INTESTINAL PHENOTYPES OF ZEBRAFISH ENTERIC NERVOUS SYSTEM DOUBLE MUTANTS

by

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The enteric nervous system (ENS) innervates the intestine and regulates the dynamic intestinal environment. In humans, ENS reduction causes Hirschsprung disease (HSCR), a genetically complex disorder that results in intestinal dysmotility and, in many patients, intestinal inflammation. The zebrafish is an excellent model in which to study the relationship between inflammation and genes linked to HSCR. Zebrafish homozygous for a mutation in one HSCR gene, *sox10*, have fewer enteric neurons and develop microbiota-dependent intestinal inflammation. Zebrafish homozygous for a mutation in another HSCR gene, *ret*, also have fewer ENS neurons but do not exhibit increased intestinal inflammation. To investigate the opposing intestinal inflammation phenotypes of *sox10* and *ret* mutants, I analyzed intestinal phenotypes of *sox10;ret* double mutants. Because *sox10* acts early in neural crest cells that form the ENS and *ret* acts later, within ENS cells themselves, I hypothesized that intestinal inflammatory phenotypes of *sox10;ret* double mutants would resemble those of *sox10* mutants. To test this hypothesis, I quantified intestinal inflammation in *sox10;ret* double mutants by counting intestinal neutrophils and enumerating intestinal bacteria and recently-proliferated intestinal epithelial cells.

Surprisingly, I observed a wild-type (WT) neutrophil abundance phenotype in *sox10;ret* mutants, suggesting that a cell type outside of the ENS is involved in determining the intestinal inflammatory phenotypes of ENS mutants. This result led me to investigate intestinal enterochromaffin cells, which express *ret* but not *sox10*. I hypothesized that *sox10;ret* double mutants would exhibit the same decreased enterochromaffin cell phenotype as *ret* mutants. However, *sox10;ret* mutants had more enterochromaffin cells than *ret* mutants and were similar to WT. This result prompts further exploration of potential interactions between the *sox10* and *ret* genes to gain insights into how these genes interact to regulate intestinal development and the role of the ENS in the maintenance of intestinal health.

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Introduction

Gastrointestinal diseases affect between 60 and 70 million people in the United States each year (Digestive Diseases Statistics for the United States, 2014). They cause 13% of all hospitalizations, approximately 50 million physician office visits, and cost adults $142 billion per year (Peery et al., 2015). Disorders involving intestinal inflammation, such as inflammatory bowel disease, are especially prominent, and their incidence continues to rise in the United States. While there has been some success in the production of therapeutic agents for gastrointestinal diseases, lack of certainty about the etiology of many gastrointestinal diseases has limited management of these health issues to symptom relief rather than effective treatment (Saha, 2014). The key to creating future therapies for gastrointestinal diseases will be an understanding of the underlying physiological causes for disease states, which will rely upon increased mechanistic understanding of the enteric nervous system (ENS) which regulates many aspects of vertebrate intestine function. Though research has made it clear that the contributions of the ENS are great in number, precise mechanisms by which the ENS interacts with many of its effector cell populations remain to be defined. Understanding how the ENS works to maintain intestinal health will therefore require further elucidation of genetic determinants that crucially underlie ENS development and function, as well as increased discernment of the complex relationships between the ENS and factors such as the host immune system and the intestinal microbiota.

The ENS at a glance

The ENS is the largest and most complex subdivision of the peripheral nervous system and is a mesh-like network of neurons and glial cells that provides intrinsic innervation to the intestinal tract from the esophagus to the most distal portion of the intestine (Furness, 2004; Figure 1). Comprised of about as many neurons as the spinal cord, the mammalian ENS contains two large divisions of interlacing nerve cell networks that control many aspects of intestinal homeostasis: the myenteric plexus, located between circular and longitudinal intestinal muscle layers, and the submucosal plexus, located in closer proximity to the intestinal epithelium (Lomax et al., 2005; Heuckeroth, 2016). Though it regularly communicates with the central nervous system (CNS), the ENS is capable of regulating the intestine in the absence of CNS input because it contains complete neural circuits made up of diverse neuron types that that transmit physiological stimuli from the intestine, including intrinsic primary afferent neurons (IPANs), motor neurons, and interneurons (Furness, 2004). In addition to communicating with other branches of the nervous system, the ENS interacts with an array of cell types, including intestinal epithelial cells, immune cells, and endocrine cells, to induce physiological responses in the intestine (Rao and Gershon, 2018).

The ENS is situated in a unique environment because the intestine receives stimuli from the external world, in the form of food for example, as well as constant signals from within the intestinal lumen, where trillions of bacteria, viruses and fungi, called the microbiota, reside (Cheesman and Guillemin, 2006). Due to the diversity of its molecular interactions with external and internal milieu, the ENS relies upon its highly organized network to rapidly integrate information and direct necessary

resources like water, ions, mucus, and hormones, to their needed location in the intestine (Margolis and Gershon, 2016). In addition to responding to cellular demands on a variable timescale, the ENS must simultaneously maintain vital processes such as intestinal peristalsis, blood flow, mucosal transport, electrolyte secretion, and secretory, immune, and endocrine functions (Costa, 2000). Given that the ENS plays an essential role in maintaining normal intestinal function, ENS abnormalities often lead to major health consequences. As evidenced by the adverse effects that occur in cases of disrupted ENS development, the successful maintenance of intestinal function by the ENS depends on normal assembly of ENS circuits before birth (Rao and Gershon, 2018).

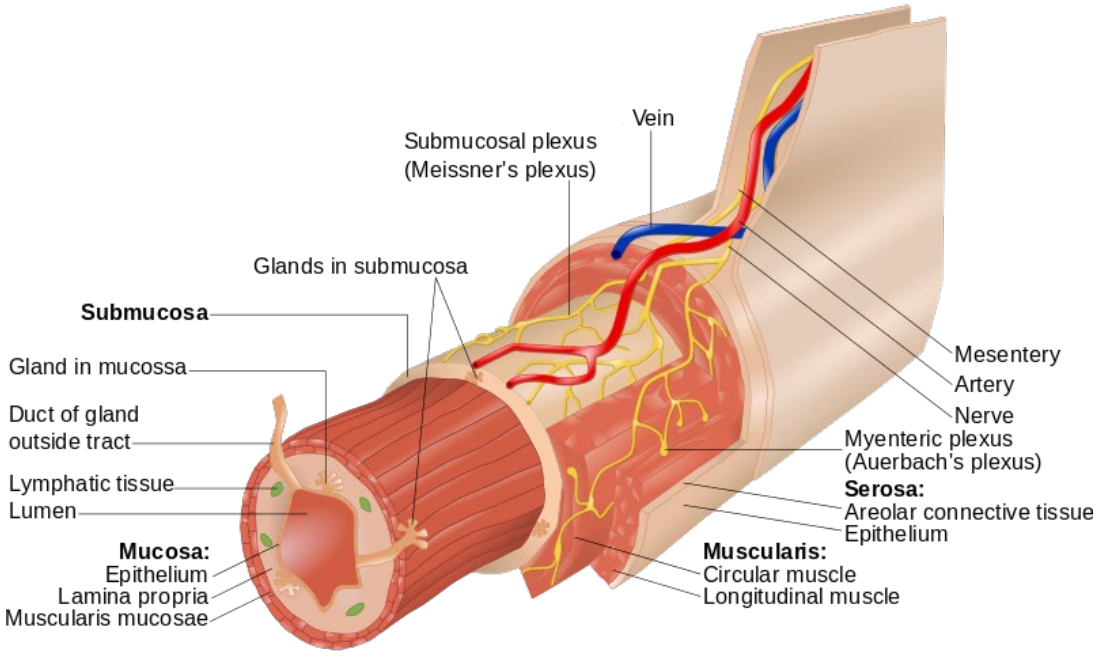

Figure 1. Anatomy of the human intestinal tract.

The wall of the gastrointestinal tract is comprised of four layers of tissue: the mucosa, submucosa, muscularis, and serosa. The enteric nervous system is embedded throughout layers of the gastrointestinal wall. Neurons of the enteric nervous system are collected into the myenteric plexus, located between the layers of the muscularis, and the submucosal plexus, within the intestinal submucosa. Image obtained from Wikimedia and reproduced under a Creative Commons Attribution-ShareAlike 4.0 International license.

Neural crest cells give rise to the ENS

Development of the ENS hinges on the activity of neural crest cells. Neural crest cells are a highly migratory embryonic cell type that gives rise to numerous differentiated cell types during vertebrate development (Rao and Gershon, 2018). Prospective neural crest cells arise from the boundary between the non-neural ectoderm and the neural plate, a thickened sheet of primordial cells situated along the dorsal midline of the embryo (Bronner-Fraser, 1993). Specification of the prospective crest cells to this border region is accomplished by combinations of signals initiated during gastrulation, an early phase in embryonic development wherein the basic anatomical axes and cellular layers of the embryo are established (Nagy and Goldstein, 2017). Neural crest precursor cells persist at the margins of the neural plate during the process of neurulation, in which the neural plate transforms to become the neural tube, the structure from which the brain and spinal cord are derived. After neural tube closure, populations of neural crest precursor cells appear as a strip of cells situated between the most dorsal aspect—the "crest"—of the neural tube and the presumptive epidermis and are identified by their expression of distinct transcription factors (Bronner and LeDouarin, 2012).

Before neural crest cells are morphologically identifiable as individual groups of migratory cells, they first sever their connections to neighboring neuroepithelial cells during delamination, a phase in neural crest development that allows physical separation of neural crest cells from the neural tube (Nagy and Goldstein, 2017). Detailed immunohistological studies in several species revealed that events leading to separation of the neural crest cell population from the neural tube are similar to those

that occur in a typical epithelial-to-mesenchymal transition (EMT), an important developmental process in which epithelial cells lose their polarity and their attachments to other cells to gain qualities of mesenchymal cells, multipotent cells that migrate to populate multiple organ primordia (Mayor and Theveneau, 2012). Once structurally distinguished from the neural tube, neural crest cells are poised to begin their migration throughout the embryo. They depart from the neural tube as a continuous wave, soon splitting into distinct streams of progenitor cells that go on to colonize a diverse range of destinations within the developing embryo, differentiate and give rise to numerous cell derivatives, including glia and neurons of the peripheral nervous system, melanocytes, endocrine cells, major components of craniofacial cartilage and bone, and cardiac structures (Kuo and Erickson, 2010; Theveneau and Mayor, 2012).

ENS development from the vagal neural crest in amniote models

The neural crest origin of the ENS was established over sixty years ago, when it was demonstrated that excision of the dorsal neural tube resulted in absence of the ENS in chick embryos (Yntema and Hammond, 1954). Further studies in which defined axial segments of the neural tube (cranial, vagal, trunk and sacral) were transplanted into host chick embryos determined that the ENS primarily arises from the vagal neural crest, with some contributions to the post-umbilical bowel by sacral crest cells (LeDouarin and Teillet, 1973). In the chick, the ENS forms from vagal neural crest cells at the level of somites 1-7 (Rothstein et al., 2018). These enteric nervous system progenitors enter the primordial foregut at a very early point in development, embryonic day 3 or 3.5, then migrate caudally, completing progressive colonization of the avian mechanical stomach (gizzard), intestine, ceca, and hindgut by embryonic day 7.5 (LeDouarin and

Teillet, 1973). Sacral crest cells corresponding to axial levels beyond somite 28 also contribute to the ENS in chicks, primarily colonizing the distal hindgut.

A similar pattern of ENS development was discovered in mouse embryos. At embryonic day 9, vagal neural crest cells from the level of somites 1-7 enter the proximal foregut and migrate in a rostrocaudal direction to colonize the foregut, midgut, cecum, and hindgut by embryonic day 14 (Heanue et al., 2016). As in chick embryos, sacral crest cells also contribute to colonization of the hindgut and caudal midgut in mice, though they migrate from the region caudal to somite 25 instead (Rothstein et al., 2018). In humans, the ENS is derived from vagal neural crest cells that arrive at the foregut around week four of gestation and migrate along the length of the developing intestine to colonize the foregut, stomach, midgut, cecum, and hindgut by week seven of gestation (Nagy and Goldstein, 2017). Based on mouse data, it is hypothesized that the sacral neural crest also contributes to the ENS in the human hindgut, though there is no experimental evidence available to confirm this as of yet (Heanue et al., 2016).

Once they have reached the mesenchyme of the foregut—a region consisting of the distal esophagus, the stomach and the proximal duodenum—from their axial level of origin, vagal neural crest cells are termed enteric neural crest-derived cells (ENCCs), which will go on to colonize the entire intestinal tube (Rao and Gershon, 2018). In humans, avian and rodent systems, ENCCs from the vagal crest are interspersed within the outer half of the fore- and mid-gut mesenchyme, which is comprised of a population of initially uniform cells that lie between the endodermal tube and the layer of squamous epithelial serosa that externally surrounds the intestine (Theveneau and Mayor, 2012). Vagal ENCCs migrate from the neural tube towards the foregut and

travel in a wave between the dermomyotome, a layer of presumptive dorsal dermis and skeletal musculature, and the sclerotome, which will form the rib cartilage and vertebrae (Bronner-Fraser, 1993). As the wave of migratory ENCCs advances caudally, circular smooth muscle layer differentiation begins and limits ENCCs to the layer of the intestinal wall between the serosa and smooth muscle, thus giving rise to the future myenteric plexus (Goldstein 2018). Once at the midgut, ENCCs advance inward from the level of the myenteric plexus towards the epithelium in a secondary migration pattern, thereby colonizing the submucosal mesenchyme and initiating development of the submucosal plexus of the ENS (Young, 2001).

ENS development relies on progenitor cell proliferation and differentiation

Of utmost importance during ENS development is the balance between ENS progenitor cell proliferation and differentiation. Proliferation of ENS precursors both generates and maintains a vital pool of precursor cells while sustaining cell mass at the migratory wavefront, thereby preserving the continued movement of precursors along the intestinal length (Rao and Gershon, 2018). Sufficient proliferation of enteric neural crest-derived cells is crucial for the migration of these cells throughout the developing intestine, but its cessation must be carefully coordinated with the onset of cellular differentiation, as diminished proliferation of ENS progenitors results in an inadequate number of cells to carry out colonization of the entire intestine (Goldstein, 2018). Differentiation of ENS progenitors importantly gives rise to neurons and glial cells, but problems arise if this differentiation is premature or delayed: premature neuronal differentiation leads to a dearth of proliferative cells, thus hindering adequate colonization, whereas delayed differentiation produces an excess of progenitor cells

unable to differentiate normally in an older mesenchymal environment (Goldstein, 2018).

Differentiation of ENS progenitors depends on critical molecular mediators

Although a variety of molecules present in the developing intestine influence ENS progenitor cell differentiation, there are three central regulatory molecules expressed by progenitors that are crucial for coordinating the processes of ENS progenitor cell proliferation and differentiation within the developing intestine: PHOX2B, SOX10 and RET. The paired-like homeobox 2b (PHOX2B) gene product is a neuronal-type specific transcription factor expressed by enteric neural crest-derived cells as soon as they enter the foregut mesenchyme. Essential for the formation of all autonomic ganglia, including those of the ENS, PHOX2B functions to promote ENCC proliferation and survival. Deletion of PHOX2B in mice results in total intestinal aganglionosis, as caused by failure of vagal ENCCs to proceed past the foregut to form the ENS (Pattyn, 1999). PHOX2B also importantly interacts with SOX10 during ENCC fate determination. The two transcription factors suppress one another to regulate the balance between neurons and glia in the developing intestine; in cells that will form neurons, PHOX2B is turned on and SOX10 is turned off, while PHOX2B is turned off and SOX10 turned on in glial cells. PHOX2B is also needed for the expression of RET (Goldstein, 2018).

The *SOX10* gene belongs to the *SOX* family of genes, which encode a variety of transcription factors that play important regulatory roles in tissue and organ formation during embryonic development. SOX10 is a high mobility group (HMG) box-containing transcription factor that is expressed by pre-migratory neural crest cells as

they distinguish themselves from the neural tube and remains expressed in migratory ENCCs. As evidenced by total intestinal aganglionosis in *SOX10* homozygous mutants, SOX10 activity is required to prevent apoptosis of neural crest cells prior to their arrival at the foregut and is thus required for ENCC survival (Paratore, 2001). In addition to serving as a survival signal, SOX10 is implicated in balancing progenitor cell proliferation and specification of differentiated cell types in the developing intestine. Studies of *SOX10* heterozygous mutant mice, which exhibited distal intestinal aganglionosis, decreased progenitor ENCC numbers, and premature enteric neurogenesis, demonstrated that SOX10 is required to maintain ENCC multipotency and turn on expression of other important transcription factors, including PHOX2B and RET (Pavan, 2003).

RET signaling is one of the most important pathways during ENS development. Across vertebrate species, the *Ret* gene encodes the RET receptor tyrosine kinase, a transmembrane cell surface receptor protein that is expressed by ENCCs. Expression of the *Ret* gene by ENCCs requires SOX10 and PHOX2B transcription factors, and the gene is activated by binding glial cell-derived neurotrophic factor (GDNF). First expressed in the developing stomach while ENCCs migrate through the distal foregut, GDNF binds to the receptor complex composed of RET and its co-receptor, the GDNF family receptor α-1 (GFRα1). This binding activates RET and leads to activation of downstream pathways that mediate GDNF-induced ENCC survival, proliferation, and migration throughout the intestine (Goldstein, 2018). One vital function of GDNF signaling through RET and GFRα1 is to promote migration of ENCCs into the

developing intestine and along its length towards the accumulation of GDNF at the cecum (Rao and Gershon, 2018).

The role of RET in ENS development was further investigated in experiments with RET-deficient mice. The intestinal tract in *Ret* knockout mouse is almost entirely aganglionic, with aganglionosis beginning at the level of the esophago-gastric junction at the most proximal aspect of the stomach, providing evidence that RET activity is critical for normal ENS development. While *Ret* homozygous mutant mice exhibit this widespread aganglionosis, mice that are heterozygous at the *Ret* locus exhibit a normal ENS, thus suggesting that the effects of RET on the developing ENS are dosage-dependent. Indeed, studies of mice in which RET expression was reduced to one-third its normal level revealed colorectal aganglionosis, further supporting the notion of dosage-dependent effects of RET on ENS development (Goldstein, 2018).

The microbiota, inflammation and the ENS

With more than 100 trillion microbes belonging to approximately 100 bacterial species residing within its lumen, the human intestine necessarily faces a tremendous microbial challenge. Our coexistence with this vast community of enteric microbes, collectively termed the microbiota, is widely regarded as an example of a mutualistic relationship in which the microbiota play key roles in the development and health of the intestine in addition to aiding with digestion and vitamin production (Turnbaugh et al., 2007; Guillemin and Cheesman, 2007). Despite having coevolved to maintain such a cooperative relationship with our indigenous intestinal microbes, under some circumstances, the microbiota can still pose a threat to the intestine at the level of the intestinal epithelium, which serves as the barrier between the microbiota and the

internal environment (Peterson and Artis, 2014). The intestinal epithelium consists of a layer of cells that provide physical and biochemical barriers to microbial invasion (Yu, 2018). These cells act to prevent microbial translocation by physically segregating or trapping microbes, and they also relay microbial signals to mucosal immune cells to initiate antimicrobial responses. A growing body of evidence suggests that dysbiotic intestinal microbial communities, along with disruptions of the intestinal epithelium, such as diminished barrier function and increased accumulation of mucosal microbes, are associated with a number of chronic inflammatory disorders, including inflammatory bowel disease (Yu, 2018).

Intestinal inflammation is initiated by immune cells that reside within intestinal tissue, such as tissue macrophages, neutrophils and dendritic cells. These immune cells are stimulated by molecular signals of danger and/or damage, such as bacterial cell wall components (Margolis and Gershon, 2016). These molecular signals are detected by specific receptors expressed by immune cells, which activates release of pro-inflammatory molecules, including secretion of interleukins, interferons, and cytokines like tumor necrosis factor alpha (Lomax et al, 2005). Release of such inflammatory mediators at the cellular level is responsible for onset of clinical signs of inflammation, which include redness, increased temperature, swelling, pain and loss of function (Hakansson and Molin, 2011). Inflammatory bowel disease (IBD) is an umbrella term referring to disorders that involve chronic intestinal inflammation, including ulcerative colitis and Crohn's disease. These diseases are characterized by flare-ups of inflammation which often warrant medication and even surgery (Margolis and Gershon, 2016). IBD etiology, though incompletely understood, is partially attributed to a

dysregulated immune response to microbiota dysbiosis, a condition in which the balance of the intestinal microbial community is disrupted (Turnbaugh et al., 2007; Hakansson and Molin, 2011). The role of the microbiota in intestinal inflammatory conditions is further evidenced by recently-discovered microbial signatures for ulcerative colitis and both ileal and colonic Crohn's disease. Halfvarson and colleagues demonstrated that the microbiota composition in IBD subjects fluctuates more than that of healthy individuals, thereby establishing an important link between the state of the intestinal microbiota and inflammatory disease symptoms (Halfvarson et al, 2017).

The relationship between the microbiota and the host intestine is a dynamic one, and a growing body of evidence suggests a primary role for the microbiota in shaping the development and homeostasis of the enteric nervous system (Obata and Pachnis, 2008; Cheesman et al., 2011). The contributions of the microbiota to enteric nervous system development are highlighted by studies of germ-free organisms. Germ-free mice have been shown to possess abnormalities in enteric nervous system structure and function that can be rescued upon colonization with a normal intestinal microbiota. Loss of the indigenous microbiota is associated with several disruptions to the enteric nervous system, including decreased enteric neuron numbers and reduced excitability of enteric intrinsic primary afferent neurons that interact with the brain, which both contribute to altered intestinal motility (Pachnis, 2016). The enteric microbiota has also been recently implicated in regulating the maturation of the enteric nervous system via mucosal serotonin signaling networks (Backhked, 2018). These findings and many more suggest that the dynamic interactions between the host intestine and the

microbiota during development of the enteric nervous system may hold important consequences for enteric nervous system function.

Enterochromaffin cells and intestinal serotonin signaling

The intestinal epithelium consists of specific cell types to enable its functions of nutrient absorption and barrier, including enteroendocrine cells, which release hormones and other signaling molecules in response to chemical and mechanical stimuli within the lumen. Enterochromaffin cells are one type of chemosensory enteroendocrine cell found in the intestinal epithelium. Though they comprise less than 1% of total intestinal epithelial cells, enterochromaffin cells produce greater than 90% of the vertebrate body's serotonin (5-HT), a neurotransmitter molecule with important activity in nearly every major gut-related function (Coates et al., 2017). Although our understanding of enterochromaffin cell function has been hindered by their rarity within the intestine, recent evidence suggests that enterochromaffin cells are polymodal sensors that detect a wide range of stimuli from exogenous and endogenous sources, including from ingested food and the microbiota (Bellono, 2018).

5-HT serves diverse signaling functions in both the brain and the peripheral nervous system. In the intestine, it is synthesized both by enterochromaffin cells and by serotonergic neurons of the myenteric plexus, though enterochromaffin cells produce and secrete 5-HT to a much greater extent than do serotonergic ENS neurons (Coates et al., 2017). Different forms of the rate-limiting enzyme in 5-HT biosynthesis are used by neuronal and enterochromaffin cells, with 5-HT release from enterochromaffin cells depending on the catalytic action of the tryptophan hydroxylase-1 enzyme, whereas its release from neuronal cells requires tryptophan hydroxylase-2 (Gershon and Tack,

2007). In addition to differing from neuronal 5-HT in its enzymatic requirements for release, 5-HT derived from enterochromaffin cells also differs from neuronal 5-HT in its enzymatic downstream effects. Enterochromaffin cell-derived 5-HT exerts largely proinflammatory effects within the intestinal mucosa, while activation of the 5-HT4 receptor expressed by enteric neurons is associated with neuroprotective and neurogenerative effects (Margolis and Gershon, 2014; Natarajan et al., 2016). Following its release from the basal enterochromaffin cell surface into the lamina propria, 5-HT interacts with a variety of neuronal cell types. The primary targets of 5-HT secreted by enterochromaffin cells are the mucosal projections of both extrinsic and intrinsic primary afferent neurons. 5-HT binding to mucosal extrinsic afferent neuronal projections transmits sensory information to the central nervous system, including sensations of nausea and intestinal discomfort, while its action on myenteric and submucosal intrinsic primary afferent neurons (IPANs) initiates giant migrating contractions and secretory reflexes and peristalsis, respectively (Gershon and Tack, 2007).

Loss of ENS function in humans causes Hirschsprung Disease

The role that the ENS plays in the maintenance of intestinal health can be appreciated through examining the consequences of ENS reduction. In humans, ENS reduction manifests in Hirschsprung disease (HSCR), the most common congenital intestinal motility disorder (Gfroerer, 2015). Occurring in 1 of every 5000 live births in the United States, HSCR is characterized by a portion of the intestine that lacks nerve activity due to the absence of an ENS. Without ENS activity in the affected segment of the intestine, the intestinal luminal contents cannot be cleared; this can, in turn, lead to

severe intestinal inflammation, and if untreated, to life threatening toxic megacolon, enterocolitis and intestinal perforation (Parisi, 2002). The extent of aganglionosis seen in HSCR cases is widely variable. The most commonly occurring HSCR is short-segmented HSCR, which affects a short portion of the colon and the rectum, though longer lengths of the intestinal tract can be involved in long-segmented HSCR. Less common HSCR subtypes give rise total colonic aganglionosis, though forms of HSCR that cause total intestinal aganglionosis are extremely rare (Ganz, 2017).

HSCR has a strong genetic component, with about 50% of familial HSCR cases and one third of sporadic HSCR cases attributed to coding mutations in the *RET* gene. Many remaining HSCR cases coincide with a single-nucleotide polymorphism (SNP) in a non-coding region of the *RET* gene that greatly reduces its expression (Goldstein, 2018). Mutations causing *SOX10* haploinsufficiency are also associated with HSCR (Paratore et al., 2002). Though human genetic studies have identified mutations strongly associated with HSCR, HSCR is a multifactorial disorder, and exhibits great variability even between individuals with the same disease alleles. Moreover, there are still many HSCR cases of unknown genetic origin (Ganz, 2017). Further identification of HSCR-causing mutations and unknown disease genes will require continued genetic studies in both model organisms as well as humans (Heanue and Pachnis, 2008).

The zebrafish as a model in which to study the ENS

The zebrafish (*Danio rerio)* has been widely used as an animal model for studying the role of the ENS in intestinal function and disease states (Ganz, 2017). Zebrafish are an ideal animal model because they are easily bred in large numbers, develop quickly, and are optically transparent, enabling observation of developmental

processes in living embryos and larvae. Additionally, the zebrafish genome is highly conserved with that of other vertebrates, including humans. Zebrafish genome sequencing has revealed that 70% of human genes are related to genes found in zebrafish, and nearly 85% of human disease genes have a zebrafish counterpart (Howe et al., 2013). Thus, findings from zebrafish research are promising for applications to human health.

The zebrafish ENS is also similar to that of humans, though less complex in structure (Wallace and Pack, 2003). As opposed to mammals, in which ENS neurons are found in two layers at the myenteric and submucosal plexuses, ENS neurons in zebrafish exist in a single layer that lies between layers of circular and longitudinal smooth muscle. As such, the zebrafish ENS lacks a submucosal plexus, and the myenteric plexus is comprised of neurons scattered or arranged in small clusters rather than in larger ganglia (Wallace and Pack, 2005). As in all vertebrate species examined to date, the ENS in zebrafish is entirely neural crest-derived and contains a diverse collection of neuron subtypes. Early studies found that formation of the intestinal tube in zebrafish occurs around the 18-somite stage, whereas in mammals this occurs at somite stages 1-2 (Heanue et al., 2016). Despite forming later, the temporal sequence of events during zebrafish ENS formation is identical to that in mammals, revealing a highly conserved developmental program. The zebrafish ENS originates from vagal neural crest cells which migrate from the level of the postotic hindbrain toward the primordial intestine in two bilaterally-paired streams. These ENS progenitor cells reach the anterior aspect of the developing zebrafish intestine by 32 hours post-fertilization (hpf), and the distal end of the intestine around 66 hpf, migrating around the intestinal

circumference and differentiating into glial cells and neurons in a rostral to caudal progression (Shepherd and Eisen, 2011). Aside from these differences in neural crest cell migration patterns and time scale, many molecular mechanisms underlying ENS development in zebrafish are conserved in humans and other amniote vertebrates, including the requirement of signaling through the RET receptor tyrosine kinase (Heanue et al., 2016).

As in humans and other vertebrate systems, the zebrafish intestine is a complex organ system comprised of a variety of cell types, including immune, neuronal, muscle, vascular, and epithelial cells (Heanue et al., 2016). However, there are some differences between the intestinal tracts of zebrafish and humans. For example, the zebrafish intestinal tract does not contain a distinct stomach, but instead has an intestinal bulb, an enlarged region of the anterior intestine (Shepherd and Eisen, 2011). Additionally, the wall of the zebrafish intestinal tract lacks a submucosal layer, and so the vascular tissue in the zebrafish intestine surrounds the mucosa and muscularis (Wallace and Pack, 2005). The intestinal epithelium of zebrafish also differs from that of humans because it lacks crypts and instead features fingerlike protrusions called folds, which decrease in size from anterior to posterior (Wallace and Pack, 2003).

Apart from these differences in structure, the zebrafish intestine is remarkably similar to that of humans (Fig. 2B). As in mammals, zebrafish epithelial cells are constantly renewed. They migrate from the base of folds to the tip of the fold, where they undergo apoptosis and are sloughed into the intestinal lumen. The zebrafish intestinal epithelium also features differentiated epithelial cells, including absorptive enterocytes, enteroendocrine cells, and mucus-producing Goblet cells (Ganz, 2017).

In total, despite differences in development and architecture, many functional aspects of the zebrafish intestine and its resident nervous system are conserved among humans and other amniotes (Shepherd and Eisen, 2011).

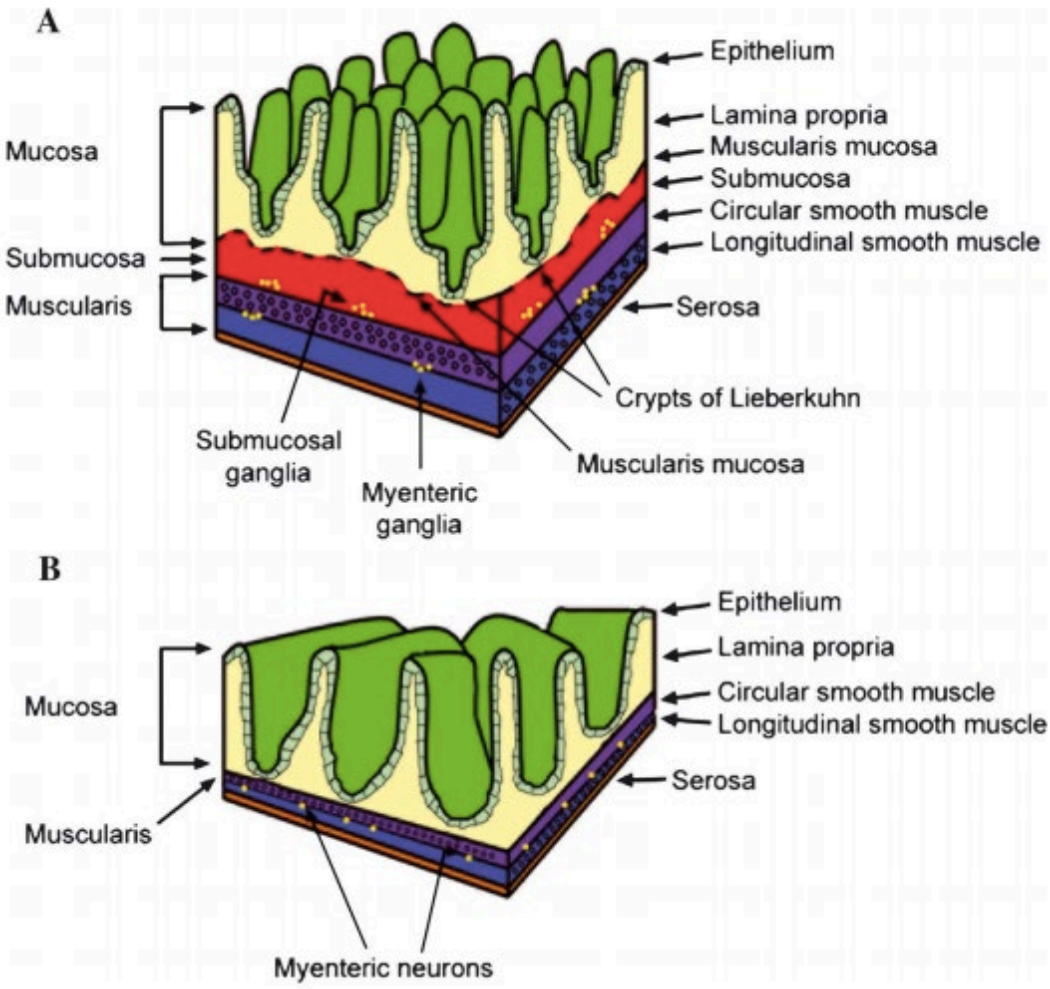

Figure 2. Architecture of mammalian (A) and zebrafish (B) intestine.

The zebrafish intestinal epithelium lacks a muscularis mucosa, which lies between the lamina propria in the submucosa in mammals, and instead, the inner intestinal smooth muscle layer is separated from the basal epithelium by a thin layer of connective tissue. Zebrafish also lack intestinal crypts (termed crypts of Lieberkuhn in this schematic) and their intestinal epithelium consists of irregularly sized folds. Image source: Wallace, K. N., Akhter, S., Smith, E. M., Lorent, K., & Pack, M. (2005).

Studies of common HSCR genes in the zebrafish model system

In a recent study from the University of Oregon, *sox10* mutant zebrafish were shown to develop microbiota-dependent intestinal inflammation due to a specific shift in community member abundance (Rolig et al., 2017). In contrast, previous research in the Eisen Laboratory found that *ret* mutant zebrafish do not exhibit increased intestinal inflammation. This finding is surprising considering that the *sox10* and *ret* mutants each lack an ENS, and as such, they each lack the same ENS cells important for modulating intestinal inflammation, thus, we expected *sox10* and *ret* mutants to exhibit similar inflammatory phenotypes. We rationalized that, because *sox10* expression is required for activation of *ret* in the same population of migrating enteric neural crest-derived cells, *ret* mutant zebrafish should exhibit the inflammatory phenotype of *sox10* mutants (Ganz, 2015; Heanue and Pachnis, 2008). To investigate the opposing inflammatory phenotypes of *sox10* and *ret* mutants and better understand the interactions of these HSCR genes, my undergraduate thesis examined the intestinal phenotypes of *sox10;ret* double mutant zebrafish. Several of my experiments lead to unexpected results, providing interesting questions to be pursued in the future.

Methods

Ethics statement

All zebrafish experiments were done in accordance with protocols approved by the University of Oregon Institutional Animal Care and Use Committee (protocol number 15–15) and conducted following standard protocols as described in The Zebrafish Book (Westerfield, 2000).

Zebrafish husbandry and phenotypic identification

sox10$^{t3+/-}$ and *ret1*$^{hu2846+/-}$;*phox2b:GFP* adult zebrafish were crossed to generate *sox10*$^{+/-}$;*ret*$^{+/-}$;*phox2b:GFP* larvae. *phox2b:GFP* is a transgenic line that expresses green fluorescent protein (GFP) in neurons, allowing identification of *ret* mutant larvae based on the absence of enteric neurons. *sox10*$^{+/-}$;*ret*$^{+/-}$;*phox2b:GFP* larvae were grown to adulthood and incrossed for experiments. The larvae produced from the *sox10*$^{+/-}$;*ret*$^{+/-}$;*phox2b:GFP* incross were identified by phenotype once they reached six days post fertilization (dpf), at which time fluorescence microscopy was used to distinguish whether a given larva appeared to be wild-type, *sox10* homozygous mutant, *ret* homozygous mutant, or a *sox10;ret* double mutant.

Dissection of larval zebrafish intestine

6 dpf larvae were euthanized with Tricaine (Western Chemical, Inc.) mounted in 4% methylcellulose (Sigma-Aldrich) and positioned to orient the intestine on the right lateral side of the body. 0.10-mm insect pins (Fine Science Tools USA, Inc.) were

loaded into micro dissecting needle holders and then sterilized with 70% ethanol. To begin the intestinal dissection, one insect pin was inserted above the most anterior portion of the intestinal bulb, near the right ear. Another insect pin was used to pare off the layer of skin covering the most ventral surface of the intestine. With one insect pin still inserted in the anterior region of the larva, the exposed intestine was dissected by inserting the other insect pin between the dorsal aspect of the intestine and the skeletal muscle layer and drawing the pin away from the body.

Polymerase chain reaction (PCR) for genotypic identification

Immediately following intestinal dissection, larvae were placed in a 96-well plate in a solution of lysis buffer (20 mM Tris-Cl; 2 mM EDTA; 2.5-mL 20% Triton X-100) and proteinase K (100μg/mL) to obtain genomic DNA. DNA was extracted from each lysed larval sample by pipetting 1uL of the supernatant from each well for use as template in a 20μL PCR. PCR reagents included GoTaq Green Master Mix (Promega) and *ret* primers. Cycling conditions: 2:00 initial denature at 94 C, followed by 39 cycles of 94 C for 30 seconds, 59 C for 30 seconds, 72 C for 30 seconds, followed by final extension at 72 C for 5 minutes, with a hold temperature of 12 C. Following restriction digest with Hpy188I (New England BioLabs, Inc.) at 37 C overnight, PCR products were run on 3% agarose gel and visualized with SYBR Safe DNA gel stain (S33102, Invitrogen) and UV transillumination (ChemiDoc MP Imaging System, BioRad).

Myeloid peroxidase

Larvae were fixed in 4% PFA overnight and washed with 1X PBS. Wholemount larvae were stained with Myeloperoxidase kit (Sigma) for histochemical identification

of neutrophils. Care was taken not to overdevelop the Myeloperoxidase (Mpo) stain because staining to prevent staining of eosinophils and erythrocytes, in addition to neutrophils (Lieschke et al., 2001). Following staining, larvae were washed in 1X PBS and their intestines dissected as previously described. Neutrophil abundance was determined via counting of cells expressing Mpo in the dissected intestine.

Microbiota enumeration

At 6 dpf, larvae were euthanized with Tricaine. Subsequently, larvae were mounted in 4% methylcellulose and their intestines were dissected using sterile technique. Following dissection, intestines were placed in 1mL sterile embryo medium, homogenized with bead beating, diluted, and plated on tryptic soy agar plates (TSA; BD). After incubation at 32°C for 48 hr, colonies were manually counted.

Quantification of proliferating cells

Larvae were immersed in 100 µg/ml EdU (A10044, Invitrogen) for 16hr then fixed in 4% PFA for 4hr at room temperature. Larvae were subsequently processed for paraffin embedding and cut into 7µm transverse sections. Slides were processed according to manufacturer instructions for the Click-iT EdU Cell Proliferation Assay Kit (C35002, Molecular Probes). EdU-labeled nuclei within the intestinal epithelium were counted over 30 consecutive sections beginning at the esophageal-intestinal junction and proceeding caudally towards the vent, as in Cheesman et al., 2011.

Whole mount larval zebrafish immunohistochemistry

At 6 dpf, larvae were euthanized with Tricaine and fixed in 4% PFA for 3 hr at room temperature. Fixed larvae were washed for 10 min in 1X PBS/0.5% Triton X-100,

permeabilized 3x for 30 min in double-distilled water, and blocked in 5% NGS, 2% BSA, 1% DMSO, 0.5% Triton-100 in 1X PBS for at least 1 hr. Larvae were then incubated for approximately 16 hr with Mouse-anti-GFP (A11120, Life Technologies) and Rabbit-anti-5-HT antibodies (#20080, Immunostar) diluted 1:1000 and 1:10000, respectively, in block solution at room temperature with rocking. Following incubation with the primary antibodies, larvae were washed 8x for 10 min in 1X PBS/0.5% Triton X-100 and incubated with goat-anti-mouse-Alexa488 (A11001, Invitrogen) and goat-anti-rabbit-Alexa546 (A11071, Life Technologies) secondary antibodies (both diluted 1:1000 in block solution), for approximately 16 hr at room temperature. Larvae were then washed 8x for 10 min before staining results were visualized with a fluorescence stereomicroscope.

Enumeration of enterochromaffin cells

Confocal images of 5-HT-positive, GFP-negative enterochromaffin cells in whole mount larvae were captured using a Leica DM6 microscope equipped with a Leica DFC365FX camera. All 5-HT-stained enterochromaffin cells within a 1 mm length of the intestinal vent were counted. Images were viewed in Imaris (version 9.2) and 5-HT-positive, GFP-negative enterochromaffin cells were manually enumerated.

Statistics

Statistical analysis was performed using Prism (Graphpad software). Statistical significance was defined as $p < 0.05$. Graphs were constructed and analyzed using GraphPad Prism (scatter plot using one grouping variable for one-way ANOVA). All experiments were performed at least twice with n=7 for each genotype (wild-type, *sox10*, *ret*, and *sox10;ret*).

Results

sox10;*ret* double mutant zebrafish retain distinct features of *sox10* and *ret* single mutants

Wild-type larvae are distinguished by several key features. In addition to the presence of GFP-expressing enteric neurons along the entire length of the intestinal tract, wild-type larvae possess neural crest-derived pigment cells, including melanocytes that are visible in a specific pattern throughout the body, as well as a swim bladder, a hydrostatic organ located between the intestinal canal and the body wall which enables the fish to maintain buoyancy (Fig. 3A). The *ret* mutant maintains wild-type pigment cells but lacks a swim bladder and enteric neurons. Notably, the *ret* mutant additionally exhibits a craniofacial deformity in which the jaw silhouette is square rather than rounded. Like the *ret* mutant, the *sox10* mutant also lacks an enteric nervous system and a swim bladder, however, it differs from the *ret* mutant in its lack of pigment cells and its maintenance of a wild-type jaw phenotype (Fig. 3C). The *sox10*;*ret* double mutant phenotype combines attributes of both the *sox10* and *ret* single mutants, as double mutant animals lack enteric neurons, pigment cells, and a swim bladder while also exhibiting the *ret*-associated square jaw phenotype (Fig. 3D). Phenotypes were confirmed with PCR genotyping (Fig. 3E).

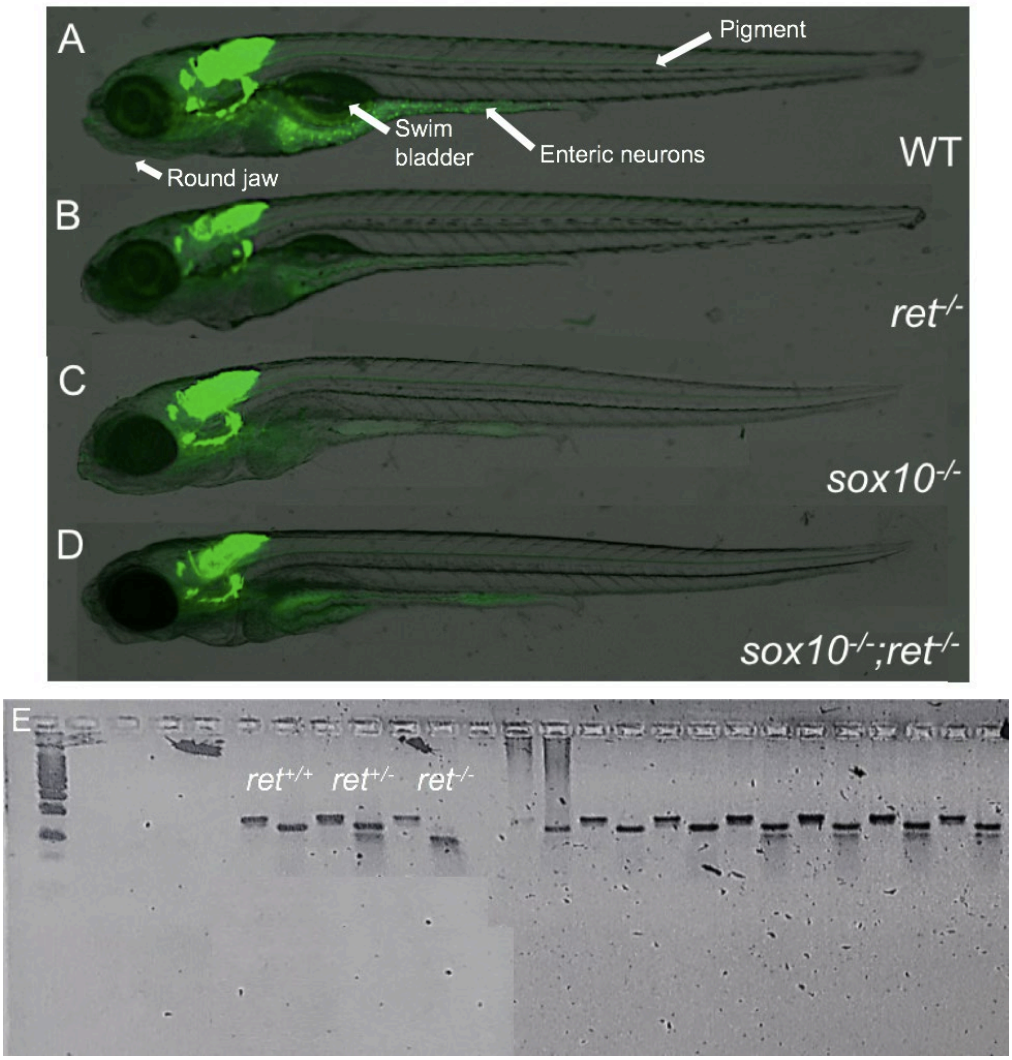

Figure 3. The *sox10;ret* double mutant phenotypically resembles both the *sox10* and *ret* mutants.

A) Representative image of a wild-type Tg(*phox2b:GFP*) zebrafish larva at 6 dpf. WT animals have an ENS, as indicated by punctate GFP expression visible throughout the intestinal tract, a swim bladder, bodily pigment, and a rounded, rather than square jaw line. (B) ret mutants lack enteric neurons and a swim bladder, have a square jaw phenotype but express WT pigment distribution. (C) sox10 mutant zebrafish lack pigment, a swim bladder and the ENS. (D) Zebrafish with mutations in both sox10 and ret possess characteristics of both sox10 and ret single mutants. In addition to lacking enteric neurons, double mutants lack a swim bladder and exhibit the absent pigment phenotype of sox10 mutants, and also maintain the distinctive ret mutant jaw phenotype. (E) Genotypes of animals at the ret locus were confirmed using PCR. Each of the lanes to the right of the labeled control lanes represent one fish.

sox10;*ret* double mutants do not exhibit the disease phenotypes of *sox10* mutants

Previous work showed that *sox10* mutants exhibit several intestinal disease phenotypes, including increased abundance of pro-inflammatory intestinal bacteria, elevated neutrophil numbers, and heightened intestinal epithelial cell proliferation (Rolig et al., 2017). In contrast, I discovered that *ret* mutant intestines are not inflamed and display neutrophil abundance, epithelial cell proliferation and intestinal bacterial load that are similar to wild-types, thus are reduced compared to *sox10* animals.

I asked whether loss of both *sox10* and *ret* resulted in the same intestinal disease phenotypes as loss of *sox10* alone. During ENS development, expression of *sox10* by migrating enteric neural crest-derived cells precedes that of *ret* in the same cell population, and, as such, a mutation in *sox10* disrupts downstream *ret* function. For this reason, I reasoned that *sox10*;*ret* double mutant intestines would more closely resemble those of *sox10* single mutants than those of *ret* mutants. Accordingly, I hypothesized that *sox10*;*ret* double mutants would exhibit increased intestinal neutrophil accumulation, bacterial abundance, and a larger population of recently proliferated epithelial cells as compared to wild-types or *ret* mutants. I quantified intestinal neutrophil abundance by staining for myeloid peroxidase, a neutrophil-specific enzyme, and subsequently determining the quantity of myeloid peroxidase positive cells in the distal intestine (Fig. 4A). Contrary to my hypothesis, I found that intestinal neutrophil abundance of *sox10*;*ret* animals was not significantly higher than that of WTs (Fig. 4B). Though variable over a large range, the intestinal bacterial load of *sox10*;*ret* mutants was also comparable to that of WT animals. Of note is our observation of similar abundances of intestinal colony-forming units across all genotypes studied, as this result

is inconsistent with previous observations (Rolig et al., 2017) that *sox10* mutants display bacterial overgrowth (Fig. 4C). Thus, we cannot conclude that our animals were reared in exactly the same conditions as those of Rolig et al. (2017), which could account for the differences in our results. Determining relative populations of proliferated intestinal epithelial cells by EdU assay, we validated the previous finding that *sox10* mutants exhibit increased epithelial cell proliferation and found that the abundance of recently-proliferated intestinal epithelial cells of *sox10*;*ret* animals resembled that of wild-types (Fig. 4D).

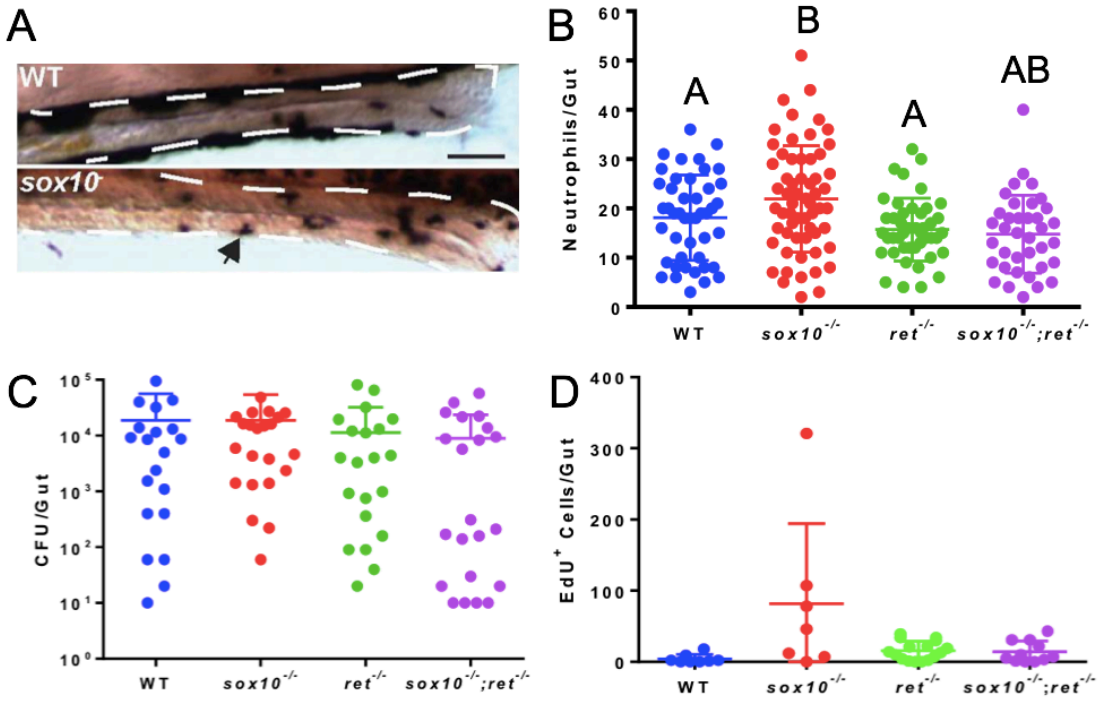

Figure 4. *sox10;ret* double mutant zebrafish exhibit wild-type intestinal phenotypes.

(A) Representative images of intestinal neutrophil accumulation in 6 dpf wild-type and sox10 mutant larvae. Arrowhead indicates an example of a neutrophil marked by expression of myeloid peroxidase. sox10 mutant is easily recognized by the lack of pigment cells. Image courtesy of Rolig et al. (2017). (B) Quantification of intestinal neutrophil numbers per fully dissected larval intestine. (C) Quantification of bacterial colonization levels in dissected intestines of WT larvae and ENS mutant siblings. (D) Total numbers of proliferating intestinal epithelial cells in 6 dpf larvae, as assessed beginning at the esophageal-intestinal junction and continuing caudally over thirty serial sections. Letters denote significance via one-way ANOVA with multiple comparisons, $p \leq 0.05$. Results are an accumulation of repeated independent experiments with n=6 or more per group.

Enterochromaffin cell abundance in *sox10*;*ret* double mutants resembles that of wild-types

The observation that *sox10*;*ret* double mutant zebrafish do not exhibit increased intestinal neutrophil accumulation prompted us to reconsider the potential interactions of these HSCR-associated genes. We rationalized that the difference in the abundance of intestinal neutrophils between *sox10* and *ret* mutant zebrafish may be due to suppression of the *sox10* inflammatory phenotype by a *ret*-expressing cell type other than enteric neurons. Thus, we sought to identify a candidate cell type for investigation, the abundance or absence of which might explain the difference in intestinal inflammation among *sox10* and *ret* mutants. Recent work in the Eisen laboratory found that *ret,* but not *sox10,* is expressed in the wild-type larval zebrafish intestine by enterochromaffin cells (ECs), a specialized enteroendocrine cell type that produces the neurotransmitter serotonin. This preliminary work also suggested that *ret* mutants have decreased EC numbers. As such, ECs seemed like an ideal candidate cell type to be further investigated in *sox10* and *ret* single mutants as well as in *sox10*;*ret* double mutants. Given our rationale that *ret* expression by a non-ENS cell type might suppress the *sox10* inflammatory phenotype in *sox10*;*ret* double mutants, we hypothesized that *sox10*;*ret* double mutants would exhibit the same EC phenotype as *ret* mutants. Surprisingly, anti-serotonin staining of *sox10*;*ret* double mutants revealed that the number of intestinal ECs (5-HT-positive and GFP-negative cells in the vent region of the intestine) in *sox10*;*ret* animals is significantly increased compared to *ret* mutants, similar to WTs (Fig. 5A-B). Specifically examining EC morphology, the cells appeared similar between wild-type, *sox10* mutant, and *sox10;ret* animals (Fig. 5C)

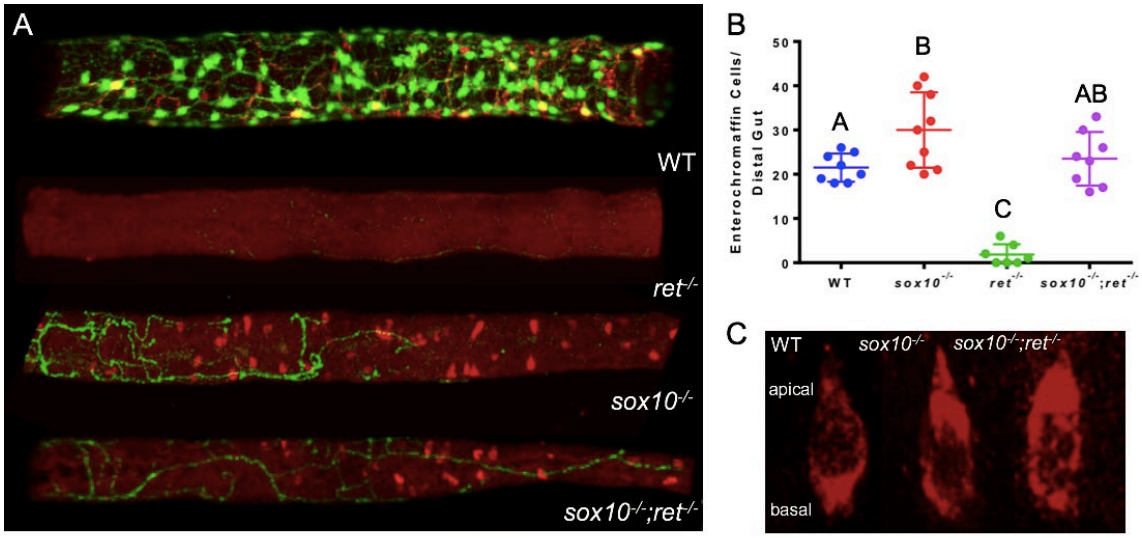

Figure 5. *sox10;ret* double mutant zebrafish display wild-type enterochromaffin cell abundance in the distal intestine.

(A) Representative images of the distal portion (approximately 500 μm) of larval zebrafish intestines stained with antibodies to serotonin and GFP. (B) Quantification of enterochromaffin cell numbers in the distalmost region of wild-type (WT), *sox10* mutant, *ret* mutant, and *sox10;ret* double mutant larval zebrafish intestines. (C) Representative images of serotonin-positive enterochromaffin cells from WT, *sox10* mutant, and *sox10;ret* double mutant larval zebrafish intestines. The apical and basal surfaces of the enterochromaffin cells are denoted. Letters denote significance via one-way ANOVA with multiple comparisons, $p \leq 0.05$. Results are an accumulation of two independent experiments with n=7 or more per group.

Discussion

In this study, I investigated intestinal phenotypes of zebrafish with mutations in two genes, *sox10* and *ret*, which each play essential roles during enteric nervous system development. During early development, these genes are expressed by the neural crest-derived progenitor cells of the enteric nervous system, and their loss of function results in a severely reduced or absent enteric nervous system in all vertebrates thus far examined, including in humans. In the present study, I describe the intestinal phenotypes of *sox10*;*ret* double mutants and compare them to the intestinal phenotypes of *sox10* and *ret* single mutants. I present two striking findings: one, that *sox10*;*ret* double mutant zebrafish do not exhibit the intestinal inflammatory phenotype of *sox10* mutants, and two, that *sox10*;*ret* double mutants exhibit wild-type intestinal enterochromaffin cell abundance, in contrast to *ret* mutants. Taken together, these results reveal a complexity in the interactions between the *sox10* and *ret* genes that we do not currently understand.

Previous work in zebrafish implicated the enteric nervous system in regulating intestinal inflammation by demonstrating that loss of *sox10* results in intestinal inflammation (Rolig et al., 2017). Prompted by our finding that *ret* mutants do not exhibit intestinal inflammation, we generated *sox10*;*ret* double mutants and analyzed intestinal inflammation. Given that *sox10* is expressed by all neural crest cells as they delaminate from the neural tube and is required for activation of *ret,* while *ret* is first expressed in vagal neural crest cells as they assemble proximal to the foregut, one might reasonably predict that loss of both *sox10* and *ret* would result in the same intestinal inflammatory phenotype as loss of *sox10* alone, because *sox10* mutants lack the cells

that would go on to express *ret*. As this prediction is based upon what is known about the time course of *sox10* and *ret* expression during enteric nervous system development, confirming this prediction would indicate that intestinal inflammation is strongly regulated by the enteric nervous system. Our observation that loss of both *sox10* and *ret* results in wild-type levels of intestinal inflammation, specifically intestinal neutrophil abundance, bacterial abundance and proliferating epithelial cell abundance, suggests that *ret*-mediated suppression of the *sox10* phenotype of intestinal inflammation occurs via the action of some cell type that exerts its function in the absence of the enteric nervous system. However, further work is required to better elucidate the effects of mutations in *sox10* and *ret* on intestinal function and their implications for the many cell types that comprise and interact with the intestine.

Though the present study further characterizes the opposing intestinal phenotypes of *sox10* and *ret* mutants, the basis of their differences in phenotype remains poorly understood. Rolig and colleagues (2017) showed that *sox10* mutant intestines exhibit microbially-induced inflammation that is caused by distinct changes to the intestinal microbial community. Surveying the intestinal microbial community of *sox10* mutants revealed an over-abundance of pro-inflammatory bacterial lineages and a shortage of anti-inflammatory bacterial lineages, a dysbiotic state which could be rescued upon restoration of the enteric nervous system or administration of an anti-inflammatory bacterial strain (Rolig et al., 2017). However, *ret* mutants do not exhibit signs of intestinal inflammation, an observation which suggests that the environment for the microbiota must differ between *ret* mutants and *sox10* mutants. The conditions that permit overgrowth of pro-inflammatory bacterial lineages within the *sox10* intestine are

incompletely defined, however, since *sox10* mutants are known to possess an overabundance of pro-inflammatory bacterial lineages while lacking anti-inflammatory bacterial lineages, it should be a priority to profile the intestinal microbial communities of *ret* mutants, as differences in intestinal microbial community membership between these mutants may account for their differences in intestinal inflammation.

In light of the observation that *sox10*;*ret* double mutants fail to exhibit the intestinal inflammatory phenotypes of *sox10* mutants, I sought to explore an alternative possibility that the loss of *ret* gene activity in another cell type extrinsic to the enteric nervous system would be more pertinent to intestinal inflammation. In the wild-type larval zebrafish intestine, *ret*, but not *sox10,* is expressed by enterochromaffin cells, which also secrete the crucial signaling molecule serotonin. This observation led to our investigation of intestinal enterochromaffin cell abundance in *sox10*, *ret* and *sox10*;*ret* mutant zebrafish, in which I observed wild-type distal intestinal enterochromaffin cell numbers in *sox10*;*ret* mutants. I also observed *sox10* mutants to exhibit increased intestinal enterochromaffin cell numbers, consistent with the increase in intestinal epithelial proliferation, while *ret* mutants display very few of these 5-HT-producing cells. Substantial evidence suggests an important role for 5-HT in intestinal inflammation, as increased enteric 5-HT levels and enterochromaffin cell numbers have been demonstrated in ulcerative colitis and Crohn's disease patients (Spiller, 2007; Coates, 2004; El-Salhy, 1997). Furthermore, enterochromaffin cell-derived peripheral 5-HT that is stored in platelets is implicated in the inflammatory immune response, as it was demonstrated to promote recruitment of neutrophils and other innate immune cells during acute inflammation (Duerschmied et al., 2013).

From this perspective, an intriguing possibility is that the difference in intestinal enterochromaffin cell abundance between *sox10* and *ret* mutants may also help explain the difference in intestinal neutrophil abundance between the mutants. Similar to human colitis patients, *sox10* mutants exhibit increased enterochromaffin cell abundance. However, they also display increased intestinal epithelial cell proliferation, thus it remains to be determined whether the increased abundance of enterochromaffin cells in *sox10* mutants is attributable to higher incidences of epithelial cell proliferation. In this paradigm, perhaps since *ret* mutants lack increased intestinal epithelial cell proliferation, and *sox10*;*ret* mutants display the *ret* phenotype for intestinal epithelial cell proliferation, they also lack increased enterochromaffin cell abundance, which leads to a decrease in inflammation. It should be noted, though, that the present study does not assess of the amount of circulating 5-HT in zebrafish enteric nervous system mutants, as I determined the abundance of intestinal 5-HT^{+} cells via immunofluorescence assay, without quantification of 5-HT expression. Future experiments should address this limitation, in addition to more detailed exploration of the intestinal secretory cell population in *sox10*, *ret*, and *sox10*;*ret* zebrafish mutants.

Intestinal homeostasis relies upon appropriate execution of interactions between the enteric nervous system, the host immune system, and the intestinal microbiota. The future development of therapeutics for gastrointestinal diseases, including Hirschsprung's disease and inflammatory conditions like inflammatory bowel disease, will depend upon an increased understanding of the dynamic contributions to intestinal health made by the enteric nervous system and its immune and microbial counterparts. I report here that zebrafish with mutations in two ortholog Hirschsprung's disease genes,

sox10 and *ret,* display intestinal phenotypes of both *sox10* and *ret* single mutants, thereby rejecting our hypothesis that *sox10*;*ret* double mutants would exhibit increased intestinal inflammation and reduced enterochromaffin cell abundance. In total, our findings in this study of *sox10*;*ret* double mutant zebrafish suggest novel complexities in the mechanisms of *sox10* and *ret* function and their interaction. Continued investigation of the interplay between host genetics, the microbiota, cells within the intestinal epithelium, and the enteric nervous system will undoubtedly improve our understanding of intestinal homeostasis and inflammation.

Bibliography

Bellono NW, Bayrer JR, Leitch DB, Castro J, Zhang C, O'Donnell TA, et al. Enterochromaffin Cells Are Gut Chemosensors that Couple to Sensory Neural Pathways. Cell [Internet]. 2017/06/22. 2017 Jun 29;170(1):185–198.e16. Available from: https://www.ncbi.nlm.nih.gov/pubmed/28648659

Bianco F, Bonora E, Natarajan D, Vargiolu M, Thapar N, Torresan F, et al. Prucalopride exerts neuroprotection in human enteric neurons. Am J Physiol Gastrointest Liver Physiol [Internet]. 2016/02/18. 2016 May 15;310(10):G768–75. Available from: https://www.ncbi.nlm.nih.gov/pubmed/26893157

Bronner ME, LeDouarin NM. Development and evolution of the neural crest: an overview. Dev Biol [Internet]. 2012/01/02. 2012 Jun 1;366(1):2–9. Available from: https://www.ncbi.nlm.nih.gov/pubmed/22230617

Bronner-Fraser M. Mechanisms of neural crest cell migration. BioEssays [Internet]. 1993 Apr 1;15(4):221–30. Available from: https://doi.org/10.1002/bies.950150402

Cheesman SE, Guillemin K. We know you are in there: Conversing with the indigenous gut microbiota. Res Microbiol [Internet]. 2007;158(1):2–9. Available from: http://www.sciencedirect.com/science/article/pii/S0923250806002439

Cheesman SE, Neal JT, Mittge E, Seredick BM, Guillemin K. Epithelial cell proliferation in the developing zebrafish intestine is regulated by the Wnt pathway and microbial signaling via Myd88. Proc Natl Acad Sci [Internet]. 2011 Mar 15;108(Supplement 1):4570 LP-4577. Available from: http://www.pnas.org/content/108/Supplement_1/4570.abstract

Coates MD, Mahoney CR, Linden DR, Sampson JE, Chen J, Blaszyk H, et al. Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome [1] . Gastroenterology [Internet]. 2004 Jun 1;126(7):1657–64. Available from: https://doi.org/10.1053/j.gastro.2004.03.013

Coates MD, Tekin I, Vrana KE, Mawe GM. Review article: the many potential roles of intestinal serotonin (5-hydroxytryptamine, 5-HT) signalling in inflammatory bowel disease. Aliment Pharmacol Ther [Internet]. 2017 Sep 1;46(6):569–80. Available from: https://doi.org/10.1111/apt.14226

Costa M, Brookes SJH, Hennig GW. Anatomy and physiology of the enteric nervous system. Gut [Internet]. 2000 Dec 1;47(suppl 4):iv15 LP-iv19. Available from: http://gut.bmj.com/content/47/suppl_4/iv15.abstract

De Vadder F, Grasset E, Mannerås Holm L, Karsenty G, Macpherson AJ, Olofsson LE, et al. Gut microbiota regulates maturation of the adult enteric nervous system via enteric serotonin networks. Proc Natl Acad Sci U S A [Internet]. 2018/06/04. 2018 Jun 19;115(25):6458–63. Available from: https://www.ncbi.nlm.nih.gov/pubmed/29866843

Digestive Diseases Statistics for the United States [Internet]. National Institute of Diabetes and Digestive and Kidney Diseases. U.S. Department of Health and Human Services; 2014 [cited 2018Nov26]. Available from: https://www.niddk.nih.gov/health-information/health-statistics/digestive-diseases

Duerschmied D, Suidan GL, Demers M, Herr N, Carbo C, Brill A, et al. Platelet serotonin promotes the recruitment of neutrophils to sites of acute inflammation in mice. Blood. 2013 Feb;121(6):1008–15.

El-Salhy M, Danielsson Å, Stenling R, Grimelius L. Colonic endocrine cells in inflammatory bowel disease. J Intern Med [Internet]. 1997 Nov 1;242(5):413–9. Available from: https://doi.org/10.1046/j.1365-2796.1997.00237.x

Furness JB, Jones C, Nurgali K, Clerc N. Intrinsic primary afferent neurons and nerve circuits within the intestine. Prog Neurobiol [Internet]. 2004;72(2):143–64. Available from: http://www.sciencedirect.com/science/article/pii/S0301008203002041

Ganz J. Gut feelings: Studying enteric nervous system development, function, and disease in the zebrafish model system. Dev Dyn [Internet]. 2017 Oct 4;247(2):268–78. Available from: https://doi.org/10.1002/dvdy.24597

Gershon MD, Tack J. The Serotonin Signaling System: From Basic Understanding To Drug Development for Functional GI Disorders. Gastroenterology [Internet]. 2007 Jan 1;132(1):397–414. Available from: https://doi.org/10.1053/j.gastro.2006.11.002

Gfroerer S, Rolle U. Pediatric intestinal motility disorders. World J Gastroenterol [Internet]. 2015/09/07. 2015 Sep 7;21(33):9683–7. Available from: https://www.ncbi.nlm.nih.gov/pubmed/26361414

Hakansson A, Molin G. Gut microbiota and inflammation. Nutrients [Internet]. 2011 Jun 3;3(6):637–82. Available from: https://www.ncbi.nlm.nih.gov/pubmed/22254115

Halfvarson J, Brislawn CJ, Lamendella R, Vázquez-Baeza Y, Walters WA, Bramer LM, et al. Dynamics of the human gut microbiome in inflammatory bowel disease. Nat Microbiol [Internet]. 2017 Feb 13;2:17004. Available from: https://doi.org/10.1038/nmicrobiol.2017.4

Heanue TA, Pachnis V. Ret isoform function and marker gene expression in the enteric nervous system is conserved across diverse vertebrate species. Mech Dev [Internet]. 2008;125(8):687–99. Available from: http://www.sciencedirect.com/science/article/pii/S0925477308000610

Heanue TA, Shepherd IT, Burns AJ. Enteric nervous system development in avian and zebrafish models. Dev Biol [Internet]. 2016;417(2):129–38. Available from: http://www.sciencedirect.com/science/article/pii/S0012160616300100

Heuckeroth RO, Schäfer K-H. Gene-environment interactions and the enteric nervous system: Neural plasticity and Hirschsprung disease prevention. Dev Biol [Internet]. 2016/03/17. 2016 Sep 15;417(2):188–97. Available from: https://www.ncbi.nlm.nih.gov/pubmed/26997034

Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature [Internet]. 2013/04/17. 2013 Apr 25;496(7446):498–503. Available from: https://www.ncbi.nlm.nih.gov/pubmed/23594743

Kuo BR, Erickson CA. Regional differences in neural crest morphogenesis. Cell Adh Migr [Internet]. 2010;4(4):567–85. Available from: https://www.ncbi.nlm.nih.gov/pubmed/20962585

Le Douarin NM, Teillet M-A. The migration of neural crest cells to the wall of the digestive tract in avian embryo. J Embryol Exp Morphol [Internet]. 1973 Aug 1;30(1):31 LP-48. Available from: http://dev.biologists.org/content/30/1/31.abstract

Lieschke GJ, Oates AC, Crowhurst MO, Ward AC, Layton JE. Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. Blood [Internet]. 2001 Nov 15;98(10):3087 LP-3096. Available from: http://www.bloodjournal.org/content/98/10/3087.abstract

Liu M-T, Kuan Y-H, Wang J, Hen R, Gershon MD. 5-HT4 receptor-mediated neuroprotection and neurogenesis in the enteric nervous system of adult mice. J Neurosci [Internet]. 2009 Aug 5;29(31):9683–99. Available from: https://www.ncbi.nlm.nih.gov/pubmed/19657021

Lomax AE, Fernández E, Sharkey KA. Plasticity of the enteric nervous system during intestinal inflammation. Neurogastroenterol Motil [Internet]. 2005 Feb 1;17(1):4–15. Available from: https://doi.org/10.1111/j.1365-2982.2004.00607.x

Margolis KG, Gershon MD. Enteric Neuronal Regulation of Intestinal Inflammation. Trends Neurosci [Internet]. 2016/07/20. 2016 Sep;39(9):614–24. Available from: https://www.ncbi.nlm.nih.gov/pubmed/27450201

Margolis KG, Stevanovic K, Li Z, Yang QM, Oravecz T, Zambrowicz B, et al. Pharmacological reduction of mucosal but not neuronal serotonin opposes inflammation in mouse intestine. Gut [Internet]. 2013/06/07. 2014 Jun;63(6):928–37. Available from: https://www.ncbi.nlm.nih.gov/pubmed/23749550

Mollaaghababa R, Pavan WJ. The importance of having your SOX on: role of SOX10^{+} in the development of neural crest-derived melanocytes and glia. Oncogene [Internet]. 2003 May 19;22:3024. Available from: https://doi.org/10.1038/sj.onc.1206442

Nagy N, Goldstein AM. Enteric nervous system development: A crest cell's journey from neural tube to colon. Semin Cell Dev Biol [Internet]. 2017/01/10. 2017 Jun;66:94–106. Available from: https://www.ncbi.nlm.nih.gov/pubmed/28087321

Obata Y, Pachnis V. The Effect of Microbiota and the Immune System on the Development and Organization of the Enteric Nervous System. Gastroenterology [Internet]. 2016 Nov;151(5):836–44. Available from: https://www.ncbi.nlm.nih.gov/pubmed/27521479

Paratore C, Eichenberger C, Suter U, Sommer L. Sox10 haploinsufficiency affects maintenance of progenitor cells in a mouse model of Hirschsprung disease. Hum Mol Genet [Internet]. 2002 Nov 15;11(24):3075–85. Available from: http://dx.doi.org/10.1093/hmg/11.24.3075

Parisi MA. Hirschsprung Disease Overview. 2002 Jul 12 [Updated 2015 Oct 1]. In: Adam MP, Ardinger HH, Pagon RA, et al., editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2019. Available from: https://www.ncbi.nlm.nih.gov/books/NBK1439/

Pattyn A, Morin X, Cremer H, Goridis C, Brunet J-F. The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. Nature [Internet]. 1999;399(6734):366–70. Available from: https://doi.org/10.1038/20700

Peery AF, Crockett SD, Barritt AS, Dellon ES, Eluri S, Gangarosa LM, et al. Burden of Gastrointestinal, Liver, and Pancreatic Diseases in the United States. Gastroenterology [Internet]. 2015/08/29. 2015 Dec;149(7):1731–1741.e3. Available from: https://www.ncbi.nlm.nih.gov/pubmed/26327134

Peterson LW, Artis D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nat Rev Immunol [Internet]. 2014 Feb 25;14:141. Available from: https://doi.org/10.1038/nri3608

Rao M, Gershon MD. Enteric nervous system development: what could possibly go wrong? Nat Rev Neurosci [Internet]. 2018 Sep;19(9):552–65. Available from: https://www.ncbi.nlm.nih.gov/pubmed/30046054

Reedy M V, Faraco CD, Erickson CA. The Delayed Entry of Thoracic Neural Crest Cells into the Dorsolateral Path Is a Consequence of the Late Emigration of Melanogenic Neural Crest Cells from the Neural Tube. Dev Biol [Internet]. 1998;200(2):234–46. Available from: http://www.sciencedirect.com/science/article/pii/S0012160698989631

Rolig AS, Mittge EK, Ganz J, Troll J V, Melancon E, Wiles TJ, et al. The enteric nervous system promotes intestinal health by constraining microbiota composition. PLOS Biol [Internet]. 2017 Feb 16;15(2):e2000689. Available from: https://doi.org/10.1371/journal.pbio.2000689

Rothstein M, Bhattacharya D, Simoes-Costa M. The molecular basis of neural crest axial identity. Dev Biol [Internet]. 2018;444:S170–80. Available from: http://www.sciencedirect.com/science/article/pii/S0012160617305985

Saha L. Irritable bowel syndrome: pathogenesis, diagnosis, treatment, and evidence-based medicine. World J Gastroenterol [Internet]. 2014/06/14. 2014 Jun 14;20(22):6759–73. Available from: https://www.ncbi.nlm.nih.gov/pubmed/24944467

Shepherd I, Eisen J. Development of the zebrafish enteric nervous system. Methods Cell Biol [Internet]. 2011;101:143–60. Available from: https://www.ncbi.nlm.nih.gov/pubmed/21550442

Spiller R. Recent advances in understanding the role of serotonin in gastrointestinal motility in functional bowel disorders: alterations in 5-HT signalling and metabolism in human disease. Neurogastroenterol Motil [Internet]. 2007 Aug 1;19(s2):25–31. Available from: https://doi.org/10.1111/j.1365-2982.2007.00965.x

Theveneau E, Mayor R. Neural crest delamination and migration: From epithelium-to-mesenchyme transition to collective cell migration. Dev Biol [Internet]. 2012;366(1):34–54. Available from: http://www.sciencedirect.com/science/article/pii/S0012160611014692

Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. Nature. 2007 Oct;449(7164):804–10.

Wallace KN, Akhter S, Smith EM, Lorent K, Pack M. Intestinal growth and differentiation in zebrafish. Mech Dev [Internet]. 2005;122(2):157–73. Available from: http://www.sciencedirect.com/science/article/pii/S0925477304002503

Wallace KN, Pack M. Unique and conserved aspects of gut development in zebrafish. Dev Biol [Internet]. 2003;255(1):12–29. Available from: http://www.sciencedirect.com/science/article/pii/S0012160602000349

Westerfield M. The Zebrafish Book. A Guide for The Laboratory Use of Zebrafish (*Danio rerio*). Vol. 385, Eugene. 2000.

Yntema CL, Hammond WS. The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chick embryo. J Comp Neurol [Internet]. 1954 Oct 1;101(2):515–41. Available from: https://doi.org/10.1002/cne.901010212

Young HM, Hearn CJ, Farlie PG, Canty AJ, Thomas PQ, Newgreen DF. GDNF is a chemoattractant for enteric neural cells. Dev Biol [Internet]. 2001;229(2):503–16. Available from: http://www.sciencedirect.com/science/article/pii/S0012160600901003

Yu LC-H. Microbiota dysbiosis and barrier dysfunction in inflammatory bowel disease and colorectal cancers: exploring a common ground hypothesis. J Biomed Sci [Internet]. 2018 Nov 9;25(1):79. Available from: https://www.ncbi.nlm.nih.gov/pubmed/30413188