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Fishing for answers: Isolating enteric neurons and identifying putative ENS mutants

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Fishing for answers: Isolating enteric neurons and identifying putative ENS mutants

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DEDICATION

I dedicate my work to my family and my friends. To my parents who have stood by me and supported me through the years, and to my little brother who with his gift of gab has kept me humble, my enduring thanks. To my friends, who have kept me sane through this process and kept me calm through the darkest of nights.

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ABSTRACT

The enteric nervous system is a network of nerves that serve to innervate the gut and control homeostasis, enzyme secretion, and motility. The enteric nervous system develops from the vagal neural crest, migrating from the area of the hindbrain to populate the gut. While we know several major genes that are involved with these processes, we understand little about what genes control the migration of neuronal precursors, what genes drive neuronal differentiation, and what genes drive enteric neuron subtype specification. To determine what these genes are, we aim to isolated enteric neurons and characterize the gene expression profiles of those neurons. To achieve this, it is first necessary to develop a protocol for efficiently and quickly removing viable enteric neurons from the intestinal wall. This thesis investigates initial experiments with enzymatic dissociation protocols to isolate enteric neurons from the adult zebrafish intestine. We describe the methodology used to test the various enzymes and protocols to ascertain which conditions are most favorable for the isolation of enteric neurons from the adult zebrafish gut. Preliminary results suggest a combination of collagenase and papain enzymatic digestion is the most successful strategy for the isolation of enteric neurons from the adult zebrafish intestine.

An alternative strategy for identifying genes required for ENS development is to do a reverse genetic screen. The second part of this thesis describes efforts to generate putative mutants using the CRISPR-Cas9 system for *ssh1a* and *coro1ca*, two candidate genes that have been shown to influence actin dynamics and cellular motility and may play important roles in neural crest migration and axon guidance during ENS development. We have established three mutant lines for *ssh1a* and two for *coro1ca* and have begun characterization of these lines.

CHAPTER ONE: GENERAL INTRODUCTION

General Purpose:

The general purpose of this thesis is twofold. First, this thesis serves as an introduction to developing protocols for the dissociation of the adult enteric nervous system in zebrafish. We are interested in finding a way to dissociate neurons to identify different molecular markers of the enteric neuronal subtypes as well as genes that drive the differentiation of enteric neurons from enteric neural crest cells (ENCC). To this end, I worked on developing an enzymatic technique that would allow for the extraction of the enteric neurons from the intestinal wall and maximize cell viability. While these studies identify some enzymatic treatments that are not successful, I was able to develop other treatments that show promise with further optimization.

The second goal of this thesis is to cover the progress that has been made in verifying two putative mutations in the genes, *coro1ca* and *ssh1a*, that may play a role in either the development or function of the enteric nervous system.

With this is mind, this thesis is divided in the following manner. Chapter 1 is a literature review, covering topics such as the development of the enteric nervous system, intestinal anatomy, dissociation procedures used in mice and rats, and other general background information. Chapter 2 covers the progress in developing a protocol for the dissociation of enteric neurons. Finally, Chapter 3 discusses the progress in identifying carriers of two putative mutants in the *ssh1a* and *coro1ca* genes.

Introduction:

Enteric Nervous System:

The enteric nervous system (ENS) is the largest subdivision of the peripheral nervous system (PNS). Containing between 200-600 million neurons in humans, the ENS is capable of operating independently of the central nervous system and functions as the overseer of intestinal activity (Furness et. al. 2014). This neuronal control is important for modulating motility, homeostatic balance, secretion of digestive enzymes, and other activities required for digestion (Heanue and Pachnis 2007). The enteric nervous system works in conjunction with the interstitial cells of Cajal (ICC) and smooth muscle to move food through the intestines (Ward and Sanders 2006). This motion, called peristalsis, is vital to the continued survival of the organism. The regular contraction of the smooth muscles propels food from the stomach through the intestine and allows for digestion. Crosstalk between the ICC's and the ENS via neurotransmitters, combined with coordination of the smooth muscle of the intestinal walls, allows for precise adjustments of these peristaltic contractions in the intestines (Ward and Sanders 2006).

The majority of the ENS arises from the vagal neural crest, in particular the neural crest adjacent to somites 1-7 (Durbec et. al. 1996). In some species, a portion of the ENS is derived from the sacral neural crest, found in the region of the 28th somite, and contributes to the postumbilical ENS (Durbec et. al. 1996). Although they contribute to the ENS, sacral crest cannot compensate for the vagal neural crest during development (Burns et. al. 2000; Douarin and Teillet 1973). In mice, migration of the vagal neural crest from the neural tube begins on embryonic day 8.5 (E8.5). By day 9-9.5 the vagal crest has begun to colonize the intestine, and by E14.5-E15.5 the entire intestine is colonized (Sasselli et. al. 2012). Once the neural crest cells have arrived in the gastrointestinal tract, they are referred to as enteric neural crest cells (ENCC).

During colonization, the ENCC will migrate down the length of the intestines and differentiate into enteric neurons, settling into place in the tunics of the intestines. These tunics are the layers of the intestines (McKinley and O'Loughlin 2012). The ENCC travel in waves via a chain like motion, with cells adhering to and crawling over one another (Avetisyan et. al. 2015). In zebrafish, the neural crest migrates from the vagal area in two parallel chains of cells (Shepherd and Eisen 2013). The endothelin/endothelin receptor (EDN/EDNR) and glia cell derived neurotrophic factor/Ret (GDNF/RET) signaling pathways are required for proper enteric neural crest migration. Mutations in endothelin converting enzyme 1 (Ece1), the ligand endothelin3 (End3), and the endothelin receptor type B (EDNRB), have been shown to affect migration of ENCC into the gut (Bondurand and Southard-Smith 2016). EDNRB specifically has been shown to affect the speed of individual ENCC's but not directionality (Young et. al. 2014). Similarly, GDNF ligand and its receptors, Ret and GDNF family receptor alpha1(GFRα1), a co-receptor to Ret, play key roles in ENCC migration, and defects in this signaling pathway result in gastrointestinal tracts with regional hypogangliosis and agangliosis (Bondurand and Southard-Smith 2016). The current model is that proliferation and a decrease in space force the ENCCs to continue the migration, until the entire gastrointestinal tract is populated (Avetisyan et. al. 2015).

In zebrafish, the first enteric neural crest cells can be detected at the anterior end of the gut via *phox2b* expression 2 days post-fertilization (Shepherd and Eisen 2011). *phox2b* is a transcription factor required for the specification of autonomic nerves (Elworthy et. al. 2005). The *phox2b* population continues to migrate over the next seventy-two hours and begins to differentiate during this same period (Elworthy et. al. 2005). By day 7, the larval gut is fully innervated by enteric neurons, though the ENS has not reached the density of neurons seen in the

adult zebrafish (Holmberg et. al. 2003). Much of our current knowledge is based upon immunohistochemistry experiments from guinea pigs, rats, and mice (Hao and Young 2009). The enteric neurons are comprised of several subtypes. In the guinea pig, ten to fifteen subtypes of myenteric neuron and four to five subtypes of submucosal neurons have been identified, but the molecular programs that drive the differences in differentiation between subtypes are less well known (Hao and Young 2009). The subtypes of neurons are often identified by neurochemical combinations; each subtype expresses neurotransmitters, neuropeptides, and enzymes that synthesize neuropeptides in unique combinations (Uyttebroek et. al. 2010; Holmberg et. al. 2004; Olsson et. al. 2016; Olsson et. al. 2011; Olsson et. al. 2008). In zebrafish, the enteric neuron subtypes have not been as extensively characterized. Uyttebroek et. al. characterized some of the enteric neuron subtypes of 3 days post fertilization (dpf) zebrafish via immunohistochemistry. Their work describes five overall neuronal subpopulations based on serotonin (5-hydroxytryptamine, 5-HT), choline acetyltransferase (ChAT), neuronal nitric oxide synthase (nNOS), and the calcium binding proteins—calretinin (CRT), and calbindin (CB) (Uyttebroek et. al. 2010; Uyttebroek et. al. 2013). Seven neuropeptides have been reported to be expressed in zebrafish: serotonin, acetylcholine, substance P, neuronal nitric oxide synthase (nNOS), neurokinin-A (NKA), adenylate cyclase activating polypeptide (PACAP), vasoactive intestine peptide (VIP), and calcitonin gene-related polypeptide (CGRP) (Heanue, Shepherd and Burns 2016). While expression patterns of these neuropeptides have been identified, the morphologies and molecular mechanisms that drive differentiation of the neurons in zebrafish remain unclear (Heanue, Shepherd and Burns 2016).

There is still much to learn about enteric neuron subpopulations, particularly the subpopulations found in the adult zebrafish and the genetic markers that can be used to

distinguish between the subpopulations. Our work to isolate enteric neurons aims to bridge this gap by isolating adult enteric neurons and characterizing their gene expression patterns. To do this, we sought to design a protocol that would allow us to isolate neurons from the adult zebrafish so we could identify genetic markers of enteric neurons and use these markers to determine if any markers show subtype specific expression patterns.

Intestinal anatomy:

In higher vertebrates, the gastrointestinal tract is comprised of the small intestine and the large intestine. Both organs are comprised of four tunics, also known as layers of tissue, in mammals. From innermost to the most external, they are as follows: the mucosa, the submucosa, the muscularis, and the serosa (Figure 1) (McKinley and O'Loughlin 2012). The mucosa, which lines the lumen of the intestine, is responsible for direct interactions with the digesting food and secretion of enzymes. Folds of villi increase the surface area of the lumen, allowing for an increase in chyme-intestinal interactions during the digestive process (McKinley and O'Loughlin 2012).

In mammals, the enteric nervous system is distributed in two neuronal plexi, or networks; the submucosal and the myenteric (Sasselli et. al. 2012). The submucosal plexus is found in the submucosal layer, which also includes structures such as the lymphatic ducts and the vasculature that fuel and derive nutrients from the intestines (Wallace et. al. 2005). The muscularis layer of the intestine is comprised of two muscle sheets: the outer longitudinal muscle with fibers that are oriented parallel to the intestine and the inner circular layer with muscle fibers that wrap circumferentially around the intestinal track (McKinley and O'Loughlin 2012). The myenteric plexus is nestled between these two layers of muscle (Wallace et. al. 2005).

However, in other vertebrates such as the zebrafish, a teleost, the gastrointestinal tract consists of a singular intestine (Heanue, Shepherd and Burns 2016). The intestinal folds of teleosts are also less complex than in their mammalian cousins; zebrafish have gentler folds that are randomly distributed throughout the gut rather than the numerous villi that are found in higher vertebrates (Wallace et. al. 2005). Additionally, zebrafish lack a true stomach but have a distension of the intestine at the rostral end of the intestine, called the intestinal bulb, which has been theorized to serve as a food reservoir (Wallace et. al. 2005; Ng et. al. 2005).

Furthermore, the enteric neurons are arranged in a different manner in zebrafish. Zebrafish lack a separate submucosal plexus but they do have a myenteric plexus, embedded within the muscularis. (Wallace et. al. 2005). The enteric neurons themselves do not form ganglia, but are arranged in singular neurons or rarely in clusters of a few cells (Figure 1B) (Heanue, Shepherd and Burns 2016).

Current dissociation protocols for enteric neurons:

Protocols for successfully isolating viable enteric neurons have been developed by several groups; however, all of these protocols have been developed for isolating enteric neurons from rat and mouse tissue (Bondurand et. al. 2003; Schafer et. al. 1997; Kruger et. al. 2002; Hotta et. al. 2013; Heuckeroth et. al. 1998; Chalazonitis et. al. 1994; Anitha et. al. 2006). To characterize the different enteric neurons and subtypes in zebrafish, we first needed to develop a protocol for isolating neurons from the intestinal muscularis of zebrafish. Using the published protocols as a starting point, we investigated the effects of different enzymes, digestion temperature, and length of digestion on the effectiveness of tissue dissociation and recovery of

viable enteric neurons. These three factors have the most effect on the viability of the cells and how well the tissue is dissociated.

A few protocols for the dissociation of enteric neurons have used mice, but the majority focus on rats during the embryonic stages (13.5-14.5) or newly born rats (Kruger et. al. 2002; Hotta et. al. 2002; Schafer et. al. 1997). At the time of this writing, no attempt to isolate adult enteric neurons from the zebrafish intestine has been reported. Thus, the aim of my project was to develop a protocol for isolating enteric neurons from adult zebrafish intestines. The isolation of these neurons would allow us to address questions about the gene expression patterns in adult zebrafish enteric neurons, which could be used to determine the genes that define enteric neurons and enteric neuron subtype specification.

Four main enzymes were used in the various tissue dissociation protocols examined: collagenase, trypsin, dispase, and papain. One of the most commonly used enzymes is collagenase. Collagenase is an enzyme that cleaves the collagen triple helix and is capable of targeting multiple types of collagen. Collagenase is particularly effective at digesting collagen rich extracellular matrix (Mookhtiar and Van Wart 1992). Multiple labs have employed collagenase as a part of their protocol, usually in conjunction with either trypsin or dispase (Table 1). Initial studies by Schafer et. al. employed a concentration of 1mg/ml of collagenase as a tissue dissociation enzyme (Schaefer et. al. 1997). This concentration of collagenase forms the basis for many protocols by subsequent researchers (Schaefer et. al. 1997; Heuckeroth et. al. 1998; Anitha et. al. 2006; Bondurand et. al. 2003).

Trypsin is commonly used in conjunction with collagenase for the dissociation of intestinal tissue (Table 1) (Huang et. al. 2010). Trypsin is a protease found in the pancreas of most vertebrates and is responsible for the cleavage of dietary proteins into peptides

(Vandermarliere et. al. 2013). It cleaves only at a c-terminal end between an arginine and lysine residue (Motyan et. al. 2013). While stronger than collagenase at dissociating tougher tissue trypsin is more capable of damaging tissues and cells due to its indiscriminate targeting of all extracellular proteins (Li et. al. 2013).

Another enzyme, dispase, has been used as a fibronectinase and a type IV collagenase for the dissociation of epithelial-mesenchymal connections (Stenn et. al. 1998). Dispase has also been used to purify Schwann Cells from the sciatic nerve of rats and to isolate immune cells (Zhu et. al. 2012; Autengruber et. al. 2012). Dispase is often used in conjunction with collagenase to aid dissociation and prevent cell clumping (Autengruber et. al. 2012).

Finally, papain is considered to be a 'middle of the road' enzyme; it is capable of dissociating tissue, but also is less destructive on neurons. Papain, a cysteine protease originally isolated from the papaya, has a broad specificity and is capable of acting as an endopeptidase, amidase, and esterase (Motyan et. al. 2013). Multiple labs have used papain to isolate neurons, ranging from the retina to neurons of the CNS (Cerda et. al. 2009; Goetz et. al. 2012; Huettner and Baughman 1986; Robinson et. al. 2014). An investigation into the benefits of using papain for a dissociation of the inferior colliculi from postnatal day 3-5 rats found that the neurons were in better condition for cell culture after a papain incubation in comparison to trypsin (Kaiser et. al. 2013). Additionally, neurons dissociated using papain have been shown to retain axonal processes that survive and after dissociation retain the capacity to form synapses in culture (Sicaeros et. al. 2007). Papain has also been successful used to isolate zebrafish spinal cord neurons for use in microarray analysis (Cerda et. al. 2009).

In addition to the type of enzyme, the temperature at which the tissue is incubated is critical. Most previously described protocols in mammals were conducted at 37^0C with a few

exceptions were the dissociation was conducted at room temperature (Table 1) (Bondurand et. al. 2003; Anitha et. al. 2006).

Dissociation protocols such as Schafer et. al.'s technique were developed to isolate neurons from the myenteric plexus layer of the rat intestine by first peeling away the unwanted muscularis prior to digestion (Schafer et. al. 1997). By mechanically removing unwanted muscle layers, they were able to greatly aid the effectiveness of the enzymatic digestion. Anitha et. al. were able to do a similar step with mice ilea (Anitha et. al. 2006). In zebrafish, enteric neurons are embedded in the muscularis of the intestines, either alone or in small groups rather than in complex ganglia, and the plexus is integrated within the muscularis, rather than being a layer between the muscular layers as is the case in mice and rodents (Wallace et. al. 2005; Ganz et. al. 2016). As such, the muscularis cannot be easily peeled away, but needs to be dissociated instead to free the embedded neurons.

In addition to anatomical differences, we also wanted to keep in mind subsequent long-term experimental goals for the isolated neurons. Firstly, as our main goal was to gather transcriptomic data from the sorted cells, we would need a protocol that worked relatively quickly. If the dissociation was left too long, while we might have gotten a large number of neurons, we risk changes in transcriptome expression. The environment the cell resides in can have a large effect on gene expression; the longer the cell is outside of normal operating conditions, the more likely that gene expression will change (Evans 2015).

Secondly, to avoid having to amplify the transcriptome at later stages using PCR, we needed to collect as many GFP-positive (GFP+) neurons as possible. Thus, while a treatment may result in a higher cell dissociation index, it is equally important to maximize cell viability. With unusually long axonal and dendritic extensions, neurons are a delicate cell type; with

dissociation comes the rick that the dissociation protocol will kill the cells (Kaiser et. al. 2013). The enteric neurons of zebrafish are embedded in the smooth muscle of the gut (Wallace et. al. 2005; Ives et. al. 1978; Liang et. al. 2015). Thus one of the biggest hurdles to isolating enteric neurons was dissociating the smooth muscle tissue to remove the embedded neurons without damaging the neurons. The goal was to find or develop a protocol that gave the highest number of viable cells after dissociation.

Additionally, we know that the age of the animal can have an effect on the efficiency of a dissociation protocol on a particular tissue. For example, a protocol for the isolation of enteric neurons from the guts of d7 zebrafish embryos doesn't work with the adult tissue (Unpublished data). As the intestinal tissue matures, there is a proliferation of muscles and an increase in maturation of the epithelial luminal environment, making it increasingly more difficult to get efficient dissociation of the muscle and recovery of neurons (Wallace et. al. 2005).

Tissue Dissociation:

In order to isolate enteric neurons, it was important to be able to identify the neurons within the intestinal tissue. To identify the enteric neurons, we took advantage of a transgenic zebrafish line in which GFP is expressed under the promoter phox2b: *Tg(phox2b:GFP)* (Nechiporuk et. al. 2007). *phox2b* is a transcription factor required for the normal development of the ENS. *phox2b* is initially expressed in ENCC and later becomes more restricted to the neuronal population (Pattyn et. al. 1999). The availability of the *Tg(phox2b:GFP)* line marking enteric neurons enabled the use of fluorescence activated cell sorting (FACS) to select GFP+ neurons. In designing the initial experiments, several requirements were considered. First, we wanted to isolate as many, healthy, living cells as possible in a short time period to ensure

minimal changes in transcription. Secondly, we aimed to recover as many viable cells as possible to maximize the amount of RNA recovered for subsequent sequencing and to facilitate collecting and surveying as many neuronal subtypes as possible for genetic marker analysis. As the *Tg(phox2b:GFP)* line marks the neural population, it allowed us to use FAC sorting to exclude the non-neuronal cells such as enterocytes and smooth muscle cells. An intestine from a non-GFP expressing wild type zebrafish line, ABC-2E, serve as a control to help identify noise and exclude unwanted cell types such as enterocytes and smooth muscle in the samples.

An alternative approach would be to use a technique like laser capture to collect the neurons of interest. Laser capture is highly accurate and capable of cutting through the tougher muscle tissue, but it can only isolate a small number of cells per section (Curran et. al. 2000). Additionally, laser capture takes considerable amount of time to embed, section, and process the tissue (Curran et. al. 2000). Finally, as our end goal was to characterize the gene expression patterns for the isolated enteric neurons, a small yield of cells would have required amplification steps for any RNA isolated from these cells for gene expression studies.

Zebrafish as a model organism:

For a number of reasons, zebrafish are an excellent model organism for studying the development of the enteric nervous system. Zebrafish are: amenable to genetic manipulations such as chemical mutagenesis, morpholinos, and genome editing; capable of laying clutches of 200-300 eggs; transparent during the early stages of development, allowing for easy observation of development during embryogenesis and organogenesis; are rapid developers; and by 5 days post fertilization are fully functioning larvae (Lieschke et. al. 2007, Dahm and Geisler 2006; Chang et. al. 2013). The usefulness of zebrafish as a model organism, has been extensively

reviewed by a number of groups: Grunwald and Eisen, *Headwaters of the zebrafish — emergence of a new model vertebrate* (2002); Dahm and Geisler, *Learning from Small Fry: The Zebrafish as a Genetic Model Organism for Aquaculture Fish Species* (2006); and Sertori et. al., *Genome editing in zebrafish: a practical overview* (2015). For these reasons, zebrafish make an excellent system for studying the molecular mechanisms of ENS development.

The generation of a better molecular profile of enteric neurons and their development may provide insight into genes that influences Hirschsprung Disease (HSCR) susceptibility. The broader aim of our research is to effectively identify enteric neuron molecular markers to better identify ENS subtypes and improve our understanding of how these subtypes are affected in HSCR-like models. To do this, we sought to identify and characterize enteric neuron subtypes in zebrafish on a molecular level and identify genes that may drive subtype specification of those neurons during development. The specific aim of this project was to develop a protocol to isolate adult enteric neurons and enteric neuron precursors so that we can begin to build a profile of the genes expressed in enteric neurons. Assembling a list of gene expressed in populations of enteric neurons could also help identify markers to distinguish between these subtypes.

To identify enteric neurons we made use of a transgenic zebrafish line *phox2b:GFP*. The Tg(*phox2b:GFP*) marks all *phox2b* expressing neurons in the ENS, allowing for us to distinguish neurons from enterocytes or smooth muscle in the intestine (Pattyn et. al. 1999). This line is particularly useful for our studies as, like the endogenous *phox2b* expression, GFP expression is initiated as the neural crest emigrate from the neural tube to become the enteric neuron precursors. The transgene, and therefore the GFP, is expressed as the ENCC differentiate into neurons and continues to be expressed in the neurons into adulthood.

In humans, *phox2b* is a transcription factor required for the enteric and sympathetic neuron lineage and mutations in *phopx2b* have been linked to HSCR (Ameil et. al. 2003; Benailly et. al. 2003; Trochet et. al. 2004; Matera et. al. 2004). Loss of function studies in mice demonstrate *phox2b* is required for *RET* expression and subsequent generation of noradrenergic enteric neurons (Pattyn et. al. 1999; Pattyn et. al. 1997). Similarly, Elworthy et. al. demonstrated in zebrafish that a knockdown of phox2b via morpholinos resulted in a reduction of enteric neurons present in the gut while the rest of the gut was unaffected (Elworthy et. al. 2005).

Hirschsprung Disease:

Hirschsprung Disease (HSCR) is a neurocristopathy of the intestines characterized by aganglionosis in the distal intestines (Goldstein et. al. 2016). In Hirschsprung Disease, the neural crest cells that would normally develop into the enteric nervous system (ENS) fail to either differentiate or migrate. Those who are born with Hirschsprung Disease suffer from multiple maladies, including restriction of bowel movement, issues with feeding, abdominal distention, blockage of intestinal lumen, and vomiting (Goldstein et. al. 2016). The disease affects 1 in 5000 births, with a skewing towards males (Passarge 2002).

There are two forms of Hirschsprung Disease, differentiated by the length of aganglionic intestine. The first, and by far the most common, is Short Segment HSCR (S-HSCR). S-HSCR is also known as Hirschsprung Type 1, and is diagnosed when the aganglionosis of the intestine extends from the rectum to the colon. This subtype represents of 60-85 percent of all Hirschsprung diagnoses. Hirschsprung Type 2, also known as Long Segment HSCR (L-HSCR), affects the remaining 15-25 percent of cases (Panza et. al. 2012). In L-HSCR, the intestinal aganglionosis can extend beyond the most posterior portion of the intestines into the rest of the

intestines (Goldstein et. al. 2016). Two other variants of Hirschsprung have been reported in the literature, but remain less common than S-HSCR and L-HSCR. One variant presents with complete intestinal agangliosis (Goldstein et. al., 2016; Kappur, 1999). The other variant is when an aganglionic segment precedes an otherwise normally enervated distal segment in the intestine (Amiel et. al. 2007).

Male preponderance is approximately 4:1 for S-HSCR and 2:1 for L-HSCR (Amiel et. al. 2007). Reasons for this skew are still unknown despite ongoing research. HSCR also shows a slight ethnic bias. Asian populations are the most often affected (2.8 per 10000 live births), followed by African Americans (2.1 per 10000 live births), then Caucasians (1.5 per 10000 live births, and finally Hispanics (1.0 per 10000 live births) (Amiel et. al., 2007). Additionally, approximately 10 percent of Hirschsprung cases are concomitant with other abnormalities (Heanue and Pachnis 2007). For example, a recent study conducted by Friedmacher and Puri examined the incidence of Hirschsprung and Down Syndrome and found that of those who were diagnosed with Hirschsprung, 7.32 percent were also diagnosed with Down Syndrome (Friedmacher and Puri 2013). The complications associated with the concomitancy of both genetic disorders include increased mortality and decreased functionality post-surgery (Friedmacher and Puri 2013). While it is most common to diagnose Hirschsprung Disease during the initial stages of life, it is not unheard of for older children and even adults to be diagnosed. The main form of treatment for HCSR is surgery. While the treatment is mostly effective, long term health is compromised (Menezes et. al. 2006).

The underlying genetic cause of Hirschsprung disease is multifactorial and nonmendialian (Heanue and Pachnis 2007). Most cases of HCSR are sporadic, with no clear pattern of inheritance. However, there are familial cases of HCSR, and these cases are usually L-

HSCR. RET, a transmembrane receptor tyrosine kinase, is the most commonly associated gene for familial inheritance of L-HSCR (Gabriel et. al. 2002). Approximately 50 percent of the familial cases of Hirshsprung's are associated with mutations in the RET gene, along with 15-35 percent of sporadic cases (Panza et. al. 2012).

While RET is the gene most commonly affected in Hirschsprung Disease, it is not the sole cause of HSCR. Mutations in the RET ligand, GDNF, have also been identified in Hirschsprung sufferers, but the segregation of the gene and the disease suggests that any GDNF mutations are modifiers rather than an outright causative gene. (Borghini et. al. 2002). Additionally, several other genes including EDNRB, GDNF, the RET ligand; and *SOX10*, a transcription factor, are known to contributed to HSCR susceptibility (Panza et. al. 2012).

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Figures:

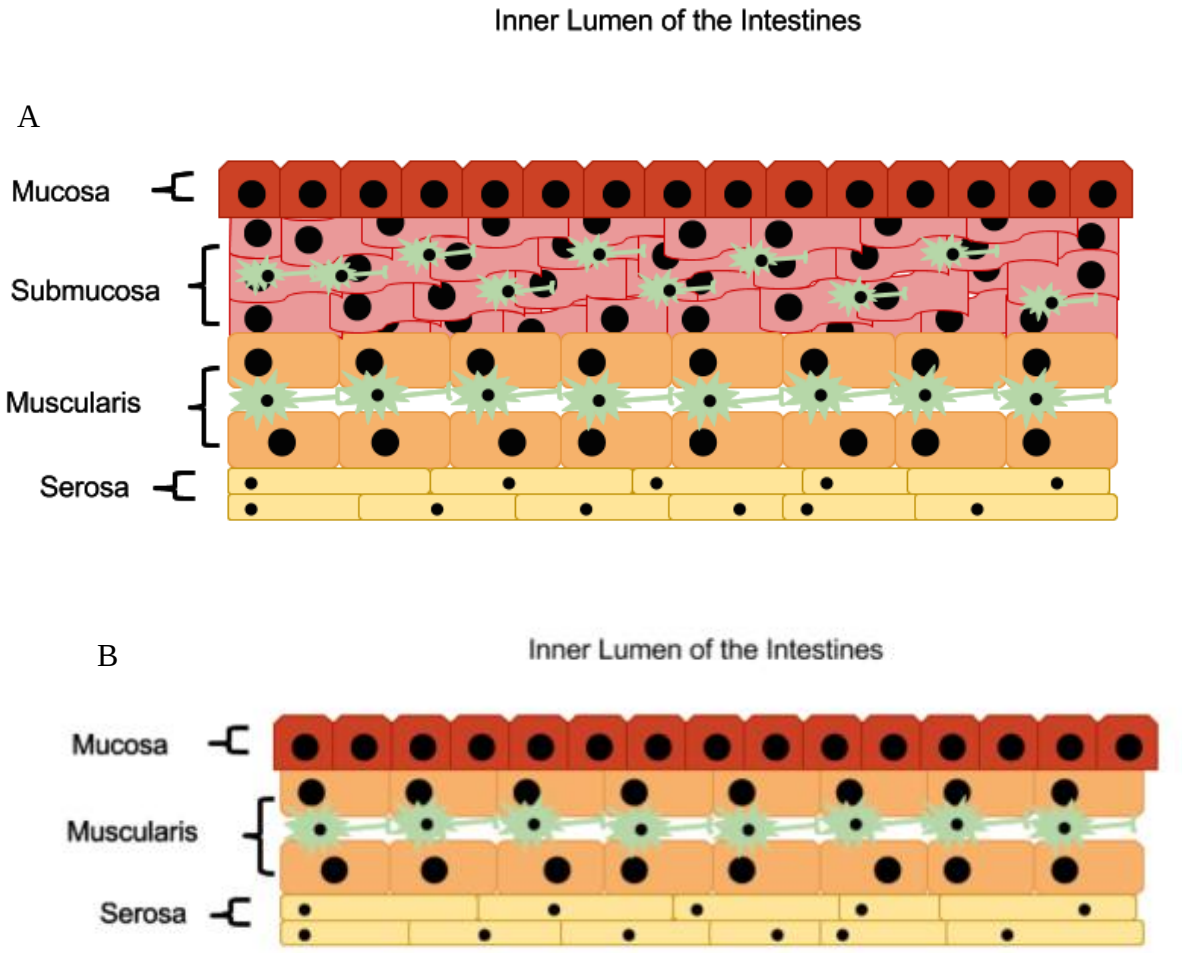

Figure 1: Representation of the layers of the higher vertebrate intestine (A) and teleost intestine (B). Derived from Wallace et. al. 2005. From inner most to outer most layers, the mucosa (red) is closest to the inner lumen of the intestines, followed by the submucosa (pink), the muscularis (orange), and the serosa (yellow). Neurons are found in the myenteric plexus (located in the muscularis layer) (small green cells) and the submucosal plexus (located in the submucosal layer) (large green cells).

CHAPTER 2:

THE ISOLATION OF ENTERIC NEURONS FROM ADULT ZEBRAFISH

Abstract:

The enteric nervous system is a subdivision of the peripheral nervous system (PNS). The ENS helps to regulate homeostasis, motility, and secretion related to proper digestive function. While many studies have focused on the neurophysiology of the enteric nervous system, the genes required for its development and the genes that define ENS subtypes are less well characterized. Our lab has been working to identify molecular markers that will help characterize the development, migration, and differentiation of the enteric neuron subtypes that make up the ENS. In order to identify genes that define different subtypes, we need to first generate a list of genes that are expressed within enteric neurons. To identify genes involved in enteric neuron specification and differentiation, we sought to isolate adult enteric neurons from the zebrafish intestine for gene expression profiling studies.

In the zebrafish, adult neurons are embedded within the muscularis of the intestine. This presents the problem of how to extract the enteric neurons from the tough smooth muscle layer without damaging the enteric neurons. To isolate intact viable neurons, we used enzymatic dissociation specifically with varying combinations of collagenase, dispase, trypsin, and papain to dissociate the intestinal tissue and isolate the enteric neurons.

Our preliminary experimental results suggest that a combination of collagenase and papain was the most successful at producing the best cell survival rates. Further optimization of this protocol could increase survival and isolation of enteric neurons by modifying incubation times and continuing to adjust enzyme concentrations.

Introduction:

To build a gene expression profile of a particular population of limited number of cells, it is necessary to isolate the cells of interest. Thus, to study the molecular signature of enteric neurons within the enteric nervous system, it is important to remove the neurons from the muscle and connective tissue layers they are embedded in, whether it's a plexus for the higher order vertebrates or from the muscularis in organisms such as the zebrafish. The isolation and expression profiling of these neurons would allow the identification of molecular markers that define the enteric neurons.

As our aim was to profile enteric neurons, we needed to find a way to isolate these neurons from the other cell types in the intestine. To do this, it was important to find a way to dissociate the intestinal tissue and differentiate between the enteric neurons and the enterocytes and smooth muscle the neurons are embedded in. To this end, we used the transgenic *phox2b:GFP* reporter line (Nechiporuk et. al. 2007). In this line, GFP expression can be observed in ENCC and most of the enteric neurons. This expression allows us to select the neuronal positive cells from dissociated tissue (Hines et. al. 2015).

Most dissociation techniques designed for the process of enteric neuron isolation focus on the isolation of enteric neurons from the rat and mice intestinal tract. An overview of these protocols for enteric neurons can be seen in Table 1. To develop a protocol for isolating enteric neurons from adult zebrafish, we based our initial experiments on these protocols. Of the eleven protocols we focused on, 6 were developed for the embryonic rat or mouse, 3 were for rodents between birth and postnatal day 14, and two were developed for the adult rat intestine.

We also considered the differences in anatomy between rat and mouse and zebrafish. Unlike the zebrafish, the rat enteric neurons are arranged in ganglia rather than as single cells or

in small groups as in zebrafish (Heanue, Shepherd and Burns 2016). Additionally, rats and mice have multiple, thicker layers of intestinal tissue that allows for muscular tissue layers to be removed by hand for easy exposure to a dissociative enzyme (Schafer et. al. 1997; Smith et. al. 2013). The layers of the zebrafish intestine cannot be easily pulled apart due to their size and the difference in the arrangement of neurons (Wallace et. al. 2005). Because of these differences, we focused on protocols that would dissociate the smooth muscle cells, without requiring manual removal of the muscularis.

We also considered the age of the tissue. The age of tissue used for enteric neuron isolations differs among the published protocols. The majority of studies utilized embryonic tissue, while a few studies used newborn or adult rat intestinal tissue (Kruger et. al. 2002; Schafer et. al. 1997; Bondurand et. al. 2003). Most of the protocols examined used embryonic day 14-14.5 (E14-E14.5) rat intestines. At this time in development, the enteric nervous system has fully colonized the developing gut and differentiation is underway, but there are still significant numbers of neural crest progenitors (Anderson et. al. 2000; Hao et. al. 2011). At E14, the small intestine of the rat is still a monolayer of endoderm; the multilayered adult form of the small intestine begins to form around E17 (Spence et. al. 2011).

The purpose of the dissociation was also a consideration. Many of the previously published protocols isolated cells for cell culture and only required a limited number of cells to survive. Because we sought to maximize the number of cells that could be used for FAC Sorting, RNA extraction, and gene expression analysis, it was important to optimize our experiments for the highest cell survival over a short dissociation time. A short dissociation time was preferential to prevent changes in gene expression that might occur over longer time scales.

Enzymatic dissociation is the preferred method for large scale isolation of enteric neurons. Other methods like laser capture, which is capable of accurate but limited extrication of neurons, are more labor intensive and costly in comparison to enzymatic dissociation for the same number of cells (Curran et. al. 2000). For gene expression profiling, large numbers of cells provide more RNA without the need for amplification and reduce potential bias introduced via amplification.

Several enzymatic combinations have been used for enteric neuron dissociation. For the dissociation of the rat intestine, many research groups used a combination of collagenase and dispase to isolate cells for cell culture (Heuckeroth et. al. 1998, Chalazonitis et. al. 1994, Anitha et 2006). Collagenase is an endopeptidase that cleaves between the glycine and the adjacent residue of the triple helix structure. It is most often used to break up connective tissue and muscle (Worthington Biologicals). Collagenase is also frequently used for the dissociation of intestinal muscle tissue to obtain cell types such as neurons, neural stem cells, and interstitial cells of Cajal (Schafer et. al. 1997; Kruger et. al. 2009; Ordog et. al. 2004). Dispase, also known as neutral protease, is capable of hydrolyzing n-terminal peptide bonds of nonpolar amino acid residues (Worthington Biologicals). It is often described as a gentler alternative to trypsin and collagenase. Together, collagenase and dispase serve to weaken both the extracellular matrix and the muscle that envelop the enteric neurons.

Another enzymatic combination used by investigators is collagenase and trypsin. This protocol is most often used to isolate cells for cell culture experiments and to compare postnatal neural crest stem cells to embryonic neural crest stem cells (Schafer et. al. 1997; Kruger et. al. 2002). Trypsin is a serine protease that cleaves on the c-terminal side of a positively charged lysine and arginine. Kruger et. al. used a combination of trypsin and collagenase to isolate

enteric neural crest cells for the characterization of antibodies that could be used to distinguish enteric neuron subtypes (Kruger et. al. 2002).

Papain, a cysteine protease, has been more routinely used for brain and retinal dissociations in rats or mice. In zebrafish, papain dissociation of spinal cord neurons was found to be a gentler alternative to enzymes like trypsin for preserving the viability of neurons (Cerda et. al. 2009). Furthermore, retinal dissociations also use papain for the dissociation of retinal neurons (Goetz et. al. 2012; Ozdinler and Macklis 2006).

The length of the experimental protocol from the beginning of dissection to collection and lysing of dissociated cells is critical. An optimal viability for RNA integrity post sorting on the FACS machine required the cells to be sorted within two hours of the beginning of the dissection (unpublished data). Therefore, we chose to focus on protocols that had shorter enzyme incubation times (Bondurand et. al. 2003, Heuckeroth et. al. 1998, Jaeger et. al. 1995).

For the above reasons, our initial experiments focused on modifying the Anitha et. al. (2006) and the Chalazonitis et. al. (1994) protocols as a starting point (Table 1). Our experiments used either a combination of two enzymes, such as collagenase and dispase or collagenase and trypsin, or each enzyme on their own (Table 2, Table 3). Beginning with collagenase, incubations were done at 37C with a concentration of 1mg/ml of collagenase, as this was the most commonly seen in published protocols (Heuckeroth et. al. 1998, Jaeger et. al. 1995, and Bondurand et. al. 2003).

Materials and Methods:

Zebrafish Care:

All fish were cared for and maintained in accordance with the guidelines laid out by *The Zebrafish Book* (Westerfield 2007). Fish were kept on a 14/10 hour light/dark cycle at 28.5C. We used the transgenic line, Tg(*phox2b:GFP)*, for all dissections (Nechiporuk et. al. 2007).

Intestinal Prep:

Twenty four hours prior to intestinal removal, zebrafish were removed from the rack and placed into a breeding tank. Feeding was restricted to limit digestive enzymes and excessive retention of food in the intestinal lumen. Two hours prior to FAC sorting, zebrafish were placed into a Tricaine Methanesulfonate (MS222) (Fluka Analytical # A5040-100G) anesthetizing solution (0.168mg/mL) and rendered unconscious. Fish were removed from the anesthetic solution and their intestines dissected out and placed in a Ca^{2+}, Mg^{2+} free Ringer's solution (0.137M NaCl, 0.004M KCl, 4.43×10^{-4}M $NaH_2PO_4 \bullet 5H_2O$, 1.83×10^{-3}M KH_2PO_4, 0.012M $NaHCO_3$, and 0.011M glucose, the pH adjusted to 7.6-7.7, and refrigerated at 4C for long term storage).

While in Ca^{2+}, Mg^{2+} free Ringer's solution, the digestive accessory organs were removed, remaining fascia extracted, and intestine longitudinally cut. In some experimental trials (where indicated), the inner lumen was lightly scraped in an attempt to aid enzymatic dissociation by removing the luminal epithelial layer. The intestine was then cut into short segments, and the fragments placed into a 1.5 ml tube containing 0.5ml of the Ca^{2+}, Mg^{2+} free Ringers solution for further digestion (See figure 2 for workflow).

Enzymes:

Enzymatic treatment varied from trial to trial (See tables 2-4), but all enzymes were diluted in Hank's Balanced Salt Solution (HBSS) (Lonza #10-508F). HBSS is a calcium and magnesium containing solution as papain is calcium dependent. The Ca^{2+}, Mg^{2+} free Ringer's was removed and replaced by 360µL of HBSS containing the enzymes.

The four enzymes used for these trials were trypsin (Thermo Scientific), dispase II (Stemcell # 07913), collagenase (Sigma Aldrich), and papain (Worthington Biologicals #LS003126). Exact concentrations and combinations of these enzymes were adjusted throughout the experiments (See tables 2-4). Enzyme incubations were either conducted in a water bath at 37^0C or at room temperature (~24C) (See Tables 2-4). Collagenase, dispase, or trypsin were diluted in 1 ml of HBSS/HEPES to reach the desired concentrations. 20 µl of papain was then added to 360 µl of this enzyme/HBSS solution and the solution added to the cells for dissociation.

Enzyme incubations were followed by one 10 minute incubation of DNase I (Promega). 10 µl of DNase 1(1U/µl) was added to the solution and incubated at 37^0C. The contents of the tube were gently mixed by pipetting up and down three to five times to disperse the settling clump of material when the DNase1 was added, and then were centrifuged at 3000g for ten minutes. Following centrifugation, the solution was removed, the pellet resuspended in 5% BSA in PBS, and the cells taken to the Iowa State University FAC Sorting Facility. Unless noted otherwise, each enzyme trial was conducted once.

FAC Sorting:

Prior to sorting, removal of any undigested clumps of intestinal tissue was done by filtering each individual sample through a filter cap that had a 35µm nylon mesh filter (Corning).

All FAC sorting was conducted on a FACSAria cell sorter (BD Biosciences). Flow cytometry was conducted on a FACSCanto II (BD Biosciences). GFP expression was used to sort the cells. GFP expressing enteric neurons from the *Tg(phox2b:GFP)* line allowed for selection of neuronal positive cells from the cell dissociation (Hines et. al. 2015). Cells were sorted into 100µl of cell lysis buffer **(**ThermoFisher Scientific AM1910). All cell lysis solutions were stored at -80C.

Total cell survival was derived as a function of the number of living cells (as defined by forward and side scatter) divided by the total events detected by the FACSAria for each trial (Tzur et. al. 2011).

Results:

Collagenase and Dispase:

Initial enzyme trials focused on the effectiveness of collagenase and dispase as enzymes to isolate single enteric neurons from intestinal tissues. The parameters we tested were concentration, length of enzymatic treatment and temperature of treatment. Based on the methods of Bondurand et. al., Heuckeroth et. al., and Hotta et. al., zebrafish intestines were treated with 0.5 – 2mg/ml collagenase and 1 mg/ml dispase for 20 min at room temperature with three additional 10 minute incubations in papain (Table 2, line 1-3). Treatments of 0.5-2mgl/µl of collagenase and dispase for twenty minutes appeared to be too harsh, resulting in little to no cells surviving (Table 2, line 1-3).

To reduce the overall enzymatic treatment, we also tested the effectiveness of collagenase and dispase in the absence of papain (Table 2, lines 4-6). We found that a 20 minute dissociation of either 2mg/ml of collagenase and 1mg/ml dispase or 0.5mg/ml of collagenase and 1mg/ml dispase at room temperature slightly increased cell survival, but also identified only a few GFP+

neurons (Table 2, lines 2-6). However, the total cell survival rates for the dissociation without the papain were 24.8 and 28.7 percent for the 2mg/ml collagenase and 0.5mg/ml collagenase respectively (Table 2, lines 5-6). Unfortunately, we were unable to obtain cell survival values for all trials due to low survival rates or due to the presence of large amounts of cell debris from damaged cells. These results suggest the combination of collagenase and dispase in the concentrations we tested may have been too harsh, even in the absence of papain.

To consolidate our efforts, we opted to focus on identifying a protocol that optimized cell survival rates, reasoning that increased cell number and survival rates would effectively increase our GFP+ population. To test if a decrease in enzyme treatment would increase cell survival, we examined the effectiveness of collagenase and dispase separately. We tried two variations on the concentration of collagenase and dispase. Each enzyme was tried separately or in conjunction with papain. We started at 0.2mg/ml of collagenase and 0.2mg/ml of dispase (Table 2, lines 8-9). As the survival rates were still in the 50-60 percent range, we lowered the concentration of both enzymes to 0.15mg/mL of collagenase and 0.15mg/mL of dispase respectively to see if we could increase survival (Table 2, lines 10-11). Survival rates for these trials improved over the combination of collagenase plus dispase dissociation: increasing from survival rates of 20-30 percent to survival rates in the mid 50's to 67 percent (Compare Table 2, lines 3-4 with lines 8-11). The results of these experiments indicate that digestion with either collagenase or dispase, in combination with papain, increases cell survival. Cell survival rates with similar concentrations of collagenase or dispase were roughly comparable; however, a decrease in collagenase concentration (Table 2, line 10) led to a higher cell survival rate. These results suggest decreasing the total amount of enzyme increases cell survival and recovery of GFP+ cells.

Trypsin and Collagenase:

In parallel to collagenase and dispase, we also examined the effects of using trypsin and collagenase as the dissociation enzymes (Table 3). Previous reports have used this combination to isolate ENCC or enteric neurons (Kruger et. al. 2002; Hotta et. al. 2013; Schafer et. al. 1997). The trypsin and collagenase combinations were tested with or without papain to determine if an alternative enzyme could improve cell survival and recovery of GFP+ cells. The initial results without papain showed a 30 percent cell survival rate; however, none of the surviving cells were GFP+ (Table 3, line 1). In a second trial incubation, a 0.025% Trypsin and 1mg/ml collagenase digestion was followed by three additional 10 minute papain incubations to see if the addition of papain would help isolate more enteric neurons. While the total cell survival rate was unavailable, there was an increase in the number of GFP+ cells isolated (Table 3, line 2). Trypsin without collagenase, in combination with the papain incubation, resulted in the recovery of some GFP cells but fewer than the combination of collagenase, trypsin, and papain (Compare Table 3, line 2 with line 8).

To eliminate the possibility that the lack of GFP+ cells recovered was due to insufficient dissociation, we also tested higher concentrations of trypsin. To investigate the effects of trypsin, we began by keeping the collagenase concentration constant at 1mg/ml of collagenase and varying the trypsin concentration at 0.025%, 0.05%, and 0.5 % (Table 3, lines 2-4). All collagenase and trypsin treatments were followed by three 10 minute papain incubations (Table 3, line 2-4). Both the 0.05% and the 0.5% trypsin treatments resulted in 0 percent cell viability, confirming our previous observations that at higher concentrations, trypsin is damaging to cells (Huang et. al. 2010). The 0.025% trypsin was the only treatment of the three to yield both a viable cell population and a small population of GFP+ cells (Table 3, lines 2-4).

Having established that a lower concentration of 0.025% trypsin gave a higher GFP-positive recovery rate, we then tested whether slightly shorter or longer trypsin incubations would improve the results (compare Table 3, lines 2, 5-7). In the next set of experiments, we kept the concentrations of collagenase and trypsin the same, at 1mg/1ml and 0.025 percent respectively. We began by assessing three periods: five, eight, and twelve minutes in length. The five minute trypsin incubation showed very few GFP+ cells (Table 3, Line 5); the eight and twelve minute incubations were not significantly different, with 140 and 147 GFP+ cells respectively (Table 3, lines 6-7). The results indicate that slightly longer incubation times gave slightly higher numbers of GFP+ cells.

Together, these results suggest that when using trypsin as a dissociation agent, a combination of all three enzymes, collagenase, trypsin, and papain is more effective at improving the recovering GFP+ cells than just two enzymes. Secondly, higher concentrations of trypsin do not result in a higher recover of GFP+ cells, but slightly longer incubations of a lower concentration of trypsin may be more effective at recovering GFP+ cells. While the combination of collagenase, trypsin, and papain yielded some GFP+ cells, the numbers remained far lower than we would expect and what would be optimal for transcriptional profiling. As the number of GFP+ neurons was below what we were obtaining in parallel experiments with papain treatment, we discontinued experiments with the combination of collagenase and trypsin.

Papain:

From our trials assessing collagenase and trypsin incubation times, we noted that additional papain incubations may aid the recovery of GFP+ cells. To test the effectiveness of papain as a dissociative enzyme, we carried out a series of experiments examining papain

digestion alone. For this incubation, we used 2.17mgp/ml of 2x papain. This dissociation produced a large, tight cluster of 1882 GFP+ cells, with a 38.2 percent survival rate (Table 4, line 1). These clusters were assessed by using the forward scatter on the FACS machine, which indicates cell size, and GFP fluorescence levels. Tight clusters of cells indicated the cells isolated are of a similar type; the tighter the cluster, the more alike the collected cells are. As this was a more promising avenue than other trials combining collagenase and papain, we decided to further investigate the effects of papain on the enteric neuron dissociation (Table 4).

To investigate the effectiveness of papain to dissociate adult zebrafish intestines, we examined how enzymatic concentration and length of incubation time affected the dissociation. To do this, we modified protocols that used papain to dissociate retinas or spinal cords (Goetz and Trimarchi 2012; Cerda et. al. 2009; Ozdinler and Macklis 2006). We first investigated enzyme concentration. From previous work using larval intestines, we found that 2.17mgp/ml of papain was optimal for the isolation of high numbers of viable GFP+ cells (unpublished data). Using this concentration as a starting point, we found that while we saw a similar cell survival rate between intestines incubated with 1.7mgp/ml and 2.17mgp/ml of papain, approximately 38 percent, we did not see any GFP+ cells for the 1.7mgp/ml treatment (Table 4, Lines 1-2). On the other hand, the 3.17mgp/ml treatment had 1206 GFP+ cells and 31.5 percent survival, which was lower than the 2.17mgp/ml outcomes for both categories. This suggested that an increase in papain concentration decreases the number of GFP+ cells, while a lower concentration of papain may be insufficient to effectively dissociate the tissue (Table 4, Lines 2-5)

In separate experiments, we also examined the effects of increasing incubation time by two minutes (two 10 minute incubations plus one 12 minute incubation). Increasing the last incubation time by two minutes resulted in 1557 GFP+ cells, but also resulted in a lower overall

survival rate at 23.4 percent. These experiments suggested three 10 minute incubations of 2.17mgp/ml of papain had the highest cell survival rate and recovery of GFP+ neurons.

During the course of the experiments, we observed that a newer batch of papain resulted in the loss of a tight cluster of cells on the FACS machine. This cluster is indicative of a cell population of a similar type, determined by their forward scatter and the GFP expression. The preparations using the new papain treatments lacked this tight cluster, and the survival rate decreased to 23.9 percent. This suggested that the newer batch was slightly stronger than our previous batch. To compensate for this, we decreased the incubation times to three sets of 7 or 8 minutes. (Table 4, lines 7-12). The shorter incubation times, three sets of 8 minutes and three sets of 7 minutes, yielded a greater survival rate than the previous incubations. The former had a cell survival rate of 55.1 percent, while the latter had a 59.9 percent survival rate (Table 4, lines 7-8). The decrease in incubation times improved cell survival and isolation of GFP+ cells. The three 8 minute incubation yielded 1358 GFP+ cells as compared to the three 7 minute incubation which yielded 615 cells. This suggested that the three sets of 8 minute incubations slightly improved the recovery of GFP+ cells. Unfortunately, the cells were not clustered into a single population, suggesting that these cells were background noise. Even a decrease in enzyme concentration failed to improve clustering (Table 4, line 9).

Reasoning that we may improve enzymatic accessibility to the tissue by reducing the number of cell layers, we also attempted to improve the intestinal preparation. As enteric neurons are embedded in the zebrafish intestinal muscularis, it is not possible to remove the muscularis layer as it in mice and rats (Schafer et. al. 1997; Anitha et. al. 2006). We hypothesized that scraping the lumen of the intestine could help the dissociation process by removing the enterocytes or absorptive epithelial layer of the intestines. We did multiple trials wherein we

manually scraped the luminal surface to remove layers of the intestine. While initial trials with a deep scrape showed a nice cluster of 2553 cells and a survival rate of about 60.8 percent, the cell survival outcomes for the scraping were highly variable, ranging from 0.65-25.3 percent (Table 4, Lines 10-11). These results suggested that scraping of the intestinal lumen, while having the potential to increase yield, also risks the chance of decrease in cell viability.

Conclusion:

Prior to this research, the only reported protocols for the isolation of enteric neurons were from rat and mice protocols (Table 1). Using those protocols as a base, we set out to determine which modifications of these protocols would enable us to isolate neurons from the adult zebrafish intestine.

Because of the elaborate axonal and dendritic extensions, neurons as a rule are one of the more challenging cell types to isolate from dissociated tissue (unpublished data). The collagenase and dispase combinations all had cell survival rates in the 20 percent range (Table 2, lines 1-7). With this low total cell survival rate, low viability decreases the chances to isolate GFP+ cells.

However, in later trials we were able to demonstrate that collagenase or dispase alone with papain can increase survival rates to the 50-60 percentages (Table 2, lines 8-12). The results from the single enzyme treatments of dispase and collagenase suggested the dissociation of the zebrafish enteric neurons was better if either enzyme, but not both, was used in combination with papain (See table 2). The 0.15mg/ml of collagenase combined with 2.17mgp/ml of papain treatment incubated for three 8 minute periods at 37C had the highest survival rate at 67.4 percent (Table 2, line 10). The increased survival rate for this treatment suggests that a lower

concentration of collagenase resulted in the highest cell survival rate. Confirmation of this result is required, but it provides a starting point for future testing. Our assumption is that by optimizing the survival rate, we would increase the probability of more enteric neurons surviving the dissociation process. Both a decrease in collagenase and dispase concentrations improved cell survival, suggesting that combinations of dispase and collagenase at lower levels may be effective at dissociating the adult tissue (Table 2, lines 10 and 11). Future modifications of this protocol to further improve the efficacy of dissociation could include extending incubation times with the lower concentration of collagenase (0.15mg/ml) or decreasing the collagenase concentration in combination with a decrease in dispase concentration. Other alternatives include adjusting the length of incubation time or the temperature at which the incubation is conducted to allow for either more or less enzyme exposure.

Unlike the protocol suggested by Hotta et. al., in which E13.5/E14.5 rats were successfully dissociated using 0.1% trypsin, we found that even a 0.05% solution of trypsin was too harsh for the adult zebrafish intestine (Table 3, line 3). The lack of GFP+ cells and the fact that any potential surviving cells were damaged indicates that these trials are above the upper threshold for cell survival in general. Even with milder trypsin treatments of 0.025% relatively few GFP+ cells were isolated (Table 3). Although we did not formally test it, based on our initial findings, further decreases in trypsin and/or collagenase concentrations in combination with papain may be effective at increasing the number of GFP+ cells recovered (Table 3, Line 7).

The final set of experiments focused on the use of papain as a dissociative enzyme. When the tissue was treated only with papain, cell survival rates were between 30 to 60 percent (Table 4). The majority of the papain trials had survival rates in the thirties. Two trials, however, had survival rates in the fifties (Table 4 line 7-8). These two trials had shorter incubation times,

indicating that their success may be due to the decreased incubation time. Taking advantage of this result, we then tested if decreasing incubation time, and therefore total length of time in papain, would increase cell survival rate. We found that by decreasing the time in papain, cell survival rate increased from 23.9 percent to 59.9 percent with shorter incubation times (Table 4, lines 6 - 8).

Unlike mice and rats, the muscularis layer in zebrafish cannot be easily removed. The reason for this is twofold: first, the zebrafish intestine is significantly smaller and therefore is technically more challenging to manipulate than the rodent intestine. Secondly, and perhaps even more challenging, is the fact that enteric neurons in the adult zebrafish are embedded in a much thinner, less developed muscularis as single or small groups of cells, rather than in ganglia as in rodent models (Wallace et. al. 2005; Kruger et. al., 2002; Schafer et. al. 1997; Anitha et. al. 2006). However, as an alternate, we attempted to improve access of enzymes to the muscularis by mechanically removing the inner luminal epithelial layer, the enterocytes.

Scrapping trials showed the enteric neuron cell survival rates could be increased by mechanically removing the luminal layer of enterocytes. The initial deep scrape showed 60.8 percent cell survival while the initial light scrape had a 74.4 percent survival (Table 4, lines 10, 12). However, repetitions of the experiments could not replicate the GFP+ cell survival rates (Table 4, line 11), suggesting that scraping the intestinal lumen may introduce more variability, depending on completeness of scraping and/or operator skill levels.

In the course of these experiments, we also became aware of potential confounding issues with autofluorescence. Particular populations of intestinal cells are highly autofluorescent and make the distinction between GFP+ cells and autofluorescent cells less distinct. To circumvent this we began to use PE:A-FITC ratios and cell survival rate to measure the effectiveness of the

dissociation. The PE:A-FITC ratio can determine whether or not a cell population is autofluorescent by examining the cell population for both RFP and GFP fluorescence. If a population of cells fluoresces under both the RFP (PE:A) channel and the GFP (FITC) channel, it is likely that the particular population is autofluorescent. We found that in our later experiments the cell populations sorted as GFP+ may exhibit such autofluorescence. This complication means that prior collections of GFP+ cells may or may not have also been autofluorescent. Unfortunately, we cannot go back and assess the PE:A-FITC ratios for the experiments prior to this discovery. The autofluorescence may confound GFP+ cell data, but it does not affect overall cell survival ratios, as these ratios were independent of the fluorescence counting. From the overall cell survival ratios, we can at least determine how effective a dissociation treatment is and make adjustments and decisions based on those parameters.

There are a number of possible lines of investigation that can be used to further this preliminary protocol, such as optimizing the number of viable dissociated cells, further adjusting the incubation times, and altering the levels of collagenase, trypsin, or papain. Adjusting incubation times should affect cell survival rates. A shorter incubation time could allow for more cells to survive the dissociation, but it also runs the risk of decreasing the completeness of the dissociation. If the dissociation is not complete, enteric neurons will be caught in the undissociated tissue and strained out prior to FAC sorting. On the other hand, increasing the dissociation efficiency increases the risk of decreased cell survival due to the harsher treatment of the cells. Similar considerations must be taken when choosing enzyme concentration(s). Lower levels of enzyme would likely decrease cell damage. On the other hand, not enough enzyme could lead to tissue that fails to be dissociated and a decrease in neurons recovered.

With these initial experiments in mind, we have laid the foundation for future studies into a protocol for the isolation of enteric neurons. Our results suggest that the concentrations of collagenase and dispase that we tested proved to be too harsh of a dissociation protocol, resulting in little to no cell survival. Our data also suggests that collagenase and trypsin, in the concentrations tested, resulted in less than optimal cell survival rates. This is in agreement with Buehler et. al. 2012, who found that trypsin resulted in low survival rates for enteric neural progenitors when using trypsin and collagenase. However, our results provide preliminary data that collagenase in combination with papain is somewhat able to improve cell viability.

With this work, we have identified multiple strategies that do not work for the isolation of enteric neurons from adult zebrafish intestine, and we have identified strategies that could be modified to further improve cell survival rates and used to isolate GFP+ cells using fluorescence activated cell sorting (FACS). We have determined that caution should be taken with respect to autofluorescence when using fluorescence to purify dissociated cells. With this as our starting point, we can continue to modify our protocol and enhance cell viability by adjusting combinations of collagenase, dispase, trypsin and papain at lower concentrations for shorter incubation times, or looking to alternative enzymes, such as accumax (Buehler et. al. 2012).

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Figures:

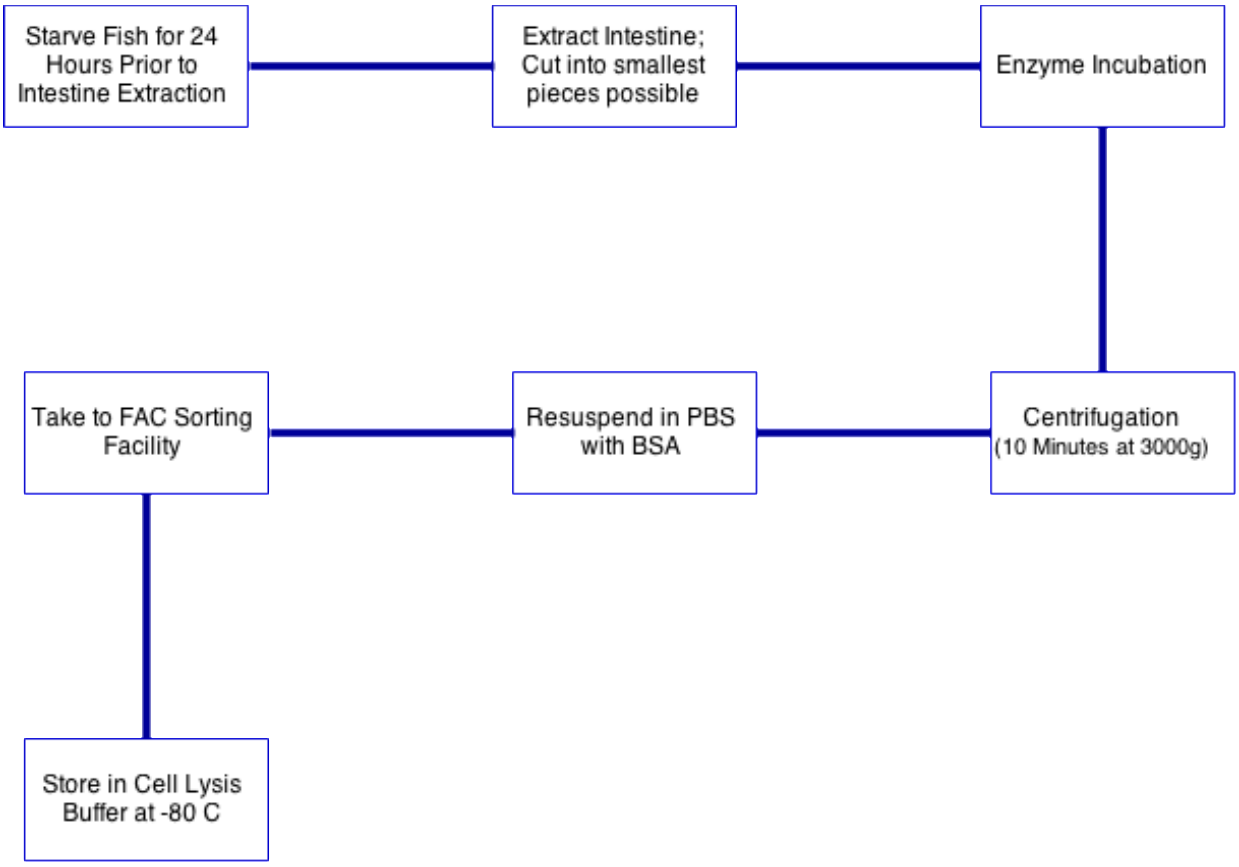

Figure 2: Diagram of procedural workflow. While the enzyme incubation varied from trial to trial, the overall work flow was similar.

Table 1: An overview of previous dissociation protocols used for the dissociation of enteric neurons from rats and mice.

Source	Enzyme	Concentration	Length of Incubation	Temperature (C)	Organism	Additional Details
Kruger et. al. 2002	Trypsin	0.025%	8 Minutes	37	Rat E14.5	Peeled Plexus
	Collagenase Type 4	1mg/ml				Used Ca2+, Mg2+ free HBSS
Kruger et. al. 2002	Trypsin	0.025%	20 Minutes	37	Adult Rat	Peeled Plexus
	Collagenase Type 4	1mg/ml				Used Ca2+, Mg2+ free HBSS
Schafer et. al. 1997	Collagenase	1mg/ml	1 hour, followed by 3-4 30 minute baths	37	Newborn Rat	Peeled Plexus
	Trypsin	0.05%	30 minutes			
Hotta et. al. 2013	Trypsin	0.10%	20 Minutes	37	Rat E13.5-14.5	
Hotta et. al. 2013	Dispase II	0.50%	30 Minutes	37	Rat: P4	
	Collagenase	0.05%				
Heuckeroth et. al. 1998	Collagenase	1mg/ml	30 minutes	37	Rat: E14.5	Placed intestines into N2 media
	Dispase II	1mg/ml				
Chalazonitis et. al. 1994	Collagenase	0.50%	30 minutes	37	Rat E14.5	Used Ca2+, Mg2+ free saline
Anitha et. al. 2006	1. Collagenase Type 2	1mg/ml	90 minutes	24	Rat E14.5	Peeled Plexus, cut into 1cm sections
	2. Collagenase Type 2*	1mg/ml	20 Minutes	37		*Repeated 2-3 times
Bondurand et. al. 2003	Collagenase	1mg/ml	6 minutes	24	Mice E11.5	Washed with Ca2+, Mg2+ free saline
	Dispase II	1mg/ml				
Bondurand et. al. 2003	Collagenase	1mg/ml	45 minutes	37	Mice P2-P14	Outer layers stripped with forceps
Jaeger et. al. 1995	Collagenase	0.10%	0.5-4 hours	37	Adult Rat	Longitudinal muscle/plexus removed prior to incubation; intestine divided into 3 parts

Table 2: Enzymatic trials testing the effectiveness of collagenase and dispase dissociation. Temperature, volume, and incubation varied between experiments and affected the overall cell survival rate and GFP+ cells recovered. In this table, papain incubation refers to three sets of ten minute incubations. 20µl of papain (2.17mgp/ml) was used for these incubations. Collagenase or dispase in combination with papain increased overall cell survival but did not improve the isolation of GFP+ neurons.

	Enzyme	Time (Min)	Total Enzyme Time (Min)	Temperature (C)	Papain Incubation	# of GFP+ Cells	Percent Cell Survival
1	1mg/mL Collagenase 1mg/mL Dispase	20	50	24	Yes	0	0
2	2mg/mL Collagenase 1mg/mL Dispase	20	50	24	Yes	0	N/A
3	0.5mg/mL Collagenase 1mg/mL Dispase	20	50	24	Yes	13	N/A
4	1mg/mL Collagenase 1mg/mL Dispase	20	20	24	No	0	0
5	2mg/mL Collagenase 1mg/mL Dispase	20	20	24	No	0	24.8
6	0.5mg/mL Collagenase 1mg/mL Dispase	20	20	24	No	0	28.7
7	1 mg/mL Collagenase	10	30	37	Yes	75	N/A
8	0.2 mg/mL Collagenase	3x8 Min	24	37	Yes	0	54.7
9	0.2 units/mL Dispase	3x8 Min	24	37	Yes	0	55.4
10	0.15 mg/mL Collagenase	3x8 Min	24	37	Yes	0	67.4
11	0.15 mg/mL Dispase	3x8 Min	24	37	Yes	0	52.4

Table 3: Experimental results testing the effectiveness of collagenase, trypsin, and papain in the dissociation of the adult zebrafish intestine. Papain incubation was the same as outlined in the legend for Table 2.

	Enzyme	Time (Min)	Total Enzyme Time (Min)	Temperature (C)	Papain Incubation	# of GFP+ Cells	Total Percent Survival
1	0.025% Trypsin 1mg/mL Collagenase	10	10	37	No	0	30.4
2	0.025% Trypsin 1mg/mL Collagenase	10	30	37	Yes	87 -168 (Two Trials)	N/A
3	0.05% Trypsin 1mg/mL Collagenase	10	30	37	Yes	0	0
4	0.5% Trypsin 1mg/mL Collagenase	10	30	37	Yes	0	0
5	0.025% Trypsin 1mg/mL Collagenase	5	35	37	Yes	6	N/A
6	0.025% Trypsin 1mg/mL Collagenase	8	38	37	Yes	140	N/A
7	0.025% Trypsin 1mg/mL Collagenase	12	42	37	Yes	147	N/A
8	0.025% Trypsin	10	30	37	Yes	22	N/A

Table 4: Experiments to determine the effectiveness of papain concentration and incubation times on dissociation and cell viability.
*Indicates that the new bottle of papain was utilized for this trial

	Concentration	Incubation Times (Min)	Total Enzyme Time (Min)	Temperature (C)	# of GFP Positive Cells	Cell Survival (%)	Notes
1	2.17mgp/ml	3x10 Min	30	37	1882	38.2	N/A
2	1.7mgp/ml	3x10 Min	30	37	0	38.9	N/A
3	3.17mgp/ml	3x10 Min	30	37	1206	31.5	N/A
4	2.17mgp/ml	2x10 Min; 1x12 Min	32	37	1537	23.4	N/A
5	2.17mgp/ml per tube	3x10 Min	30	37	954	33.5	Intestine split into two tubes
6	2.17mgp/ml	3 x 10 min	30	37	0	23.9	New Papain Bottle Used*
7	2.17mgp/ml	3 x 8 min	24	37	1,358	55.1	No Tight Cluster*
8	2.17mgp/ml	3 x 7 min	21	37	615	59.9	No Tight Cluster*
9	1.7mgp/ml	3 x 8 min	24	37	931	5.1	No Tight Cluster*
10	2.17mgp/ml	3 x 7 min	21	37	2553	60.8	Intestinal Lumen scraped; myenteric plexus left *
11	2.17mgp/ml	3 x 7 min	21	37	65	75.4	Intestinal Lumen scraped; myenteric plexus left *
12	2.17mgp/ml	3 x 7 min	21	37	382	74.4	Light Scrape*

CHAPTER 3:
THE CHARACTERIZATION OF *SSH1A* AND *CORO1CA* MUTANTS

Abstract:

With the advent of new genetic engineering technology, genome editing in zebrafish has become increasingly easier to conduct and manipulate. In particular, the discovery of the CRISPR-Cas9 system has allowed scientists to target their gene of interest (Charpentier and Doudna 2013). We have taken advantage of this technology to induce mutations in candidate genes that may play a role in the development or function of the enteric nervous system (ENS). Two genes, *coro1ca* and *ssh1a*, have demonstrated roles in actin dynamics, but their exact role in ENS development remains unclear (Williamson et. al. 2015; Niwa et. al. 2002). To examine a role for these two genes in ENS development, CRISPR-Cas9 constructs were designed and generated to cut within the 5' and 3' regions of these genes, with the aim of disrupting gene function.

Zebrafish embryos were injected with the respective CRISPR-Cas9 constructs, raised to adulthood, and the mosaic founders (G0) and G1 outcross fish screened for genomic mutations. Three *ssh1a* lines and two *coro1ca* lines showing CRISPR-induced insertions or deletions (indels) in these genes were identified.

Aim:

The aim of this study was to identify carriers of mutations induced by CRISPR-Cas9 to establish stable lines and characterize the roles for *ssh1a* and *coro1ca* in the development of the enteric nervous system.

Introduction:

Based on a gene expression analysis of day 7 zebrafish larval intestines, two genes, *coro1ca* and *ssh1a*, were identified as putative molecular markers of enteric neurons (unpublished data). To test a role for these genes in ENS development, CRISPR-Cas9 genome editing technologies were employed to create mutations in the coding sequence of these genes (data to be described elsewhere).

The CRISPR-Cas9 system used for genome editing has two major components: an endonuclease and a guide RNA. The endonuclease serves to create a double stranded break in the genome (Sander and Joung 2014). The Cas9 endonuclease is guided by a gRNA that can be tailored to whatever target gene one is desirous of cleaving (Jinek et. al. 2012). This gRNA is synthesized from two other RNA's: a CRISPR RNA and a trans-activating crRNA (tracrRNA) (Jinek et. al. 2012). The gRNA targets what is known as a proto-spacer adjacent motifs (PAM) on the genome and guides the Cas9 endonuclease to the target sequence. The Cas9 endonuclease will make a cut at the target sequence location (Charpentier & Doudna 2013). The mechanism of action and application of CRISPR-Cas9 system as a genome editing technology has been extensively reviewed by Charpentier and Doudna (2013) and Sander and Joung (2014).

After the creation of a double stranded break, there are two different pathways that can be activated to repair the break in the DNA. The first is homologous recombination (HR) and the second is DNA repair is nonhomologous end joining (NHEJ). NHEJ is prone to misrepair (Liu et. al. 2012; Hagmann et. al. 1998). Unlike HR, no template is specifically required for NHEJ repair, which often leads to insertions and deletions (van Gent et. al. 2016). This can be useful for the creation of mutants lines, as even a single base pair insertion or deletion can disturb the translation reading and potentially disrupt gene function.

Coronin, actin binding protein, 1ca, (*coro1ca*), also known as *coro1c*, is a protein that helps to prevent destabilization of actin chains (Insall and Machesky 2009). Several studies have linked *coro1ca*'s actin modifying activity to the Arp2/3 complex, which facilitates actin branching by creating new nucleation cores, the starting points for actin polymerization and branching, on preexisting actin chains (Insall and Machesky 2009, Chan et. al. 2011). *Coro1ca* has also been associated with the migratory behavior of neural crest (Xavier et. al. 2008; Williamson et. al. 2014). A study conducted by Williamson et. al. demonstrated a requirement for *coro1ca* in the migration of cranial neural crest. Knockdown of *coro1ca* with morpholinos resulted in malformed pharyngeal arches and the mismigration of the first and second arch due to a decrease in cranial neural crest migration. (Williamson et. al. 2014).

Coro1ca plays a role in the distribution and redistribution of Rac1-GDP. Rac1-GDP is a member of the Rho GTPases required for cell movement (Liu et. al. 2009). *Coro1ca* assists cytoskeletal restructuring by moving Rac1 from the lateral membrane to the ruffling membrane to destabilize the actin filaments (Williamson et. al. 2015). The role of *coro1ca* and other coronins has been well described in a series of recent reviews; see Chan et. al. (2011) and Insall and Machesky (2009).

Less is known about the slingshot phosphatase protein 1a, or *ssh1a* gene, a member of the *slingshot (ssh)* family. The *ssh* family is a family of phosphatases that act to dephosphorylate cofilin and interact specifically with cofilin and actin depolymerizing factors (Torres et. al. 2011; Niwa et. al. 2002). Cofilin is a part of the Actin Depolymerizing Factor family, a family of proteins that act to depolymerize F-actin (Carlier et. al. 1997). Cofilins localize to the ruffling membranes of leading cells and have been shown to be required for cell movement and actin filament destabilization (Aizawa et. al. 1995). The phosphorylation state of cofilin helps to

control how cofilin interacts with the actin cytoskeleton of cells. When cofilin is phosphorylated, its ability to bind to F-actin is inhibited, and when cofilin is dephosphorylated cofilins can bind to F-actin and help to destabilize the actin cytoskeleton (Rheenen et. al. 2009).

Recent research suggests that the *ssh* family can also interact with LIM-kinase 1 in a regulatory fashion (Huang et. al. 2006). Cofilin is phosphorylated by LIM-kinase 1 (LIMK1) and dephosphorylated by *ssh* (Niwa et. al. 2002). Together, the kinase and the phosphatase work in a cycle: when the cofilin is phosphorylated by LIM-kinase 1, cofilin is deactivated (Niwa et. al. 2002). When the *ssh* family dephosphorylates cofilin, cofilin can bind to actin once more and begin to depolymerize actin. This allows for actin to be disassembled and the cytoskeleton rearranged (Niwa et. al. 2002).

What remains to be determined is if there is a role for *coro1ca and ssh1a* in the development of the enteric nervous system. Based on current understanding of *coro1ca's* mechanism of action, we would predict *coro1ca* may be required for proper neural crest migration. While we know less about *ssh1a,* we would predict that it too may play a role in the migration of neural crest or in axon pathfinding by modifying the actin cytoskeleton. To test this, we aimed to establish a mutant line that would allow us to examine the roles of *coro1ca* and *ssh1a* in the development of the ENS.

Materials and Methods

Fish Lines:

CRISPR-Cas9 constructs were injected into the ABC-2E wild type one cell zebrafish embryos within 20 minutes of fertilization. CRISPR-Cas9 guide RNAs targeting two locations, a

5' target site and a 3'target site, were designed for each of the *ssh1a* and *coro1ca* genes. Further details on the design of the gRNAs will be described elsewhere.

Zebrafish were raised to adulthood using standard zebrafish husbandry as outlined in *The Zebrafish Book* (Westerfield 1993). G0 mosaic embryos were created by injecting both the 5' and 3' gRNA into one cell staged embryos. G0 individuals were outcrossed to wild type ABC-2E to create a G1 generation. This G1 generation was screened to identify individuals with potential mutations. G1 fish that were positive for the mutation were selected and bred again, either as an incross (for all three *ssh1a* alleles and the $coro1ca^{Allele\ 1\ -\ Ins}$) or as an outcross to wild type ABC-2E ($coro1ca^{Allele\ 2\ -\ Del}$). F1 larvae from an incross of the G1 siblings were phenotypically screened by immunohistochemistry to determine possible changes in the number and location of enteric neurons. The same embryos were then genotyped by PCR to determine possible correlations between genotype and phenotype.

Fin Clipping:

Fish were placed in a petri dish and anesthetized with Tricaine Methanesulfonate (MS222) (Fluka Analytical # A5040-100G). While the fish were anesthetized, tips of the tail fin were clipped, placed in a sterile PCR tube, and then kept on ice for genomic DNA extraction. Individual fish were placed into small tanks and kept separate until PCR identification was complete.

Genomic DNA Extraction:

20 µl of 50mM NaOH was added to each tube to the fin clip and heated to 95^0C for 15 minutes. The tubes were then vortexed, spun down, and returned to 95^0C for 15 more minutes. The tubes were placed on ice and 2µl of Tris pH 8.0 was added to each tube to neutralize the NaOH. All tubes were stored at -20^0C until required for further analysis.

Genotyping:

Genotyping was done by PCR amplification. GoTaq Green Master Mix was used for all PCR reactions (Promega #M712). 2µl of template DNA from the fin clip was used per reaction, 1µl each for the forward primer and the reverse primer, 8.5µl of water, and 12.5µl of the GoTaq Master mix. For PCR, an initial denaturing step at 95^0C for two minutes was followed by thirty five cycles of the following: a denaturing step at 95^0C for 30 seconds, an annealing step at 55^0C for 30 seconds, and an elongation step at 72^0C for 20 seconds. A five minute 72^0C incubation for final elongation was added as a final step.

To determine whether or not the gene was successfully cut at the 5' and/or the 3' end, two sets of primers were used for each gene. The first set of primers targeted an upstream 5' region of the gene, while the second set targeted a 3' region of the gene (See Figure 3 for an overview). To assess whether there was a full allele deletion, a 5' forward primer and a 3' reverse primer were used to PCR the entire locus. Primer sequence details will be described elsewhere. *ssh1a* 5' primers were designed to amplify a 120bp region from the 5' region wild type *ssh1a* alleles and the *ssh1a* 3' primers were designed to amplify a 123 bp 3' region. The *coro1ca* 5' primers were designed to amplify a 150 bp from the 5' region and the *coro1ca* 3' primers amplified a 130 bp region from the 3' region. If a mutated allele was present and NHEJ had inserted or deleted any number of base pairs, the mutant allele could be distinguished from the wild type allele by a change in the size of the PCR product. PCR reactions were run on a 3% Agarose gel and gel images documented using a Gel Doc™ XR+ system (Bio Rad).

Immunohistochemistry:

Five day old larvae were fixed in 1% paraformaldehyde for two hours. After two hours, the fish were washed for 5x 10 minute baths in PBS/0.1% Triton-X-100 (PBTx) followed by one

10 minute wash in PBS/1%DMSO/0.1%TritonX-100 (PBDTx). They were then placed in blocking solution (PBDTx/1%BSA/2% goat serum) for 45 minutes and then rocked overnight at 4^0C in primary antibody HuC/D (elav-like 3/4) (Invitrogen #A-21271), 1:1000 dilution in blocking solution.

Following the overnight incubation, larvae were washed eight times for fifteen minutes, each in PBDTx. The last wash solution was removed and replaced with a 1:1000 dilution of goat anti-mouse Alexa^{488} secondary antibody (Invitrogen A11029) in blocking solution and rocked overnight at 4^0C.

On the third day, the samples were washed in PBSTx eight times for 15 minutes. At the end of these washes, all samples were examined under a compound microscope for phenotypic alterations. After phenotypic observation, larvae were genotyped by PCR.

Results:

The enteric nervous system arises from the migration of neural crest into the intestine, where the neural crest then differentiates into enteric neurons. As *coro1ca* and *ssh1a* are known to have a role in actin dynamics, in particular the destabilization of actin filaments, it is possible that both genes play a role in the development of the ENS by affecting neural crest migration.

To determine whether CRISPRs to either *ssh1a* or *coro1ca* generated insertion or deletion mutations, fish were fin clipped and PCR used to determine whether there were mutations in *ssh1a* and *coro1ca*. Three generation 0 (G0) fish were positive for mutations in *ssh1a* and two G0 fish were positive for a mutation in *coro1ca*. Injection of gRNA has the potential to generate genetic mosaic zebrafish, in which certain cells may have the mutation while other cells do not. As the initial G0 animals are likely mosaics for mutations, G1 offspring

were generated by outcrossing the G0 fish to wild type fish and screened to determine if the mutation was transmitted through the germline. The identification and presence of mutations in the G1 generation indicated germline transmission.

Analysis of *ssh1a* mutant alleles:

G0 Founder fish:

Three founder fish were identified as having mutations in *ssh1a* in at least some percentage of their cells. The PCR primers were designed to examine whether or not there was a cut made at the 5' end, the 3' end, and if the full allele had been deleted by using a combination of the forward 5' and the reverse 3' primers (Figure 3). If the entire allele has been deleted by the CRISPR Cas9, the PCR procedure will be able to amplify a small fragment using the 5' forward primer and the 3' reverse primer, as the distance between the two primers will be short enough to be amplified during our extension time (data not shown). If the entire allele had been successfully deleted, a small PCR product would be generated, the exact size being dependent on the cuts produced by the CRISPR. In the absence of a deletion, there would be no PCR product as size of the space between the two primers would be too large for successful elongation during the PCR cycle.

From our results, one founder G0 initially tested positive for a full allele deletion of *ssh1a* and for 5' and 3' mutations. Due to the mosaicism of the fish, it is possible for cells to be affected by the CRISPR and the subsequent repair in different ways. This allowed for multiple mutations to be present in the G0 found fish. Two other founder fish tested positive for an insertion or deletion (data not shown). These fish were outcrossed to ABC-2E, and the G1 progeny raised to adulthood.

Recovery of *ssh1a* mutations in G1 generation:

The progeny of the G0 outcross, the G1 generation, were fin clipped and screened for carriers of putative mutations using the same PCR assay used to screen the G0 generation (Figure 3). Primers were designed to assess whether the CRISPR-Cas9 construct was successful in generating mutant alleles *ssh1a* at either or both of the 5' and 3' target sites. In the G1 generation, we identified three distinct mutations for *ssh1a*: two unique 5' insertions, $ssh1a^{Allele\ 1\ -\ Ins}$ and $ssh1a^{Allele\ 2\ -\ Ins}$ (Allele 2 has two 5' insertions – PCR bands running above the 120bp wild type product), and one 5' deletion, $ssh1a^{Allele\ 3\ -\ Del}$ (Figure 4, lanes 2 and 6, 3, and 5 - PCR bands running below the 120bp wild type product). Unfortunately, the full allele deletion failed to be transmitted to the G1 generation, but the same G0 founder transmitted $ssh1a^{Allele\ 1\ -\ Ins}$. The failure to detect the full deletion in the G1 progeny is likely due to mosaicism of the founder fish; the full allele deletion was a somatic mutation, while the 5' mutations were present in the germline.

The other two founder fish successfully transmitted insertions or deletions of about 5-15bp: the $ssh1a^{Allele\ 1\ -\ Ins}$, $ssh1a^{Allele\ 2\ -\ Ins}$, and $ssh1a^{Allele\ 3\ -\ Del}$ alleles (Figure 4, lanes 2, 3, 5, and 6). An exact size of the insertion or deletion cannot be provided at this time as further testing is required. Approximate sizes have been used to classify mutations until further molecular characterization of the mutations can be conducted.

The first incross of the G1 generation, $ssh1a^{Allele\ 1\ -\ Ins}$ presented a mutant phenotype (Figure 5). The phenotype looked very similar to another mutant known as *ichabod* (Kelly et. al. 2000). The *ichabod* mutants fail to gastrulate properly and the causative mutation has not been identified (Kelly et. al. 2000). To determine if the phenotype we observed was linked to mutations in *ssh1a*, we genotyped 5 mutant phenotype and 6 wild type embryos (Figure 6). The

PCR results demonstrated the phenotype and the genotype were not related. Both phenotypically mutant and wild type embryos demonstrated heterozygosity and/or homozygosity at the *ssh1a* locus, suggesting the phenotype and genotype were not linked. (See figure 6; lanes 3, 4, and 5 showing the heterozygosity at the *ssh1a* locus of the mutant progeny while lanes 8, 9, and 10 are their wild type siblings). As the insertion allele did not segregate with the *ichabod*-like phenotype and resulted from a G1 incross of the same G0 founder, the most likely explanation is that the *ichabod* phenotype represents a mutation in the background of our G0 founders.

Having identified G1 carriers of different *ssh1a* alleles, $ssh1a^{Allele\ 1-Ins}$, $ssh1a^{Allele\ 2-Ins}$, and $ssh1a^{Allele\ 3-Del}$, we were interested in further characterizing these mutations. To determine if these mutations resulted in phenotypic changes in the ENS, we incrossed the G1 generation to obtain embryos homozygous for *ssh1a* mutation(s). To examine if there were changes in enteric neuron number or enteric neuron distribution along the intestinal tract, we assayed the F1 generation (G1 offspring) using the panneuronal marker HuC/D (*elavl3*) at 5 dpf. After screening the larva for phenotypic changes in enteric neuron number, PCR was performed on the same larvae to assess the genotypes. HuC/D staining revealed the overall enteric neuron number of the larvae appeared normal; however, PCR genotyping of the larvae revealed that of the 3 alleles, only the $ssh1a^{Allele\ 3-Del}$ incross had larvae homozygous for the 5' deletion. PCR results from the two 5' *ssh1a* insertion alleles revealed only homozygous wild type or heterozygous genotypes (Figure 7, compare lanes 2-6 with lanes 8-19). The lack of homozygous larvae suggested two possibilities: a. either more F1 need to be screened, as small clutch sizes meant only 5 larvae from each incross were screened, or b. the homozygous mutants are dying before 5dpf, the time of the HuC/D assay.

Analysis of *coro1ca* mutant alleles:

G0 alleles and recovery of *coro1ca* mutations:

As with *ssh1a*, G0 founders from CRISPR targeting of *coro1ca* were assessed for the presence of mutations using primers that targeted the *coro1ca* alleles (Figure 3). Two founder G0 fish were identified; one positive for the full allele deletion, along with several 5' and 3' mutations, and the other only positive for the 5' and 3' insertions. The G0 fish were bred, and the G1 generation assessed by PCR to determine germ line transmission of the deletions or insertions. Only one founder fish transmitted mutations in *coro1ca*; the full allele deletion founder failed to transmit the full gene deletion to the G1 generation, but transmitted two 5' mutations. The two mutations identified from this founder were a small 5-15 bp 5' insertion, $coro1ca^{Allele\ 1\ -\ Ins}$, and a small 5-15bp 5' deletion, $coro1ca^{Allele\ 2\ -\ Del}$ (Figure 8, lanes 3, 5, 6). The second founder failed to transmit any 5' and 3' deletions. Unfortunately, as there were only male G1 carriers for $coro1ca^{Allele\ 1\ -\ Ins}$, further analysis was limited, and the G1 fish were outcrossed to ABC-2E wild type to raise a new generation for further analysis. The $Coro1ca^{Allele\ 2\ -\ Del}$ deletion G1s were incrossed to obtain F1 larvae for initial characterization studies.

To investigate the phenotype of the CRISPR generated $coro1ca^{Allele\ 2\ -\ Del}$ mutants, HuC/D immunohistochemistry was performed as described above to assess the number of enteric neurons in the *coro1ca* F1 larvae. The 5 dpf $coro1ca^{Allele\ 2\ -\ Del}$ larvae showed no discernable difference in the HuC/D expression. A PCR analysis run to assess the genotype demonstrated the presence of a $coro1ca^{Allele\ 2\ -\ Del}$ homozygous offspring suggesting this deletion allele does not affect the overall number or distribution of enteric neurons in the larval intestine (data not shown).

Conclusion:

CRISPR-Cas9 was used to generate mutations in the *ssh1a* and *coro1ca* genes. We have identified three *ssh1a* lines: two 5' insertions alleles (*ssh1a*$^{Allele\ 1-Ins}$ and *ssh1a*$^{Allele\ 2-Ins}$) and one 5' deletion allele in the *shh1a* gene (*ssh1a*$^{Allele\ 3\ -\ Del}$). For *coro1ca*, we have identified one 5' insertion allele (*coro1ca*$^{Allele\ 1\ -\ Ins}$) and one 5' deletion allele (*coro1ca*$^{Allele\ 2-Del}$). Founder G0 fish in both *ssh1a* and *coro1ca* that initially tested positive for a full allele deletion did not transmit the full allele deletion through the germline, but did transmit as a number of 5' insertion and 5' deletion alleles (5'indels). The original positive result for the full allele deletion and the subsequent failure to transmit is likely due to the mosaicism of the G0 fish. In both cases, the G0 fish that were positive for the full allele deletion were also positive for the 5' and 3' mutations. None of the G1 progeny were positive for the full allele deletion, but were positive for mutations at the 5' end, indicating only the 5' mutations were successfully transmitted to the germline.

For *ssh1a*, we identified three mutations in the G1 generation: *ssh1a*$^{Allele\ 1-Ins}$, *ssh1a*$^{Allele\ 2-Ins}$, and *ssh1a*$^{Allele\ 3\ -\ Del}$. We incrossed carriers of all three mutations and examined the F1 generation for phenotypic changes in the number of enteric neurons at 5dpf. From our preliminary HuC/D immunohistochemistry results, the *ssh1a*$^{Allele\ 3\ -\ Del}$ homozygous larvae show no changes in the number of enteric neurons in their intestines at 5 dpf and are indistinguishable from their wild type siblings. This could be for a multitude of reasons. One of the possible explanations is the nature of the deletion mutation: we do not yet know the nature of the insertions or deletions. The deletion may be a multiple of three base pairs resulting only in the loss of a small number of amino acids. The loss of a few base pairs may not be sufficient to cause a frame shift and subsequently disrupt the protein expression or function. If this is the case, small amino acid deletions or insertions may have little to no effect. However, in order to assess

this, we will need to sequence the *ssh1a* alleles and determine the exact size and nature of the deletion. This will allow us to determine the nature of the deletion and the effect it may have on protein function.

Although we do not see overt changes in the number or distribution of enteric neurons in the *ssh1a*$^{Allele\ 3\ -\ Del}$ larvae, we have not assessed the possible effects the mutation may have on the function of the neurons. Endo et. al. found that *ssh1* affects the motility of axonal growth cones and extensions. Thus, rather than eliminating the entire neuron, we may have an issue with the neuronal connections that may not be apparent by looking at the overall neuron presence (Endo et. al. 2003). Therefore, future directions for this allele would be examining the neurophysiology or neuron function using intestinal transit assays to determine if the gut is affected by the mutation. To further assess the *ssh1a* mutations and phenotypes, we can test functionality of the gut, by utilizing a feeding or transit assays. These assays will allow us to examine the passage of food through the gut. The neurons may appear normal by immunohistochemistry, but there may be issues with axonal connections that lead to immotility. Together, the immunohistochemistry and the feeding assay would allow us to assess both for the presence and functionality of the neurons

A third possibility is redundancy. Due to a duplication of the genome, zebrafish often have duplicates of genes that may only be present in mammalian genomes as a single gene (Woods et. al. 2005; Postlethwait et. al. 1998). The result of this duplication is that the duplicate gene could compensate for the loss of the other gene's function. Zebrafish have both a *ssh1a* and a *ssh1b,* along with a *coro1ca* and a *coro1cb* (zfin.org), whereas in mouse there is only *ssh1* and *coro1c* (MGI). All of these genes are unique, and it is unlikely that the gRNA targeted *ssh1b* or *coro1cb*, however, it is possible that *ssh1b* and *coro1cb* are compensating for the loss of *ssh1a*

and *coro1ca*. Further research would be required to determine what sort of role, if any, these duplications play.

As we were unable to identify larvae homozygous for the *ssh1a*$^{Allele\ 1-Ins}$ and *ssh1a*$^{Allele\ 2-Ins}$, it is possible we have not screened enough larvae (6 per mutation in figure 7; data not shown) or that these mutations cause early lethality. A critical requirement for this gene in the homozygous mutants prior to day 5 may prevent these mutants from surviving. In order to determine if this is the case for *ssh1a*$^{Allele\ 1-Ins}$ and *ssh1a*$^{Allele\ 2-Ins}$, we could examine *ssh1a* larval clutches at earlier time points, such as day 1, 2, or 3. This would allow us to assess if and how long the embryos homozygous for the *ssh1a* mutations are surviving. If the embryos are not making it to day 3, it is more likely that the homozygous mutation is affecting an earlier developmental process. Should this be the case, we may be able to assess the HuC/D staining at earlier time points. HuC/D staining in the zebrafish intestine can be observed as early as 3dpf. Alternatively, if the embryos are dying earlier, it may be possible to use *crestin* to assess the migration of the neuronal progenitors to determine if there is an issue with neural crest migration to the gut. *Crestin* is a gene that is expressed in neural crest and is commonly used to assess the presence and migration patterns of neural crest cells.

Two *coro1ca* founders were identified; one that was positive for a full allele deletion and one that was positive for 5' and 3' mutations (data not shown). The full allele deletion was not transmitted to the G1 generation. Currently, we are working on establishing an F1 generation for both *coro1ca*$^{Allele\ 1-Ins}$ and *coro1ca*$^{Allele\ 2-Del}$ of the *coro1ca*. As with the *ssh1a*$^{Allele\ 3-Del}$ allele, our results suggest the number of enteric neurons in the *coro1ca*$^{Allele\ 2-Del}$ homozygous larvae are normal and phenotypically indistinguishable from their wild type siblings. We have neither

determined the nature of the deletion mutation nor assessed the possible effects on neuron function and functional gut motility.

With this work, we have begun the establishment of two independent mutant lines for *coro1ca* and three independent lines for *ssh1a*. In the coming months, new generations will be raised and progeny examined to determine any phenotypic or functional changes in the ENS that may have resulted from these mutations.

Finally, to better understand the molecular nature of the induced mutations, it will be important to clone each of the five mutations from *coro1ca* and *ssh1a* to determine exactly how large or how small each mutation is. This would also let us make predictions about how the mutations may or may not affect the development of the enteric nervous system.

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Figures:

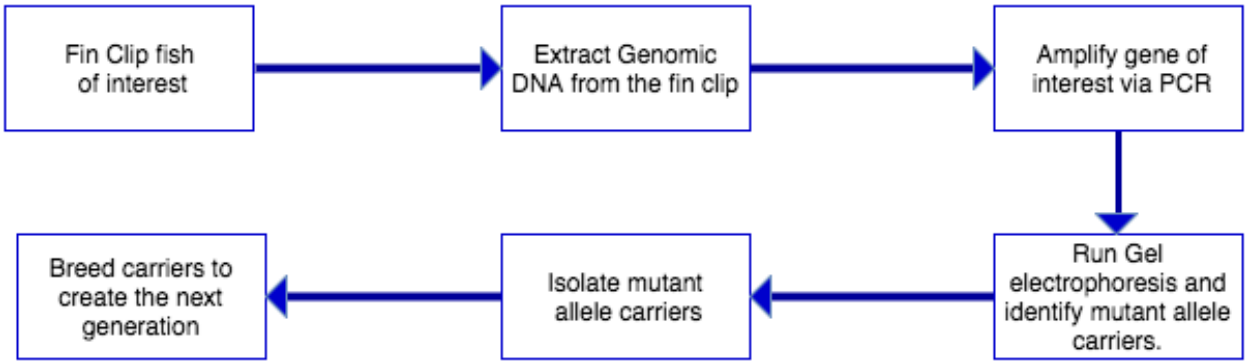

Figure 2: Overview of the protocol for the identification of CRSPR generated *coro1ca* and *ssh1a* alleles.

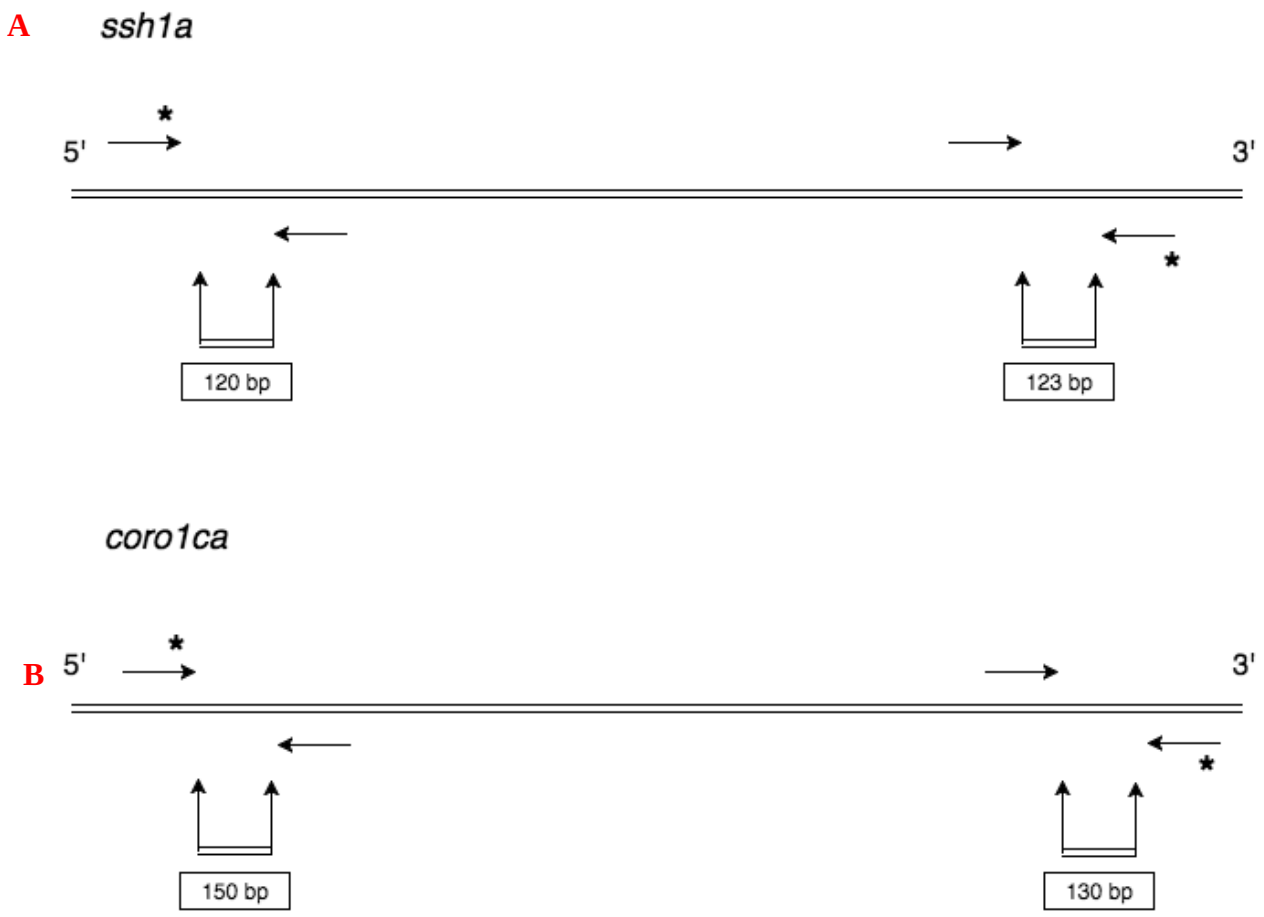

Figure 3: Schematic of primer position and expected wild type PCR products for genotyping *ssh1a* (A) and *coro1ca* (B). The primers targeted the 5' and 3' end of the gene. The size of the expected wild type PCR products for the CRISPR-targeted 5' and 3' *ssh1a* (A) and *coro1ca* (B) genes are indicated. To assess for a full allele deletion, the forward 5' primer and the reverse 3' primer were used to amplify across the gene (asterisked primers). If present, a full allele deletion would result in a small PCR product of 128bp or 180bp for *ssh1a* and *coro1ca* respectively.

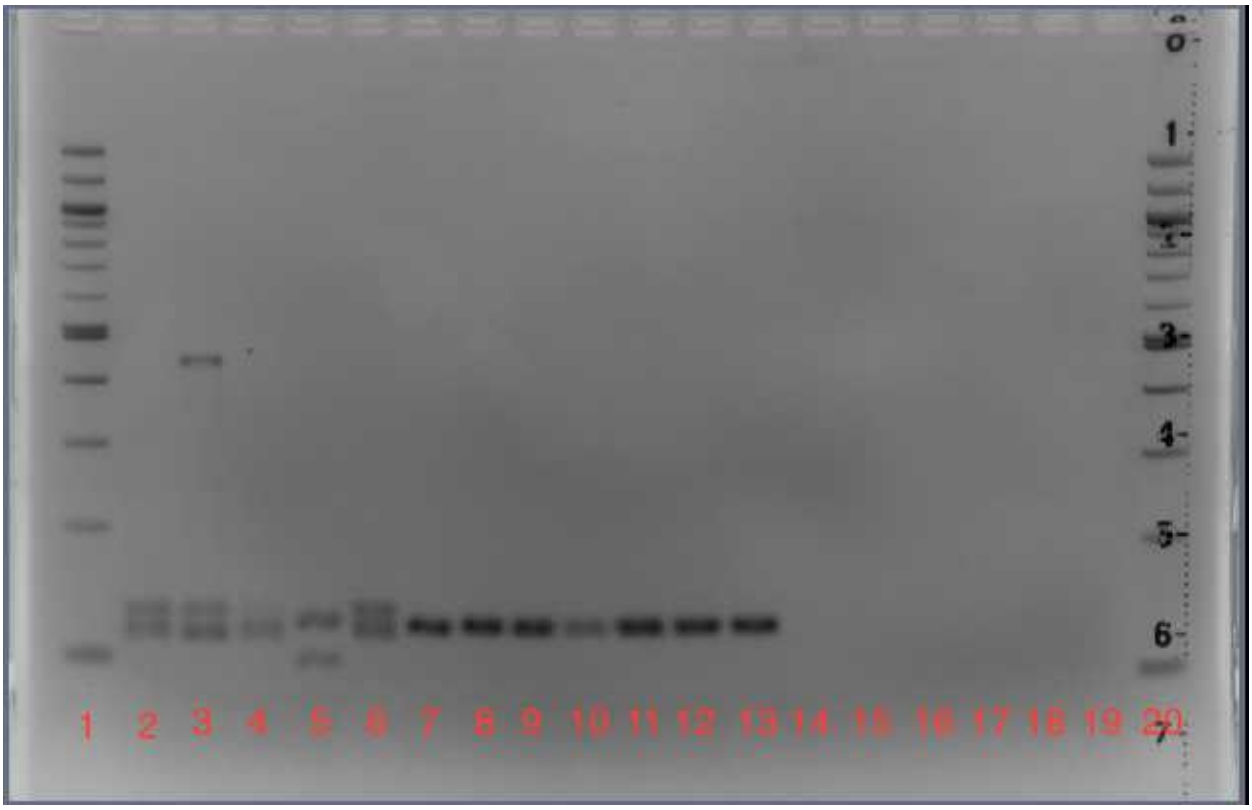

Figure 4: PCR identification of mutant and wild type *ssh1a* alleles. DNA from individual G1fish from the same G0 founder fish (positive for the full allele deletion) were tested for 5' end mutations (lanes 2-6), 3'end mutations (lanes 8-12) and full allele deletion mutations (lanes 14-19). Lanes 1 and 20 are 100bp DNA ladders. Lanes 2-6 and 14-19 G1 progeny. Lane 7 is a 5'wild type control (120bp). Lanes 2, 3, and 6 have small 5' insertion ($ssh1a^{Allele\ 2-Ins}$), while lane 5 has a small 5'deletion ($ssh1a^{Allele\ 3-Del}$). Lane 3 has a second larger insertion that has not been characterized at this time. Lane 13 is a 3' wild type control, (123bp). Only the wild type allele was observed at the targeted 3' end of the gene due (lanes 8-12). In lanes 14-19, the 5' forward primer and the 3' reverse primer (figure 3) were used to test a full allele deletion. The absence of any band in lanes 14-19, suggests the full deletion was not transmitted to the G1 progeny.

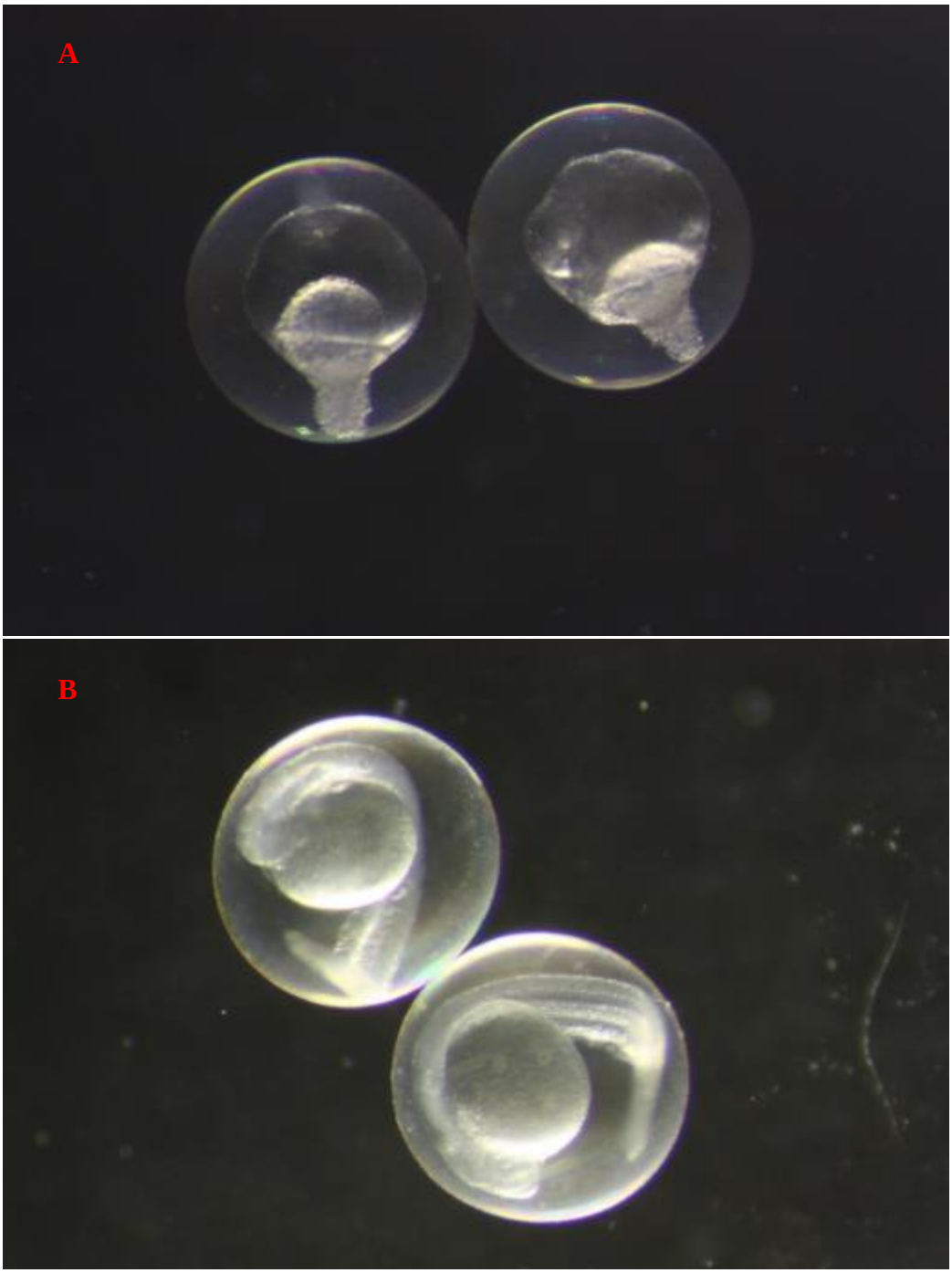

Figure 5: Phenotype observed within *ssh1a*$^{Allele\ 1-Ins}$. The mutants (A) failed to gastrulate correctly and fail to develop most dorsal structures, including the head in comparison to their phenotypically wild type siblings (B).

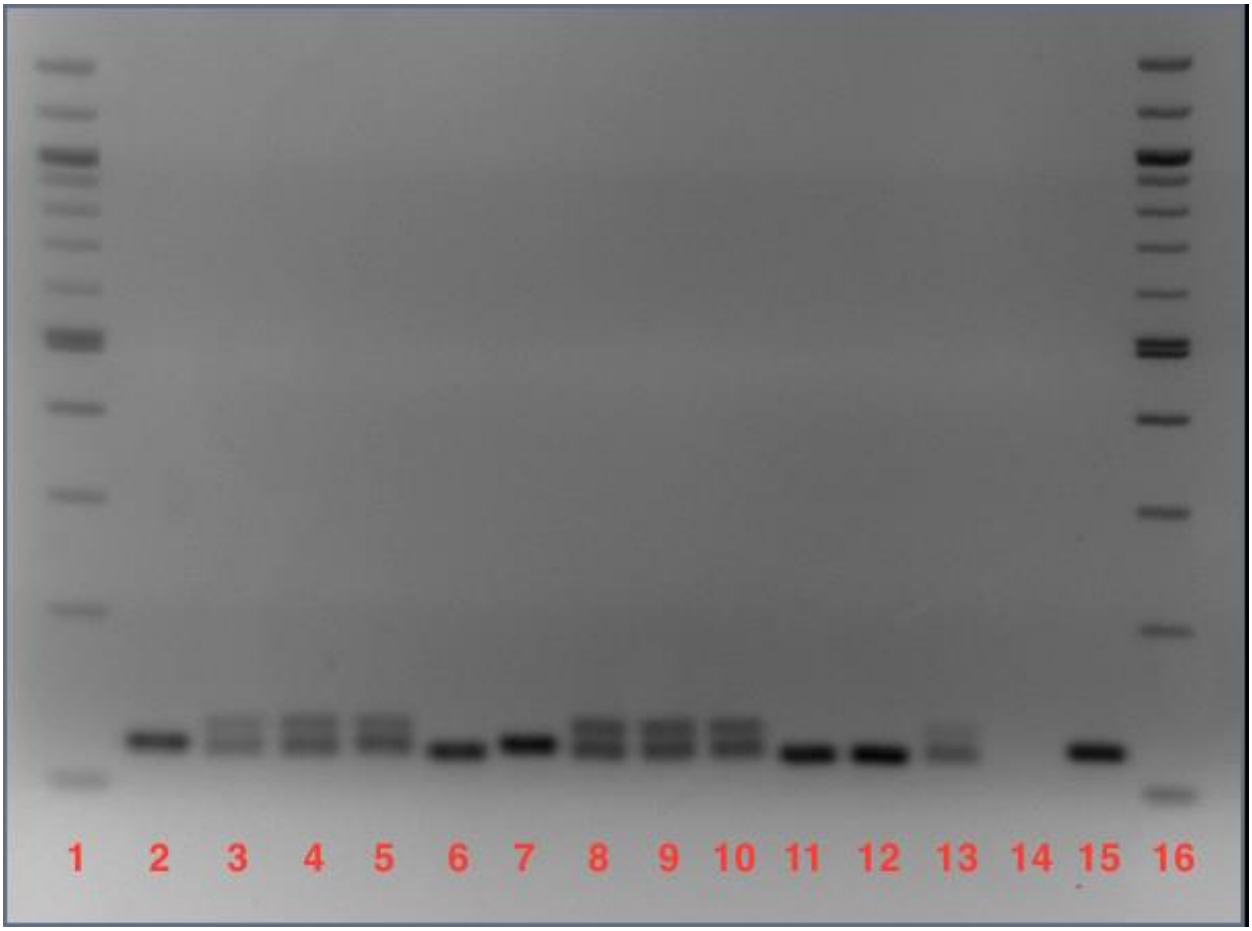

Figure 6: PCR analysis of the phenotypic mutation seen in the *ssh1a*$^{Allele\ 1-Ins}$ (See Figure 5). Lanes 1 and 16 are 100bp ladders. Lanes 2- 6 are the F1 offspring that displayed the mutant phenotype while lanes 7-12 are the F1 siblings who displayed a normal phenotype. Lane 13 is a control of the G1 parents, while lane 15 is from a wild type ABC-2E fish. All wild type alleles are 120 bp in length (5' primer set). Both the phenotypically wild type and mutants tested positive for the wild type the insertion alleles leading to the conclusion that the phenotypic mutation is not linked to the *ssh1a* gene.

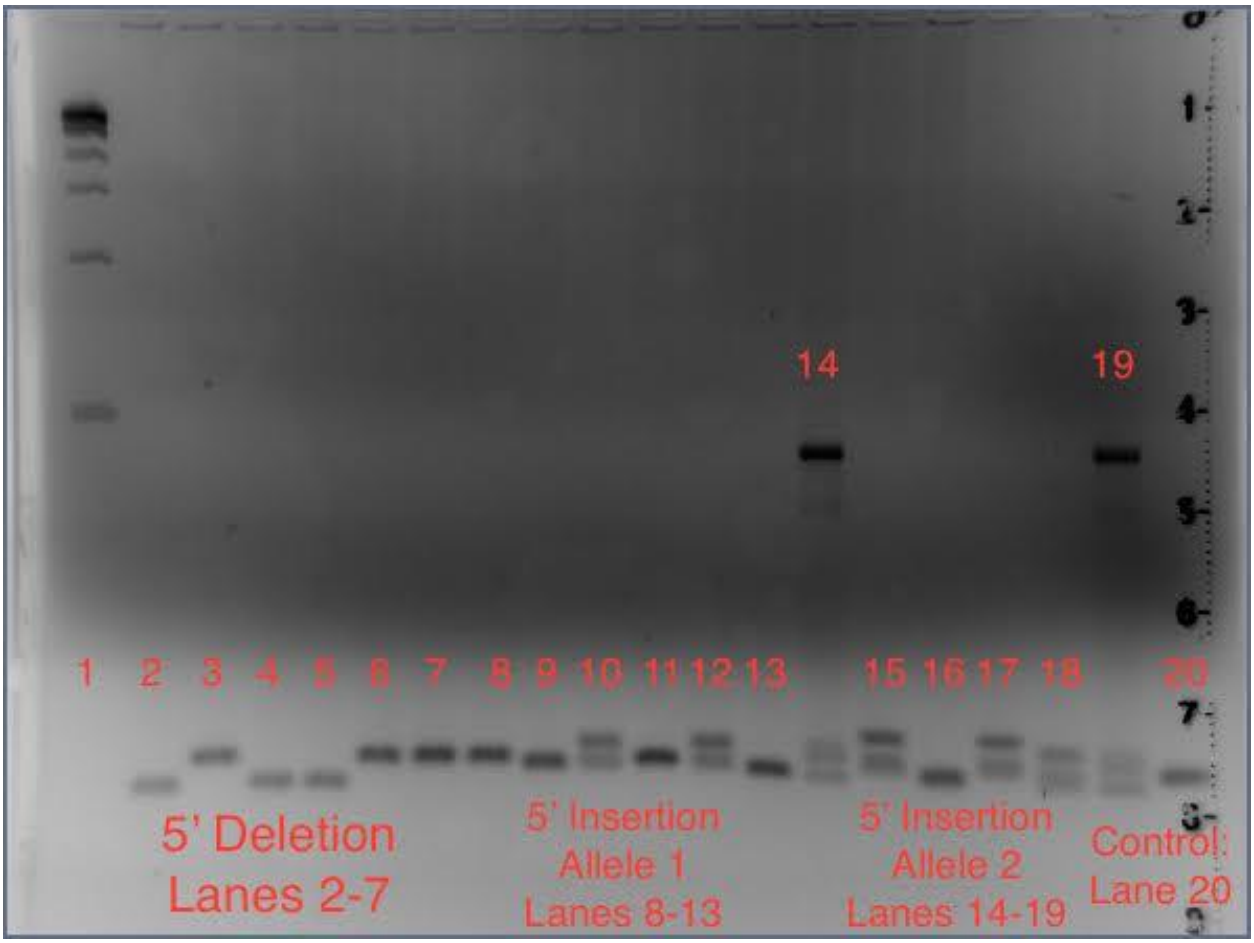

Figure 7: PCR identification of the *ssh1a* F1 generation to determine phenotypic and genotypic linkage. Lane 1 is a 1000bp ladder. Lanes 2-19 represent individual F1 offspring. Lane 20 is a control from ABC-2E fish. The *ssh1a* primers amplified 120bp at the 5' end of *ssh1a*. Multiple bands in a single lane indicate heterozygosity. Lanes 2-7 represent offspring of the 5' deletion ($ssh1a^{Allele\ 3\ -\ Del}$). Both wild type homozygous and mutant homozygous genotypes were detected. For both 5' insertion alleles, $ssh1a^{Allele\ 1\ -\ Ins}$ (lanes 8-13) and $ssh1a^{Allele\ 2\ -\ Ins}$ (lanes 14-19) no homozygous mutant larvae were recovered.

Figure 8: PCR analysis of *coro1ca* G1 fish from G0 full gene allele deletion founder. Lane 1 and 20 are 100bp ladders. Lanes 2 through 7 PCR to examine the 5' end of the *coro1ca* gene (expected product size 150bp). Lane 7 is a wild type ABC-2E control. Lanes 2-6 are individual G1 fish. A band above or below the 150 bp band is considered an insertion or a deletion respectively. Lane 3 appears to have a small insertion at the 5' end ($coro1ca^{Allele\ 1-Ins}$) (additional band running slightly faster than wile type), while lanes 5 and 6 have a small deletion ($coro1ca^{Allele\ 2\ -\ Del}$). Lane 8-12 are individual fin clips of the G1 fish, using the 3' primer sets for *coro1ca* (expected product size 130bp). Lane 13 is a wild type ABC-2E control, where the 3' primers amplified 130bp. The presence of only the wild type band, indicate no 3' indels were transmitted. Similarly, lanes 14-19, PCRs from G1 fish to test for the presence of a full gene allele deletion using the 5' forward *coro1ca* primer and the 3' reverse *coro1ca* primer to amplify across the allele (Figure 3). The absence of any bands suggests a failure to transmit a full allele deletion. Lane 19 is a wild type control.