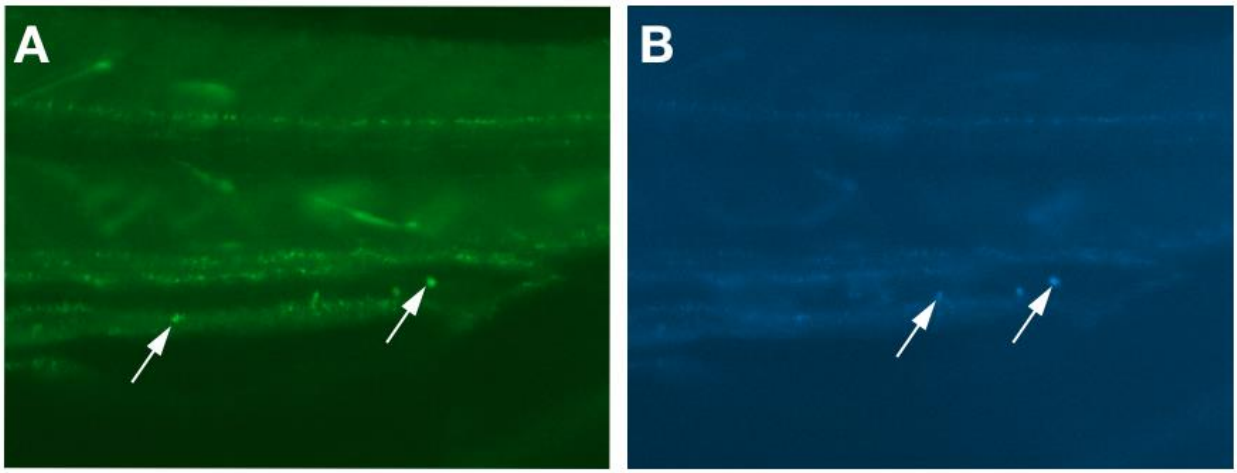

Figure 14. YFP and CFP recombination in enteric neurons of a tamoxifen treated *Tol2phox2b8.3::CreER* injected *ubi:Zebrabow* embryo at 7dpf.

Recombination is observed in the punctate cells (arrows) under YFP (A) and CFP (B) filters. This indicates successful CreER expression in enteric neurons and induction after tamoxifen treatment. Note: the anterior of the fish is to the left, and dorsal towards the top.

Discussion

Zebrafish lines

Initially, our study used the *PriZm* line which expresses the Brainbow fluorochrome construct using a *β-actin* promoter. The *PriZm* line was developed by Gupta & Poss (2012) to study lineage relationships in cardiomyocyte development.[20] When we analyzed the expression of the Brainbow cassette in *PriZm* control embryos, it became apparent that the cassette, as judged by RFP expression, was predominately seen in the heart. Expression of RFP in other organs and tissues was much lower indicating that the Brainbow cassette was not strongly expressed off the *β-actin* promoter in these tissues and therefore the *PriZm* line of zebrafish may not be particularly useful after analyzing lineage relationships in the ENS (Figure 8). Since the *PriZm* line is heavily incrossed, we expected there to be ample copies of the Brainbow cassette and hypothesized the problem to be from activity of the *β-actin* promoter. For this reason, we obtained the *ubi:Zebrabow* line that expresses the Brainbow cassette driven by the *ubiquitin B (ubi)* promoter developed by Pan et. al (2013).[16] By contrast to the *PriZm* line, the *ubi:Zebrabow* line showed strong RFP expression in all tissues, including the gut of developing embryo (Figure 8). This indicates that with the appropriate tissue specific driver we should be able to induce recombination within the ENCC and be able to study lineage relationships in descendants of recombined cells.

Wildtype stains of zebrafish are pigmented. In order to image fluorescently labeled ENCCs and enteric neurons, zebrafish ideally need to have pigmentation development prevented. This is can be achieved by raising embryos in an embryo medium containing 1-phenyl 2-thiourea (PTU). PTU hinders the production of melanin by inhibiting tyrosinase, a critical enzyme in the melanogenic pathway.[15] This inhibition is readily reversable, with removal

of PTU leading to melanization. PTU can be toxic at high concentrations, but the long term effects of using PTU on normal ENS development is not well-studied. To eliminate the need for PTU, we have crossed the *ubi:Zebrabow* with the *Casper* line of zebrafish with the aim of developing a double transgenic line which is pigment-less. *Casper* zebrafish are a double mutant in for the *nacre* and *roy* genes and was developed by White et al. (2008). *Casper* mutants lack melanocytes and iridophores and remain "transparent" into adulthood (Figure 15).[23] While our parental generation cross progeny are still phenotypically pigmented, further crossing should allow us to develop a transparent *ubi:Zebrabow;Casper* transgenic.

Figure 15. Pigmentation differences between adult wild-type *D. rerio* and the *Casper* line.

The *Casper* line of fish (bottom) lacks non-retinal melanocytes and iridophores, leading to most tissues being transparent from embryogenesis to adulthood. A normally pigmented WT (top) is shown for comparison.

Image source: White et al., 2008

Genomic CreER Incorporation and Expression

One concern about our genomic PCR experiments we had was that due to the short time span of each trial, it is possible that PCRs using genomic DNA isolated from 2-5dpf embryos could result in a band being amplified from residual *CreER* plasmid remaining in the embryos at that the time of genomic DNA isolation (Figure 10). To lower the probability of this false positive we performed *CreER* PCRs on genomic DNA isolated from 10dpf injected and control embryos. These PCRs still show *CreER* bands, indicating that the *Tol2phox2b8.3::CreER* had successfully incorporated within the host genome (data not shown). A final future confirmation will require *CreER* PCR using genomic DNA isolated from adult injected fish via fin clip. Additionally, the presence of *CreER* in the offspring of injected fish in the future would indicate that we have successfully created a germline transgenic and solidified this line.

In order to determine the presence and production of Cre recombinase, we performed reverse transcription polymerase chain reaction (RT-PCR) to identify *CreER* mRNA. We chose a 48-hour post-injection time point for the RT-PCR because there is evidence that the *phox2b* promoter controlling our injected *CreER* is active within the gut after 2dpf.[28] An early *PriZm* trial showed *CreER* mRNA expression rates to be 33% (data not shown) while two later trials using *ubi:Zebrabow/Casper* offspring showed *CreER* mRNA amplified bands on the majority of injected embryos (Figure 11). While more RT-PCR trials are needed, detection of *CreER* mRNA does further indicate successful incorporation of *CreER* within the genome.

Myocyte Recombination

Although most recombination was visualized in myocytes, the large amount of recombination seen confirms the success of previous steps used in the creation of the transient *CreER* transgenic and its activation using tamoxifen (Figure 12, 13, 14). Using 12µL of 26mM tamoxifen in ethanol per 3mL of embryo medium to create a 100µM working solution induced the most amount of recombination. The lack of recombination seen in earlier experiments was most likely due to the lower tamoxifen concentration used (10µM working concentration) and the difficulties of measuring and dissolving 0.2mg of solid tamoxifen.

Recombination occurred resulting in both the YFP and CFP proteins being synthesized with some cells expressing only one type of fluorescent protein and others expressing a combination (Figure 13, 14). This suggests that the Cre recombinase is able to induce different degrees of recombination in each of the three Brainbow cassettes within the genome. This aligns with the mosaic pattern of expression in an array of colors we would expect to see with successful recombination using the Brainbow cassette (Figure 7).

The *phox2b* promoter drives the expression of the *CreER* that was injected and incorporated into host embryos' genomes. We used this promoter because there is evidence that is active in the ENS precursors and required for their proper development.[28] Additionally, the Shepherd Lab has shown previously that Tol2 transgenesis made using a *phox2b* element can drive the expression of Kaede fluorescent protein in enteric neuronal precursor cells and be passed down to progeny to create a transgenic line.[29] Due to these reasons, we utilized the *phox2b8.3* promoter fragment instead of other transcription factors often studied within the

ENS.[30] *Phox2b8.3* is not normally expressed in myocytes. This ectopic expression which is seen in the majority of our injected embryos is likely due to the Tol2 transposition method we used to generate the transgenic line. Ectopic activation of transgenes in myocytes is a common non-specific phenotype seen F0 of Tol2 transgenics.[31] The fact that we were able to see some recombination in what appear to be ENS neurons is encouraging; although, we may consider trying other promoter fragments that are potentially are expressed in ENS precursors if the ectopic myocyte expression does not diminish in the F1 offspring.

The method to generate the *phox2b8.3::CreER* transgenic line using TOL2 means that the F0 potentially is a genetic mosaic where that some cell lineages in the injected embryo will have incorporated the *phox2b8.3::CreER* while other may not. Potentially F0 transgenics may not fully incorporate *phox2b8.3::CreER* within the ENS lineage. As a result, we are raising a set of F0 injected embryos in order to obtain a germ-line transgenic *phox2b8.3::CreER* line that would incorporate this construct in every cell of the resulting F1. Using this line for future experiments would result in consistent expression of the *phox2b8.3::CreER* construct within ENCCs.

In the future, we hope to use this stable *phox2b8.3::CreER;ubi:Zebrabow* line of fish to further study the course of ENCC specification and determine lineage relationships. Induction of recombination using tamoxifen can be performed at different embryonic ages such as 48, 60, 72, and 96hp. The resulting number and morphological phenotypic subtypes of neurons observed could then be used to determine whether ENS specification follows a fated or stochastic model (Figure 4). In both models, tamoxifen treatments undertaken at older embryonic ages should result in smaller clones than those undertaken at earlier treatments;

this is because adding tamoxifen in later stages of development would induce recombination in cells that are expected to undergo less mitosis and have fewer progeny. If ENS populations specify via a fated model, later induction times would give rise to clones with fewer neuronal subtypes (Figure 15). It might be possible to visualize clones expressing only one neuronal subtype depending on the timing when tamoxifen treatment occurs. This would allow us to conclude the time that ENCCs would be committed to becoming a specific ENS neuronal subtype and would be consistent with the fated model of ENS neuron specification. If ENS specification follows a stochastic model instead, we would expect all clones to have a diverse range of subtypes regardless of the timing of tamoxifen treatments. This result would suggest that ENS precursors remain uncomitted throughout the developmental period tested and specification could occur via non-predetermined factors.

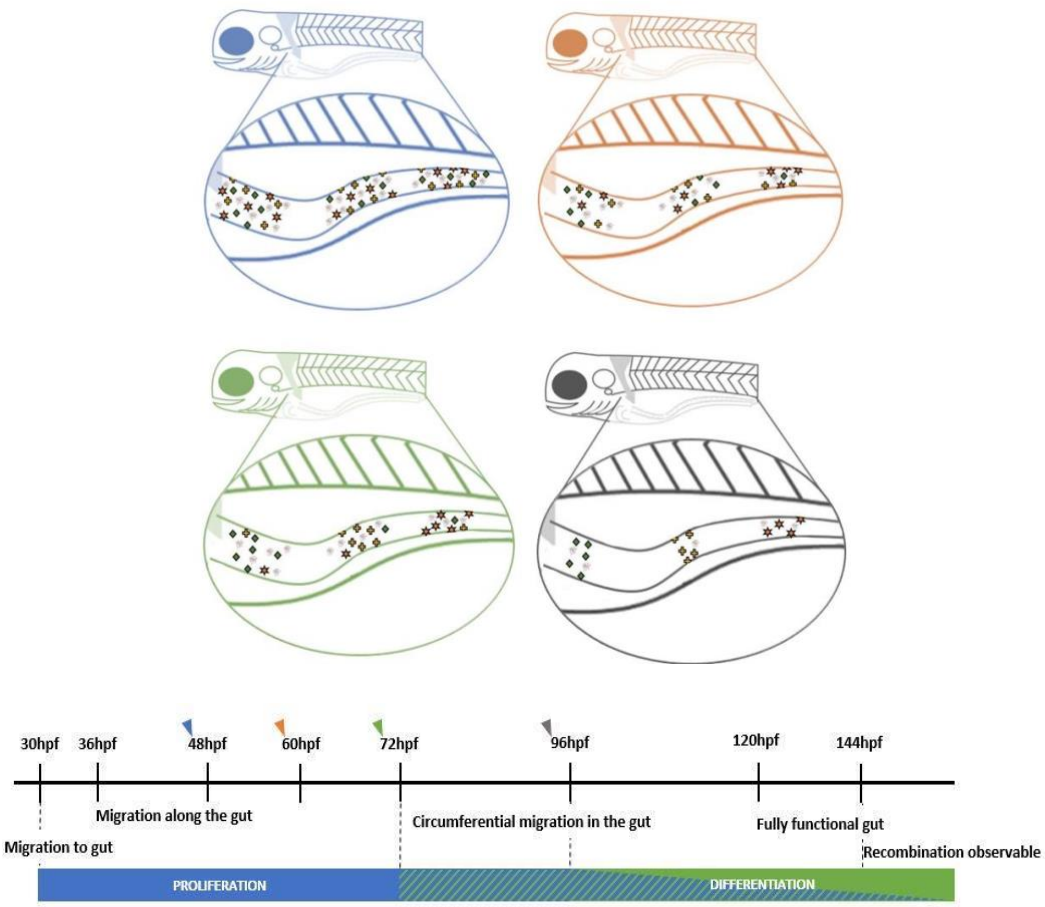

Figure 16. Expected recombination results following the fated model at time-dependent 4-OHT treatments.

Early induction of *CreEr* at 48hpf (blue) affects younger progenitor cells and is expected to result in large, clones that contain a wide variety of enteric neuronal subtypes. At 60hpf (orange), smaller clones are expected that contain fewer neuronal subtypes. According to the fated model, this trend would continue at 72hpf (green) and yield the smallest and least phenotypically diverse clones when induction occurs at 96hpf (gray).

Image Source: Amarath-Madav, 2018

C**onclusions & Future Directions**

The primary goal of this study was to create a transgenic line of zebrafish that will permit the temporally study of ENCC specification. We made many developments to generate consistent recombination results, and this project shows promise in generating a line capable of ENCC specification study. The increased injected survival rates, consistent *CreER* incorporation and expression, and effectiveness of the tamoxifen treatments indicate that significant progress has been made towards finalizing this lineage. Recombination can be seen in the ENS of some injected and tamoxifen-treated embryos, but the majority of recombination occurred in myocytes instead of the intended enteric neural precursors in F0 injected embryos. This was not unexpected given that this is a well-known non-specific ectopic expression as a consequence of TOL2 transposition. An important finding is that the *ubi:Zebrabow* line has the potential to be used to study ENCC specification due to its uniform and consistent Brainbow construct expression in the gut as compared to the *PriZm* zebrafish line. Our results also show that Tol2 incorporation is a viable method to incorporate and drive expression of Cre recombinase in this line. A 100 µM tamoxifen treatment was able to induce recombination in the majority of embryos, further indicating the viability and success of our methods to generate a double transgenic that allows recombination of the Brainbow multicolor construct. More work is required to get consistent recombination in enteric neuronal precursors as opposed to myocytes. This could be due to the mosaic incorporation pattern of Tol2 incorporation or the activity profile of our *phox2b* promoter used. The generation of a *phox2b8.3::CreER;ubi:Zebrabow* double transgenic line will be key to our long term studies. This

line will enable us to achieve our ultimate goal to draw conclusions about the nature of ENCC specification and lineage relationships in the developing ENS.

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