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Differentiation of the Enteric Nervous System in *Danio Rerio*

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Differentiation of the Enteric Nervous System in *Danio Rerio*

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B.S., University of Wisconsin, 2007
Advisor: Iain Shepherd, Ph.D.

An abstract of
A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies
of Emory University in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in
Graduate Division of Biological and Biomedical Sciences,
Program in Genetics and Molecular Biology
2015

Abstract
Differentiation of the Enteric Nervous System in *Danio Rerio*
By Colin Harrison

The enteric nervous system (ENS) is an intricate web of neurons and glia that control the digestive functions of the gut. There are 17 different, identified subtypes of neurons in the ENS. The ENS is made from a small population of neural crest cells that migrate to and along the gut to populate it. Because such a small migrating population of cells is responsible for the entirety of the ENS, it is vital that the migrating enteric neuron precursor cells (ENPCs) be maintained in an undifferentiated, proliferative state. Errors in ENS development can lead to gut aganglionosis, as is seen in the human disorder Hirschprung's Disease (HSCR). Here we establish a transgenic line, *Tg(-8.3bphox2b*:*Kaede*), to further explore a zebrafish model of HSCR based on the *lessen* mutant. Using this mutant, we found that the aganglionosis seen is due to a decrease in the proliferative potential of the ENPCs. One gene that is frequently mutated in HSCR patients is *EDNRB*, a gene responsible for keeping ENPCs in a proliferative undifferentiated state. We identified two functional variants of *ednrb* in zebrafish that have overlapping function in ENS development, and knockdown of these two genes using morpholinos causes aganglionosis phenotypes. Each initially undifferentiated ENPC matures into neuronal subtypes, which appear at different times in ENS development. Control of this differentiation requires that expression of pro-subtype genes be repressed until the right time. We found that expression of *id2a* was important for maintaining a pool of neuronal nitric oxide synthase (nNOS) neurons. Taken together these data shows that maintaining ENPCs potential to proliferate and differentiate is required to properly pattern the ENS. Understanding how ENS patterning is regulated will allow us to identify new strategies for treating various gastrointestinal disorders.

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Table of Contents

Chapter 1: Introduction to Enteric Nervous System Development and Differentiation

(Adapted from Colin Harrison and Iain T. Shepherd. Choices Choices: Regulation of Precursor Differentiation during Enteric Nervous System Development. Neurogastroenterol Motil. 2013 Jul;25(7):554-62.)

Introduction

The enteric nervous system (ENS) is the largest subdivision of the peripheral nervous system. The ENS is responsible for regulating peristalsis, blood flow, and water and electrolyte transport in the gut (1, 2). ENS development is a coordinated process in which neural crest derived ENS precursors must migrate from specific axial locations to and then along the gut. Subsequently they differentiate into the various types of neurons and glia that make up the ENS. Defects in ENS precursor differentiation have been shown to lead to aganglionosis phenotypes in several model systems. In humans this aganglionosis, when present in newborns, leads to the gastrointestinal disorder Hirschprung's Disease (HSCR) (3-7). In this chapter we review the relevant information on ENS differentiation and analyze its role in ENS development from a cellular and molecular level.

HSCR and other GI Disorders

When errors occur in ENS development it can lead to a range of gastrointestinal (GI) disorders. One of the most well characterized of these disorders is HSCR. HSCR is a developmental disorder in which there are too few neurons in the distal portion of the colon causing an aganglionosis. This colonic aganglionosis is variable in length and dependent on a host of factors (8). HSCR occurs in around 1:5000 live births and the short segment version presents more frequently in males (7, 8). HSCR occurs in both familial and sporadic cases. Several genes are known to be mutated in HSCR cases but only around 50% of familial and 20% or sporadic cases can be linked to identified genes (8-10). The only current treatment for the disorder is a surgical removal of the affected

section and reattachment of the unaffected portions (11). Other disorders such as Mowat-Wilson and Waardenburg syndrome display HSCR like aganglionosis as well (12-16).

Other GI disorders have much subtler but significant effect on ENS development and function (17). Hypo- and hyperganglionosis are both difficult to diagnose and there is some disagreement as to what is the normal range of neuronal numbers (18, 19). In both Crohn's and ulcerative colitis, expression of choline acetyltransferase (ChAT) and neuronal nitric oxide synthase (nNOS), two neuronal subtype-specific genes, are misregulated (20, 21). There also appears to be an overall change in neuronal numbers in inflammatory bowel patients (22). Diverticular disease also has a decrease in the overall ENS neuronal numbers (23). Even a disease not traditionally thought of as an intestinal disorder like Parkinson's Disease has an ENS phenotype (24-26). The wide range of disorders that have ENS phenotypes makes it vital that we understand its development.

Neural Crest Formation

The formation of the neural crest is the initial developmental process in the generation of the ENS. It is in the neural crest that a subpopulation of cells will form that will ultimately migrate to the gut. Functionally the neural crest is a group of cells that form at the boundary between neural plate and the non-neural ectoderm. These cells are known to express a specific set of markers that distinguish them from other cells present in the region.

This initial patterning of the neural crest seems to be due to a combination of signals that intermingle at this distinct border region. Specifically the three key control factors that seem to establish the neural crest as a functional unit appear to be FGF, WNT, and BMP (27-33). In early gastrulation WNT and BMP signal the differentiation of the non-neural ectoderm (34-36). At the same time, FGF signals and BMP and WNT antagonists are expressed in the neural plate (27, 29, 30, 37). At the border of these two regions FGF and WNT signaling begin to induce the expression of neural crest genes (27, 29, 32, 38). As gastrulation continues, FGF and WNT continue to promote the expression of neural crest genes and BMP switches from being an inhibitor of neural crest formation to being required for its generation (32, 34, 39). This alternative effect of BMP on neural crest induction may be the result of a dose-dependent response in these cells (34, 39, 40).

This interplay between FGF, WNT, and BMP establishes the neural crest by the induction of a set of genes referred to as the neural border specifiers that, in turn, help induce the expression of neural crest specific genes (41, 42). Two of these genes, pax3 and zic1, are necessary in neural crest development and can induce neural crest development on their own (43-46). pax3 and zic1 are influenced by a specific combination of FGF, WNT, and BMP at their conserved enhance region (28). Pax3 and zic1 are upstream of several neural crest-specific inducers including Snai1/2, FoxD3, and Twist1 (43, 47, 48). Expression of both pax3 and zic1 is induced by another neural border specifier, msx1 that is, in turn, influenced by FGF signaling (47, 49). Msx1 also appears to be influenced by the gradient of BMP present in the neural border region, as an intermediate level of BMP is needed to induce Msx1 expression (50). Gbx2, which also

interacts with zic1 and is upstream of pax3 and msx1, is directly under the influence of WNT signaling and is one of the earliest identified neural crest inducers (51). Neural crest induction also acts through Ap2 to activate pax3 but later Ap2 expression promotes neural crest formation downstream of pax3 and zic1 (52, 53). Further downstream FoxD3, Snai1/2, Twist1 and other genes act to further delineate the neural crest from the surrounding tissues (48, 54-58). These genes are important not only for the maintenance of the neural crest but several of the properties that give neural crest its unique characteristics (54, 59). This complex web of transcription factors interacts to establish the neural crest and subsequently all its cell type derivatives.

ENS Differentiation

Once the neural crest is established there are multiple steps that must occur in ENS development. Differentiation of the enteric neuron precursor cells (ENPCs) is one of the key processes in the formation of a fully functional ENS. While studies have begun to elucidate the complexities behind this process there are still significant gaps in our understanding. The ENS is a complex system that has at least 17 different subtypes of neurons (1). How this diversity in neuronal subtypes is generated is one the central unanswered questions in the field. Several studies have shown that ENS neurons and glia can be traced back to specific axial populations of neural crest cells but it is unclear exactly when the fate of these neural crest cells becomes determined to generate ENPCs (4, 60-63). It is also unclear at what stage during ENS development these ENPCs become fated to generate a specific subtype of enteric neuron or glia. Potentially, this could be a stochastic process only occurring when ENPCs migrate along the gut and completely

dependent on the ENPCs' final location within the gastrointestinal (GI) tract. At the other extreme, individual ENPCs could be fated to become specific subtypes during the formation of the ENPC population in the pre-migratory/ pre-enteric neural crest (Fig 1.1).

Understanding the processes and mechanisms that regulate differentiation of the various ENS subtypes will not only help us to understand the development of the ENS but may also help us gain a better understanding of the pathologies of various gastrointestinal (GI) disorders. While human aganglionosis disorders have been extensively studied, pathological analysis of patients has been limited to looking for the presence or absence of neurons in the gut. Because there are a large number of GI motility disorders that present in the clinic with no obvious underlying cause, it is quite plausible that some of these conditions result from the absence/ loss of a specific subset of ENS neurons or glia (64-66). If we can better identify subtypes of enteric neurons and glia, we will have a clearer understanding of how these ENS cell subtypes develop and this may give significant insights into the etiologies of these currently unexplained GI motility disorders.

Early ENS Specification of the Neural Crest

One major unanswered question is how early during embryogenesis does ENS specification occur? The key event appears to be the formation of a specific axial population of neural crest cells (NCCs). Classic chick-quail chimera studies indicate that cells within the vagal neural crest are sufficient to form most of the ENS (67, 68). In the zebrafish model system, the ENS is completely formed from vagal neural crest cells,

however in mammals and chicks the ENS is formed not only from the vagal cells but is also derived in part from sacral neural crest cells (69-72). These vagal and sacral neural crest cells appear to develop in a semi-cell autonomous manner indicating there is some level of specification that occurs early on during neural crest formation. When vagal and sacral neural crest cells were reciprocally transplanted to the other axial location, transplanted cells went on to form structures appropriate for their new axial location. However sacral crest cells were not as efficient at generating ENS neurons and glia as vagal crest cells (60, 61). Similarly, when vagal NCCs were transplanted to the sacral region, the transplanted cells followed the normal migration route of sacral cells to the gut but did so earlier and in a much greater number (62). It appears that while there is some flexibility in the axial origin of the neural crest that gives rise to the ENPCs, vagal NCCs are the preferential axial source of NCCs for the ENS and a critical number of NCCs are necessary for normal formation of the ENS (73). When the number of vagal NCCs is reduced so that only a small number of ENPCs enter the gut, the rate of ENPC migration along the gut proceeds at a much slower rate. This may be due to a lack of cell-cell contact between the low numbers of ENPCs (74). Mathematical modeling of the process of ENPC colonization of the gut suggests that proliferation differences between the different axial neural crest populations determine their ability to generate a complete ENS. These models show that cranial neural crest have a spatially determined proliferative advantage in forming ENS (75). Given these results, it is clear that distinct populations of NCCs are determined to be ENPCs within the vagal neural crest and these cells are needed to correctly populate the gut with neurons and glia.

It is clear that the spatial organization of the neural crest is important for determining the eventual fate of the ENPCs. One key anatomical structure that affects the early specification of specific vagal neural crest derivatives is the dorsal aorta. Expression of BMP4 and 7 from the dorsal aorta induces the expression of pro-sympathetic neural genes. Inhibition of BMP expression from the dorsal aorta prevents sympathetic neuron formation (76, 77). The expression of BMP by the dorsal aorta gives rise to a concentration gradient of BMP ligand in this region and this means that as neural crest cells migrate towards the gut they are exposed to varying BMP concentrations. As a result, NCCs may acquire different cell fates within the ventrally migrating stream depending on the length of time and the concentration of BMP ligand to which they are exposed to during their migration. Similarly, there is a gradient of Wnt expression extending from the neural tube laterally. NCCs that express β-catenin, a down stream signaling component of the canonical signaling pathway, at high levels have been shown to form sensory neurons (78, 79).

These embryological studies suggest that there is some level of fate determination that is occurring early on in the formation of the neural crest but the molecular basis behind this fate determination is unclear. At the earliest stage of neural crest specification, the transcription factors FoxD3 and Sox10 are expressed by the neural crest at the stage when it arises from the neuroepithelium (80, 81). Functionally, FoxD3 is important in the early selection of neuronal cell fates as opposed to non-neuronal cell fates within the neural crest (81). Sox10 is also expressed throughout neural crest development in the vagal and sacral regions and continues to be expressed in ENPCs when they reach the gut and begin

migrating along it (63, 70). Sox10 along with Pax3 induce expression of the tyrosine kinase RET, a key gene in ENS development and an early ENPC marker that is the primary gene associated with HSCR (70, 82-84). RET's initial function is to promote the survival of ENPCs, acting as the signal transducing component of the GDNF receptor along with its co-receptor GFR(alpha)1 (85, 86). Phox2b is another early marker whose expression is dependent on Sox10 (87, 88). Not only is Phox2b Sox10-dependent, but it also is expressed by ENPCs throughout their migration along the gut (88). Phox2b also appears to be important for the expression of RET as well as for the expression of the basic helix-loop-helix (bHLH) transcription factor Ascl1 (87). Furthermore, in addition to the previously mentioned transcription factors, Hox genes are involved in the fate determination of the different axial neural crest populations. Studies have shown that the different axial populations of NCCs express different Hox genes dependent on their axial origin and this NCC Hox gene expression affects their cell fate (89, 90). With respect to the vagal neural crest and ENPC fate, these cells express HoxB3 and it may play a role in the determining this fate (91).

Most of the identified transcription factors are expressed in both the pre-migratory and migratory vagal neural crest precursors, where they clearly have an important role in ENPC/ENS specification. However, the full complement of transcription factors that are responsible for the selection of ENS precursor cell fate as opposed to other neural crest cell derivatives has not been elucidated. In addition, while these transcription factors are required for ENS development, it is not clear whether any of these genes confers a

specific ENPC cell fate for an individual NCC or whether their function is simply to confer a commitment to a neural/glia cell fate.

Post Neural Crest Differentiation Control

Once the ENPCs begin to enter the gut their environment changes significantly and the signals they receive become even more important for maintaining their proliferative potential and for determining their eventual ENS cell fate. As the ENPCs enter the gut, they continue to express Sox10, RET, and Phox2b (63, 70, 85-87). Sox10 is critically important for maintaining the progenitor state of ENPCs (92). Sox10 also influences the expression of several other proteins shown to be involved in ENPC development including Ascl1, a bHLH transcription factor that actually represses Sox10 expression (80). This Sox10 driven expression of Ascl1 appears to be modulated by the notch pathway as a component of the notch signaling pathway, Hes1, represses Ascl1 expression (39). As a result, notch signaling is therefore important in maintaining the ENPCs progenitor potential (93, 94).

Sox10 also influences the expression of the G-Protein coupled receptor EDNRB, which has been shown to be important in the survival of ENPCs once they reach the gut (95, 96). EDNRB, and its ligand endothelin-3 (ET-3), seem to prevent neuronal differentiation in ENPCs and helps maintain their potential to colonize the rest of the gut (96). Another gene that appears to prevent neuronal differentiation in favor of proliferation is sonic hedgehog (shh). shh is expressed in the developing gut endoderm. shh modulates ENPC responsiveness to GDNF, promoting cell proliferation and migration while attenuating/

inhibiting neuronal differentiation (97, 98). A second hedgehog ligand, indian hedgehog (ihh), is also expressed by the gut endoderm and it also helps promote proliferation of ENPCs (99).

Many of the genes that are critical to ENS development appear to be expressed throughout this developmental process. Interestingly, many of these signaling pathways involved early in neural crest development often have additional/alternate functions later in ENPC specification. One example is the BMP family of proteins, specifically BMP4. BMP4 is involved in differentiation of neural crest-derived cells into neurons *in vitro* (100-102). It appears that ENPCs have a dose dependent response to BMP signaling, as low concentrations of BMP promote an ENPC to stay in an undifferentiated proliferative state while high concentrations promote neurogenesis (101, 102). One way in which BMP may influence neurogenesis is through its interaction with Ascl1 (103, 104). Ascl1 appears to promote the expression of the transcription factor Phox2a and together these two transcription factors promote the expression of a subset of pro-neural genes. Previous studies looking at autonomic nervous system development have shown Ascl1 couples the expression of general neuronal markers with subtype specific markers (105, 106). Ascl1 is also critically important for the development of esophageal neurons as $Ascl1^{-/-}$ mice have perturbed gangliogenesis in the esophagus. This suggests that other basic helix-loop-helix pro-neural transcription factors are also involved in ENS development (107).

Another gene that appears to have a bimodal role in ENS development is FoxD3. While the ENS glial population comes from the same ENPC precursor pool as the ENS neurons,

glial development lags behind the development of the neurons as glial markers are not seen in the early migrating ENPC chains as opposed to neuronal markers, which are observed (108, 109). FoxD3 promotes gliogenesis in the ENS, as well as influence proliferation and neural patterning (110). Notch signaling and the bHLH transcription factor Hand2 also affect ENS glial cell development (111). However Hand2 appears to indirectly affect glial development, because the glial phenotype seen in Hand2-/- mice results from an overall reduced size of the initial ENPC progenitor pool rather than any specific effect on gliogenesis cell fate determination (112). While its precise mechanistic function in ENS development remains controversial, Hand2 seems to have a more general role in ENS formation rather than specifying a specific ENS cell type fate (112, 113). These results clearly indicate that there are several regulatory pathways that function to maintain the cell fate potential in the ENPCs. However, it is less clear when these cells become further committed to a specific enteric neuron subtype or glial cell fate and how the switch from proliferating ENPC to committed neural or glial precursor is regulated.

Subtype Specification

Depending on the species, differentiation of ENPCs begins at different times during the migration process. In zebrafish, there appears to be two main waves of neuronal differentiation that occur at 72 and 96 hours post fertilization (hpf), respectively. This brings up an interesting problem as at 72 hpf the ENPCs have migrated along the length of the gut, but a subset continue to proliferate and another group needs to circumferentially migrate around the gut to completely populate it. This means that only a

specific subset of cells differentiate in this initial wave. While differentiation does not begin until after the anterior-posterior migration along the gut is completed in zebrafish, mouse ENPCs in the anterior portion of the gut begin to differentiate before ENPCs have migrated to the posterior end of the gut (114). In both fish and mice, differentiation of all neuronal and glial subtypes does not occur at the same time. This indicates that ENPCs must be temporally restricted in the cell types to which they can give rise.

While there are specific waves of differentiation seen in the ENPCs of model organisms, pan-neuronal markers appear much earlier in ENS development. These markers begin to appear as early as E10.5 in mice and in zebrafish between 24 and 48 hpf (115-117). These early cells are electrically active as early as E11.5 in mice, indicating that these cells have begun exhibiting both the molecular and physiological characteristics of neurons (118).

While certain pan-neuronal markers begin to show up early in ENS development the presence of specific markers for nitrergic (nNOS), serotonergic (5-HT), cholinergic (ChAT, VAChT), dopaminergic (DBH, TH) neurons appear at varying times during ENS formation (119-124). Similarly various other neuronal and pan-neuronal markers (IkCA, CGRP, calbindin, calretinin, VIP, substance P) appear at varying times in ENS development (Fig 1.2) (119, 121, 122, 124). The earliest expressed neuronal cell type specific marker in mice and zebrafish is nNOS, appearing around E11.5 in mice and between 48 and 72 hpf in zebrafish (119, 120). Calbindin and IkCA channels also appear at E11.5 in mice, but many other differentiation markers are absent (119). Substance P,

VIP, and 5HT neurons appear around E14 in mice while CGRP is not present until E17 (121, 122). Molecules involved in the synthesis of the cholinergic markers for ChAT and VAChT are present in mice between E10-12 but the ChAT and VAChT markers themselves do not appear until around E18.5 (123). Similarly in zebrafish expression of the markers for VIP, calbindin, CGRP, 5-HT and others do not appear until later in development between 72 and 96 hpf (120, 124).

While it is clear that the markers of these different subtypes begin to appear at different times, it is less clearly understood how a particular subtype is specified within the ENS. So far, only a few genes and signaling pathways have been shown to have a specific role in the specification of a specific ENS neuronal subtype. One of these genes, Ascl1, which appears to regulate 5-HT ENS neuronal differentiation, as this type of neuron is particularly affected in Ascl1-/- mice (107). However Ascl1 does not appear to be solely responsible for 5-HT ENS neuron development as not all Ascl1 expressing ENPC/ ENS neurons are 5-HT positive (125). Neutrophin-3 (NT-3) appears to affect submucosal intrinsic primary afferent neurons expressing CGRP because there are fewer of these neurons in TrkC -/- mice, which is the receptor for NT-3 (126). Hand2 -/- mice have a reduction in the number of nNOS, VIP, calretnin, ChAT enteric neurons but this might result from a smaller ENPC progenitor pool rather than any specific effect on ENPC cell fate determination (112, 113). Together these results indicate that it is a combination of extrinsic and intrinsic signals that lead to ENPC/ ENS neuron and glial cell fate determination and differentiation.

Further insights into ENS cell subtype specification can be gained from examining specification in other parts of the peripheral nervous system. Sympathetic neuron expression of DBH is influenced by a combination of factors including Phox2a and Hand2 (127-129). Similarly, Phox2a and Hand2 influence sympathetic neuron expression of TH, however TH neurons respond differently to protein kinase A (PKA) activity as compared to DBH neurons. In TH neurons, Phox2a and PKA act independently but in DBH neurons Phox2a and PKA synergistically (127, 130). Finally the cholinergic markers ChAT and VAChT in the parasympathetic system appear to be influenced by the expression of PKA as well as the transcription factor REST (119, 131).

Subtype specification in other neuronal systems is a complicated process involving the interaction of multiple transcription factors and signaling pathways. To fully understand the specific molecular combination of signals and factors that influence terminal cell fate specification in the ENS will require the generation of more conditional knockout and over-expression animal models. In addition, a better understanding of the precise lineage relationship between the different ENS neuronal and glial subtypes is still needed if we are to get a more complete understanding of this process. Recent technological advances such as the development of the brainbow lineage reporter system will potentially allow us to gain a much clearer understanding of the lineage relationship within the developing ENS (132, 133).

Perspectives

The enteric nervous system is a complex, dynamic circuit of neurons and glia that are necessary for normal, healthy digestion. Any errors in ENS formation can have drastic consequences for the development of an individual, as evidenced by the aganglionosis phenotype seen in Hirschprung's disease. Furthermore we have barely begun to understand the neuronal basis of many gastrointestinal disorders. The complex circuit that is the ENS is made up of multiple different subtypes of neurons, each of which are necessary for normal ENS function. Should the proper equilibrium of neuronal subtypes not form there is a risk the whole circuit will not work properly. While we can characterize the aganglionosis phenotype in HSCR comparatively easily, detecting GI disorders that affect only a specific subpopulation of ENS neurons and glia is far more challenging. Clinically however the specific loss/ reduction in number of a specific ENS neuronal subtype potentially could be the cause of many GI disorders that as of present have no known cellular basis.

While our understanding of the differentiation and development of the ENS has grown greatly over the past few years, there is still much that still needs to be determined. As we have pointed out in this introduction, while the appearance of subtypes has been well characterized, the actual molecular basis for how this specification process occurs both cellularly and mechanistically is not well understood. How early does enteric neuron and glia subtype specification begin? When are ENPCs committed to a specific neuronal subtype fate? Is it extrinsic or intrinsic factors that determine a specific subtype

specification? All of these questions and more will need to be answered in order to begin to gain a more complete understanding of the complex process of ENS formation.

In the following chapters we will begin to tease apart the answers to some of these questions. We will begin by gaining a better understanding of ENS development through the creation of a transgenic line, *Tg(-8.3bphox2b::kaede)*, which allows us to follow the development of ENPCs in new and exciting ways. We will then show how specification might begin as early as ENPCs migration to the gut and why it is vital that ENPCs are kept in a proliferative, undifferentiated state by two *ednrb* paralogues. The following chapter will examine differentiation of a specific subtype and how an intrinsic factor, *id2a* plays a vital role in the maintenance of the nNOS pool of ENPCs. The focus then shifts to how control of differentiation can influence the human disorder HSCR. Finally we will examine where our knowledge and understanding of these various ideas can go in the future.

Figure 1.1

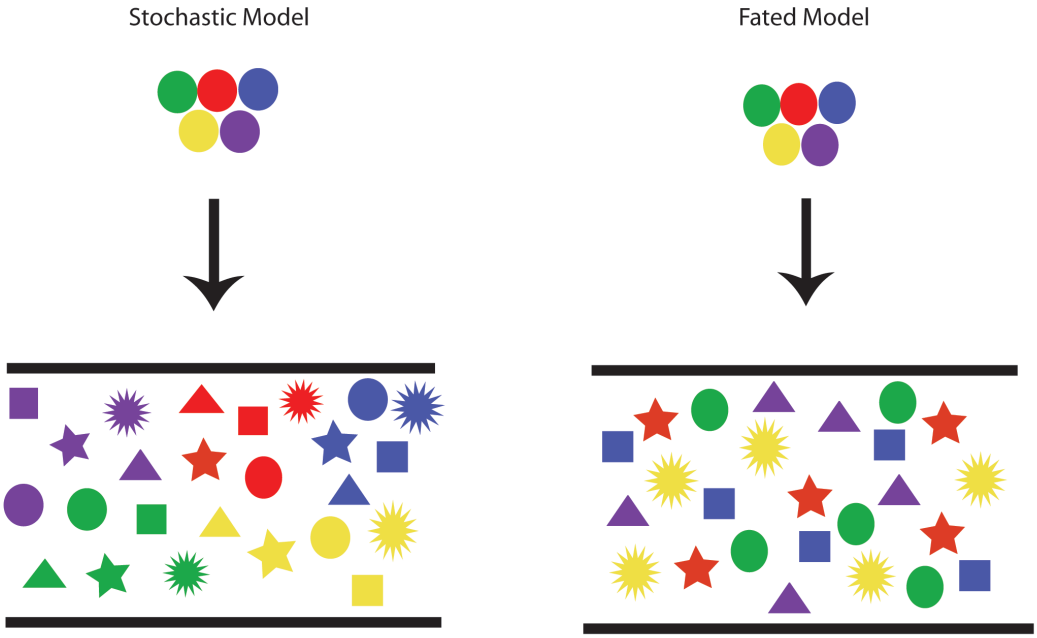


Figure 1.1 There are two models that could explain the differentiation of the ENPCs into the various subtypes in the gut. The first is a stochastic model in which the fate of the ENPCs is not specified until they reach the gut. In this model any kind of ENS cell subtype can come from any ENPC depending on where it ends up in the gut. In the fated model ENPCs are fated to become specific subtypes early in development and cells derived from one specific ENPC are all fated to become a specific subtype.

Figure 1.2

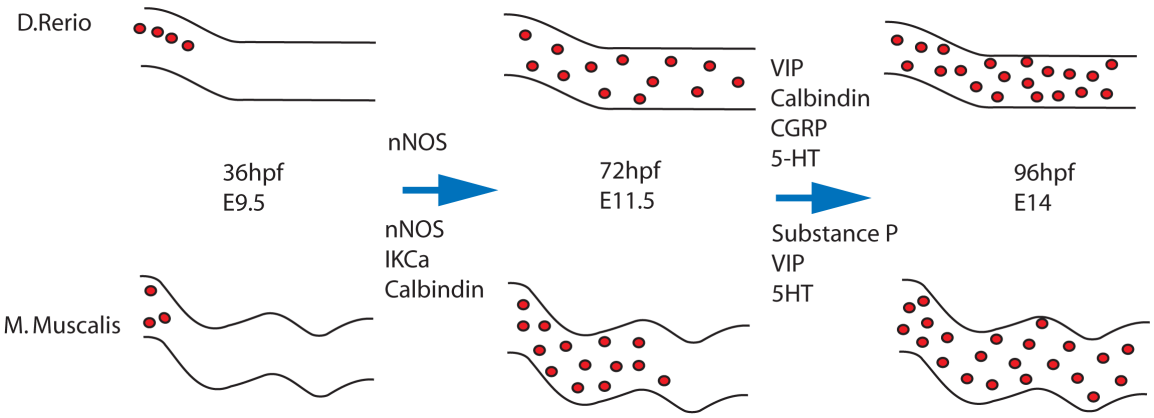

D.Rerio
36hpf
E9.5
nNOS
72hpf
E11.5
VIP
Calbindin
CGRP
5-HT
96hpf
E14
M. Muscalis
nNOS
IKCa
Calbindin
Substance P
VIP
5HT

Figure 1.2 Schematic diagram of ENPCs populating the guts of *D. rerio* and *M. musculis*. ENPCs enter the gut at 36 hpf and E9.5 in mouse and zebrafish respectively. nNOS appears in zebrafish between 48-72 hpf and around E11.5 in mice. IKCa and calbindin also appear in mice around E11.5. at 72 hpf ENPCs have populated the posterior of the gut in zebrafish and a first wave of differentiation has occurred while ENPCs are still migrating to the posterior in mice. A 96 hpf the second differentiation wave has occurred in zebrafish while at E14 ENPCs have populated the posterior gut in mice. Between 72 and 96 hpf VIP, calbindin, CGRP, and 5HT appear in zebrafish. Substance P, VIP, and 5HT appear at E14 in mice while CGRP doesn't appear until much later at E17. ChaT and VAChT also do not appear until later in mouse development at E18.5

Chapter 2: Functional Analysis of ENS Development

(Adapted from Harrison C, Wabbersen T, Shepherd IT. In vivo visualization of the development of the enteric nervous system using a *Tg(-8.3bphox2b:Kaede)* transgenic zebrafish. Genesis. 2014 Dec;52(12):985-90.)

Introduction

As outlined in the previous chapter, ENS development is a complex process. Normal function of the ENS is essential to maintain proper function of the digestive system (134). The development of a fully functional ENS requires enteric neuronal crest cells (ENPCs) to migrate to and along the gut while proliferating appropriately. Any errors in this developmental process can lead to aganglionosis of the gut, a condition commonly seen in the human clinical condition Hirschprung's disease (135, 136).

While the general course of ENS development in many vertebrate species is known, better tools are needed to help understand all the intricacies of the process (135, 137, 138). Zebrafish are an ideal model system to develop these tools, given the ease with which one can image live embryos *in vivo*. Zebrafish embryos are externally fertilized, are transparent, and have a relatively fast generation time. Genetic tools are freely available that make it possible to generate transgenic animals in a time efficient and cost effective manner.

While green fluorescent protein is an excellent tool for visualizing developmental processes, alternative fluorescent proteins have shown great potential in unlocking new ways to observe biological phenomenon. One such protein, the Kaede protein, has the ability to shift its emission spectra when exposed to UV light (139, 140). Normally this protein fluoresces green, but when exposed to UV light the protein shifts to expressing red fluorescence (139, 140). We have utilized this property of the Kaede protein, along

with the live imaging benefits of the zebrafish, to develop a transgenic line to visualize development of the ENS *in vivo*.

Results

To create this transgenic line we utilized the Tol2 transgenic system (Kawakami et al. 141, 142, 143). In this system, plasmids are generated with two Tol2 transposition sites flanking the target sequence of DNA to be integrated. When this Tol2 construct is injected into one-cell embryos along with the TOL2 transposase mRNA, the target sequence gets efficiently integrated into the host's genome. To express the Kaede protein in the ENPCs we utilized a zebrafish ENPC specific enhancer. The transcription factor Phox2b has previously been shown to be an excellent marker for ENPCs in zebrafish and other vertebrate species (87, 88). Subsequent studies have shown that specific regions of the zebrafish *phox2b* enhancer are sufficient to drive GFP expression in ENPCs (144, 145). Utilizing the -8.3b*phox2b* enhancer element we generated a plasmid containing the Kaede protein under control of this enhancer flanked by Tol2 sites (Fig 2.1). Following injection into one-cell embryos, potential transgenics were isolated and raised to adult-hood. These adults were then outcrossed with wild type adults and their progeny were re-screened for Kaede expression. One male was identified and was further outcrossed to establish the line. These transgenic embryos show stable expression of the Kaede protein in the neural crest and the ENPCs with the ability to shift from red to green fluorescence after exposure to UV light (Fig 2.2).

To demonstrate the utility of this line in studies of ENS development we carried out live imaging experiments with the transgenic embryos. We had previously identified a zebrafish ENS mutant in the lab, named *lessen,* that has a significantly reduced number of enteric neurons (146). This phenotype is caused by a null mutation in the *med24* gene, a component of the mediator co-transcriptional activation complex (146). Subsequent experiments showed that *med24* translation blocking morpholino produced a similar ENS phenotype to that seen in *lessen* mutants (146). The ENS phenotype in the *lessen* mutant has been shown to be due to a decreased proliferation rate in the ENPCs (146). Utilizing the *Tg(-8.3bphox2b*:*Kaede*) transgenic line we were able to look more closely at the *med24* knockdown ENPC cellular phenotype in live imaged *med24* morphant embryos.

We examined *Tg(-8.3bphox2b*:*Kaede*) expression in uninjected control embryos and embryos that had been injected with the *med24* morpholino. We compared both the migration and division rates between the two sets of embryos during the period ENPCs migrate along the gut (36-60 hours post fertilization (hpf)) (88, 147). We analyzed, in detail, a specific time period during this migration, comparing control and *med24* morphant embryos from 50-56 hpf (Fig 2.3). From simple observation it is apparent that already at 50 hpf ENPC migration in *med24* morpholino injected embryos lags behind control embryo ENPC migration and the rate of this lag continues to increase over this time period. *med24* morphant embryos show a 37.3% (+/- 6.7%) reduction in migration rate as compared to age-matched controls (Fig 2.4).

From observation of our control and *med24* morphant embryos, we also see that there appears to be a decrease in division rate in the *med24* morphants and that more division occurs at the leading edge than elsewhere in the migrating chain (Fig 2.4). In photoconverted ENPCs, each cellular division decreases the amount of red photoconverted Kaede that is present in the cells. As these cells are dividing they are also continuing to make new green Kaede due to the continued expression of the *phox2b* enhancer. Cells that are dividing rapidly will have higher levels of green fluorescence and lower levels of red fluorescence than cells that are dividing more slowly. To quantitate these data, we analyzed the levels of green and red fluorescence in the ENPCs over time. If the -*8.3bphox2b* enhancer is equally active in control and *med24* morphants we would expect to see ENPCs in both strains to gain green fluorescence back at the same rate after photoconversion. However, control embryos gain green fluorescence back faster than the *med24* morphants (Fig 2.5A). Some of this difference in the rate of change in color of the Kaede fluorescence could be due to higher -*8.3bphox2b* activity in our control embryos than compared to morphants. Alternatively, if the level of enhancer activity is the same in morphants and control embryos and the rate of ENPC cell division is less in *med24* embryos we would expect to see the amount of red fluorescence in ENPCs to decrease at a slower rate than in control embryos. We do indeed see a significant decrease in the rate of red fluorescence loss in our *med24* morphants (Fig 2.5B). This result is further validated when the number of divisions per cell is calculated, as we see a drop in this rate of ENPC cell division in our *med24* morphants (Fig 2.5C). This cell division count also confirms that division rates in the first two cells of the migrating chain are higher than the general rate for all ENPCs (Fig 2.5C). This apparent proliferative migration driving the

migration along the gut is consistent with our previous results and with experiments and observations in other species, as well as mathematical modeling of this developmental process (73, 74).

Discussion

In summary the *Tg(-8.3bphox2b*:*Kaede*) transgenic line provides a powerful tool for studying ENS development. The ability to observe ENS development *in vivo* as well as selectively label specific cells will allow for a wide range of applications. In addition to what we have highlighted here, there are other potential uses for this transgenic line (Sato et al. 148, Pan et al. 149). One such use is the ability to label individual ENPCs and perform lineage analyses to determine if specific ENPCs give rise to specific ENS neuronal lineages at different time points during ENS development.

Methods

Construct Generation

The -8.3b*phox2b* sequence was PCR amplified from genomic DNA and subcloned into the MCS of pBtol_*cfos*EGFP, a modified PBluescript vector containing the human *cfos* promoter and tol2 transposition sites, using EcoR1. The Kaede sequence was PCR amplified from a PCS2 Kaede containing plasmid and subcloned into pGW_*cfos*EGFP using BamH1 and Cla1 sites to attach SV40 poly A (150). Kaede SV40 was then subcloned into pBtol_*cfos*EGFP-8.3b*phox2b* by excising the EGFP in pBtol_*cfos*EGFP with BamH1 and Xba1.

Tg(-8.3bphox2b:*Kaede*) *Construct Injections*

Wild type embryos were collected and injected at the one cell embryo stage. We established the line by injecting 400 embryos. The injection solution was prepared with 25 ng/µl of *Tg(-8.3bphox2b*:*Kaede*) transposon construct, 35 ng/µl of transposase RNA, phenol red for visualization, and RNase-free water and kept on ice (151). One nl of solution was injected into one cell embryos and embryos were raised at 28.5° C.

Transgenic Screening and Raising

Injected *Tg(-8.3bphox2b*:*Kaede*) embryos were screened for integration of the targeting construct by looking for Kaede fluorescence protein expression at 48 hpf. Twenty positive embryos were identified, raised to adulthood and outcrossed with wild type adults in the hope of generating germ line transmission of the transgene. Positive embryos showed tissue specific expression consistent with wild type Phox2b expression as seen in other studies (144, 145). One adult male with germ line transgenic integration was identified and isolated. This transgenic male was outcrossed to establish the line. The pattern of fluorescence seen in positive embryos has remained unchanged over four generations.

Med24 Morpholino Injections

Tg(-8.3bphox2b:*Kaede*) transgenic adults were outcrossed with wild type adults and their embryos were injected at the one cell stage. One-cell embryos were injected with 1 ng of a *med24* morpholino at a concentration of 5 ng/µl. The sequence for the translation blocking morpholino was +1/+25, CCTGTTTCAGATTCACCACCTTCAT (146).

Stop Motion Imaging

Embryos were raised to the desired age at 28.5° C. *med24* morphant and control embryos were then embedded in 0.6% agar (Ringer's solution) in 35 x 10 mm filming dishes (Falcon) and covered with embryo media and mesab. Embryos were then exposed to UV light (358 nm) for ten minutes to convert the Kaede protein from fluorescing green to red. Z-stacked images were captured in Slidebook™4.2 every fifteen minutes for 6-10 hours (Olympus IX81). Subsequent images and movies were analyzed using NIH image J software (1.46r) (152). To calculate the migration rate, 24 control embryos and 20 *med24* morphant embryos were analyzed. Migration distances were obtained using the particle tracker plug-in for Image J to follow the path of the leading edge cell of the migrating ENPC chain (153). To calculate florescence and division rates, 64 starting ENPCs were analyzed. Fluorescence was measured by calculating fluorescence intensity of the individual cell over the cell area. The particle tracker plug in was again used to follow the paths of ENPCs and new appearances of cells from previous cells to calculate division rate (153). P-values were calculated with a t-test for division rate and within subjects ANOVA for all other cases.

Figure 2.1

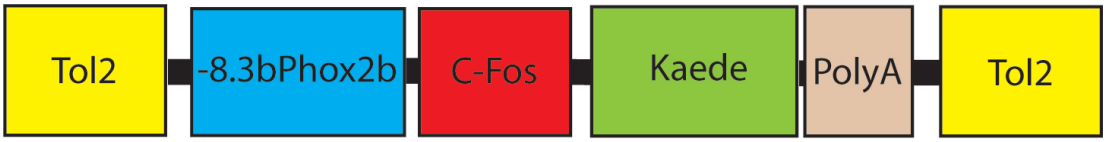


Figure 2.1 Orientation of the *Tg(-8.3bphox2b:Kaede)*. The -8.3bphox2b enhancer controls expression of the Kaede protein. The flanking tol2 sites allow for integration of the construct into the genome when in the presence of the transposase.

Figure 2.2

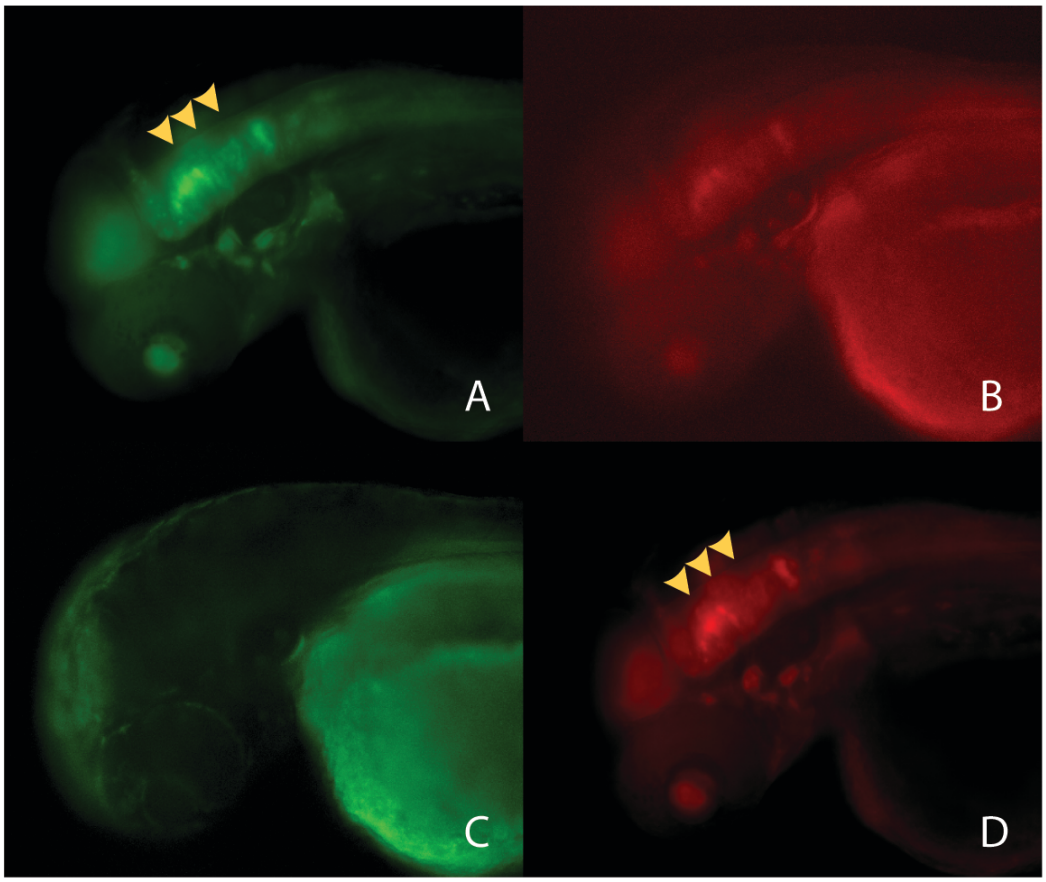


Figure 2.2 After exposure to UV light, green fluorescence converts to red fluorescence. A) *Tg(-8.3bphox2b*:*Kaede*) embryo expressing green fluorescence pre-conversion, arrowheads indicate expression in the hindbrain. B) *Tg(-8.3bphox2b*:*Kaede*) embryo expressing red fluorescence pre-conversion, there is a small amount of converted Kaede protein. C) *Tg(-8.3bphox2b*:*Kaede*) embryo expressing green fluorescence post-conversion, there is no green fluorescence in the neural crest after UV exposure D) *Tg(-8.3bphox2b*:*Kaede*) embryo expressing red fluorescence post conversion, arrowheads indicate significant red fluorescence expression after UV exposure.

Figure 2.3

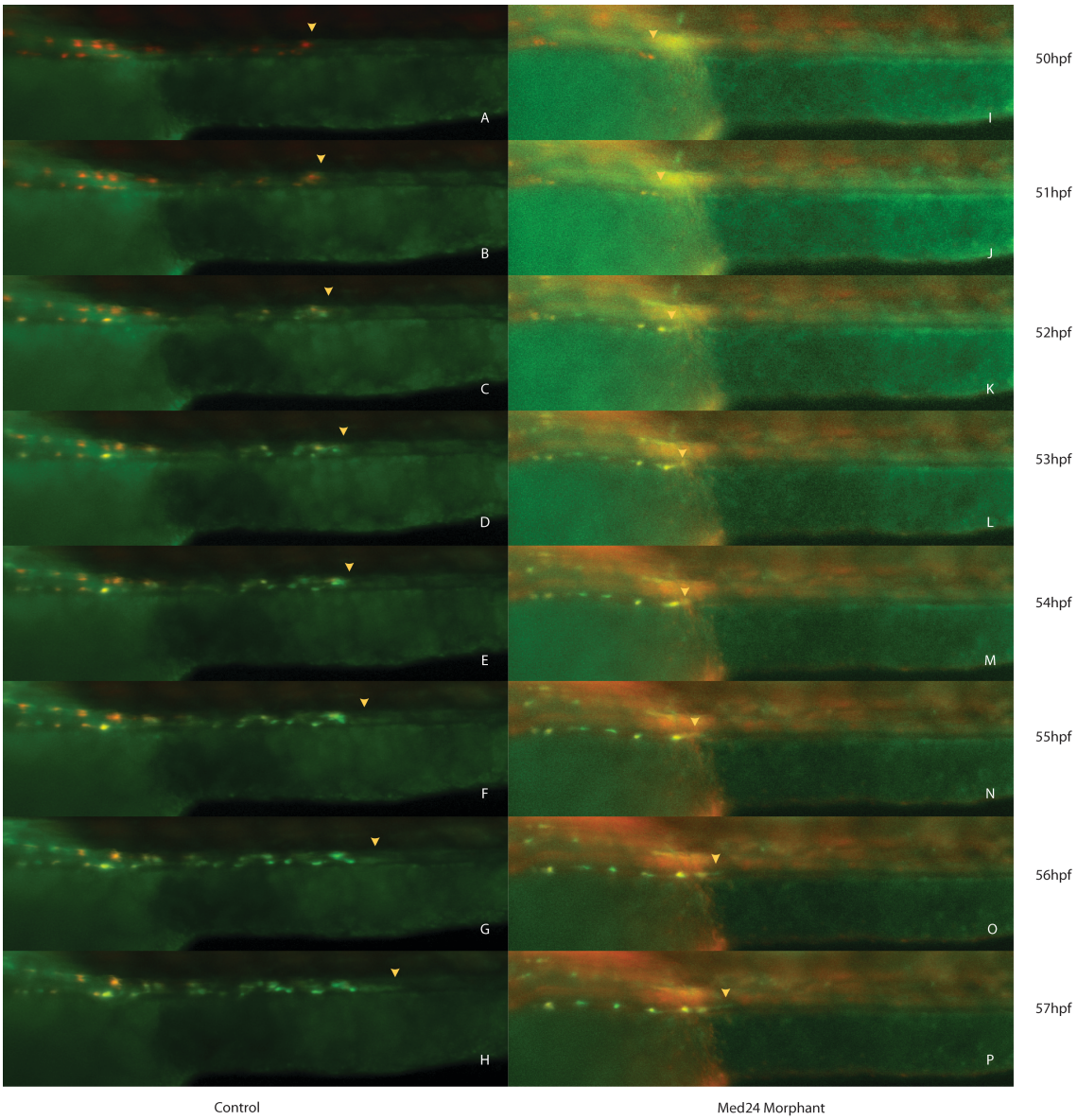

A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
50hpf
51hpf
52hpf
53hpf
54hpf
55hpf
56hpf
57hpf
Control
Med24 Morphant

Figure 2.3 Control and *med24* injected *Tg(-8.3bphox2b*:*Kaede*) transgenic embryos over the time period of 50-57 hpf. Images A-H from control embryos while J-P are from *med24* morpholino injected embryos. Each image is a stop motion image of the embryo on the hour. The left side of the each photo is the anterior portion of the gut and the yolk sac is at the bottom left of each image. The arrowheads indicate the leading cell in the migrating chain. The *med24* embryos are initially lagging in the migration of the ENPCs and continue to lag further over the course of the time period. ENPCs show green fluorescence more rapidly in control embryos over *med24* embryos.

Figure 2.4

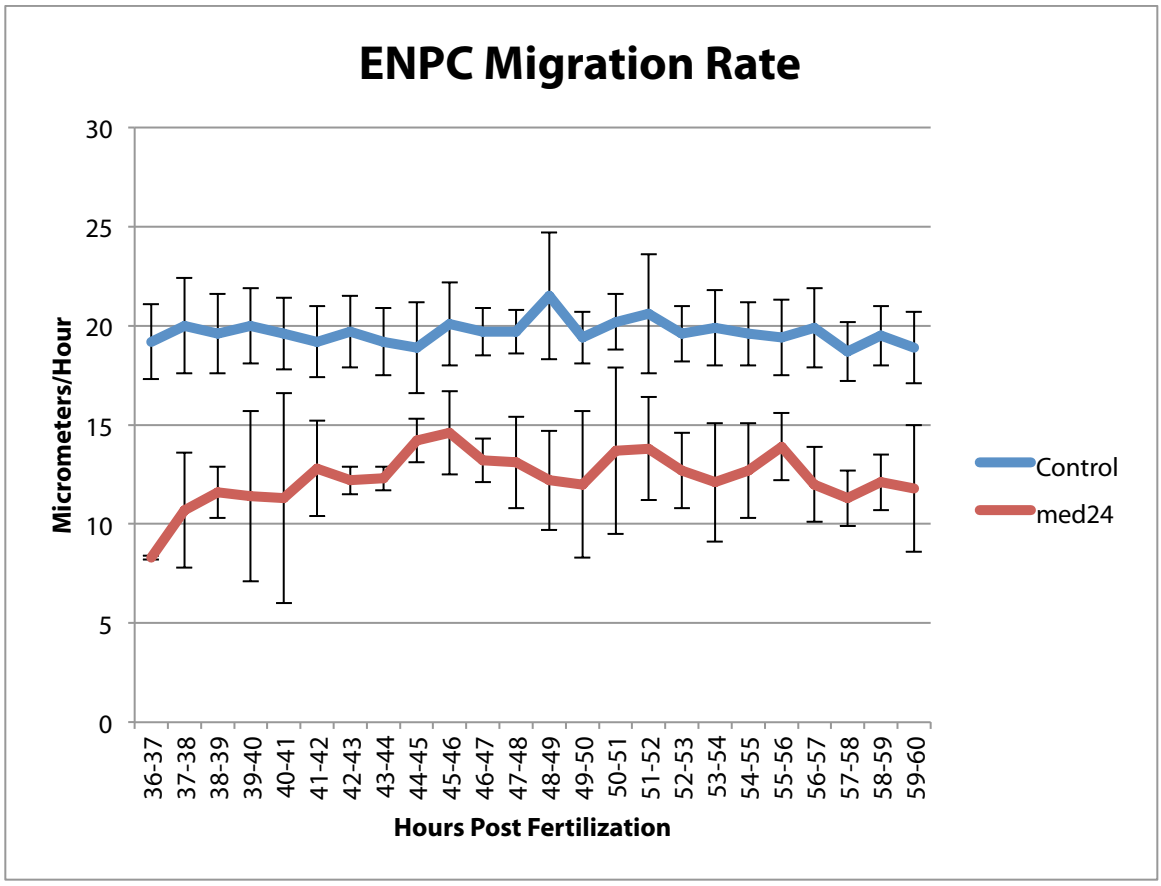
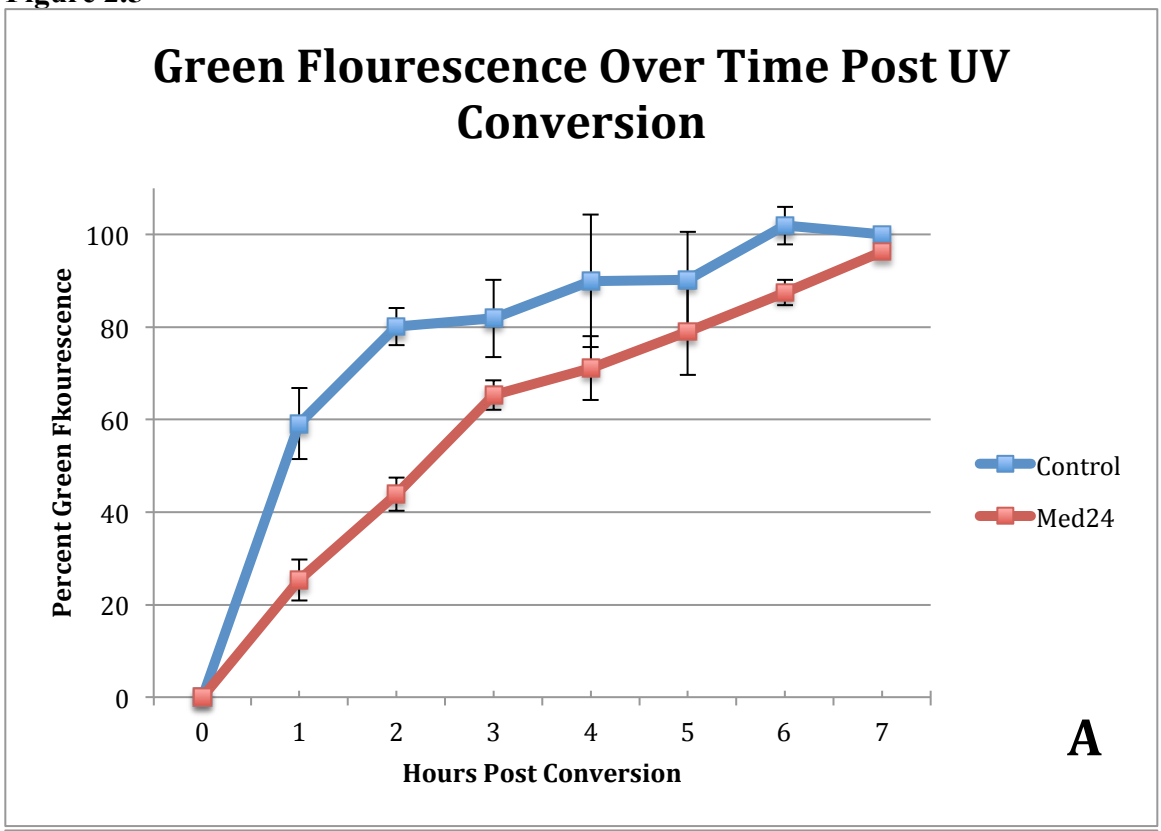
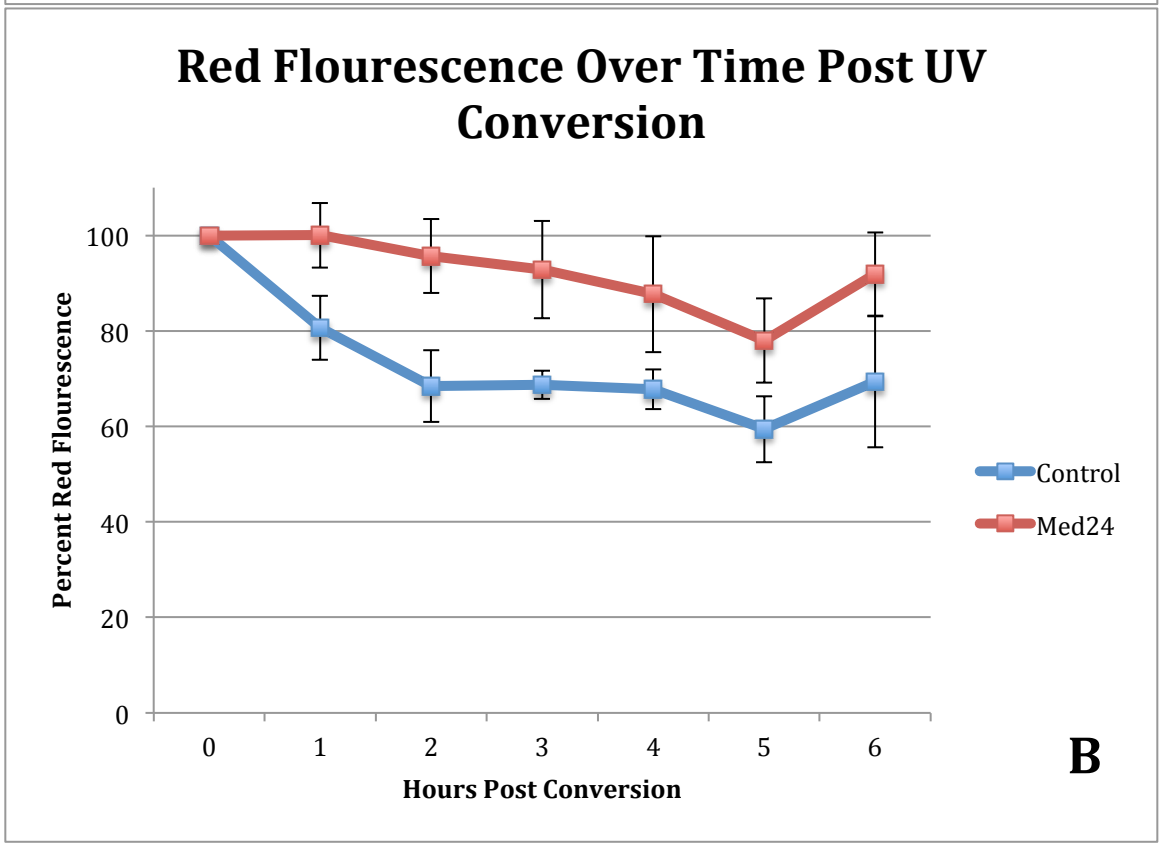

ENPC Migration Rate
Micrometers/Hour
30
25
20
15
10
5
0
36-37
37-38
38-39
39-40
40-41
41-42
42-43
43-44
44-45
45-46
46-47
47-48
48-49
49-50
50-51
51-52
52-53
53-54
54-55
55-56
56-57
57-58
58-59
59-60
Hours Post Fertilization
Control
med24

Figure 2.4 Migration rate of the enteric neuron crest cell chain for control and *med24* morpholino injected *Tg(-8.3bphox2b*:*Kaede*) embryos. The migration rate for *med24* injected embryos is significantly less than that of the control embryos. Error bars are one standard deviation. ($p<.0001$)

Figure 2.5


Green Flourescence Over Time Post UV Conversion
Percent Green Fkourescence
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Hours Post Conversion
Control
Med24
A


Red Flourescence Over Time Post UV Conversion
Percent Red Flourescence
100
80
60
40
20
0
0
1
2
3
4
5
6
Hours Post Conversion
Control
Med24
B

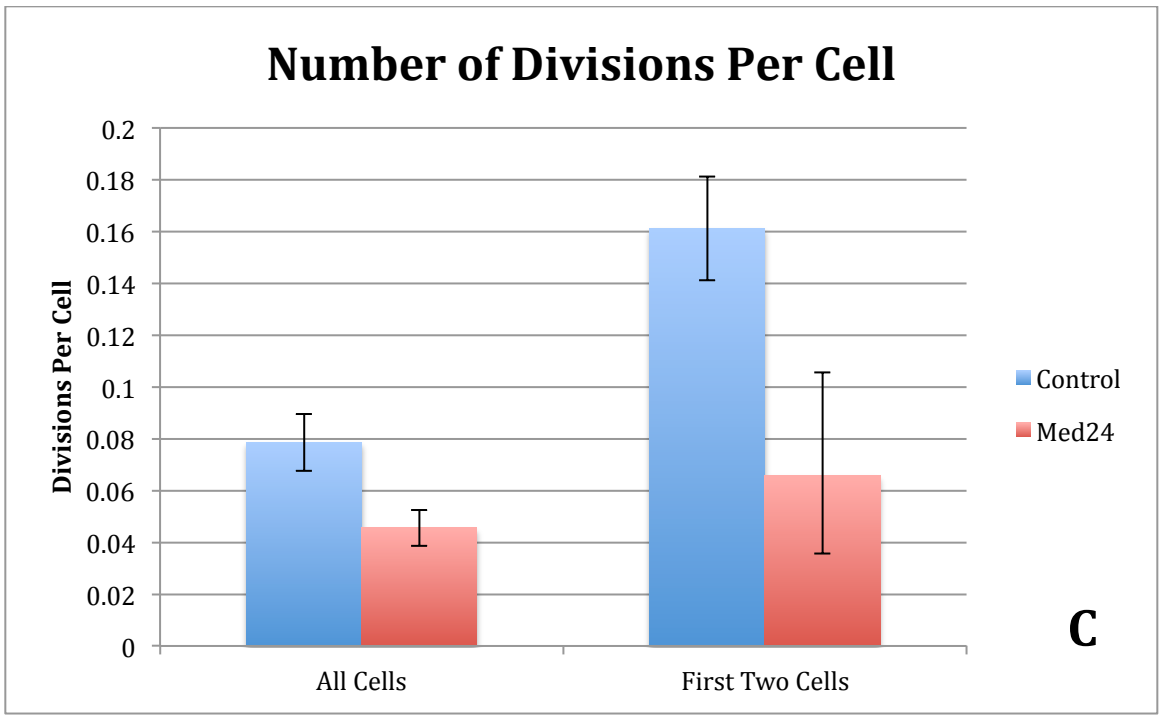


Figure 2.5 Change in green and red fluorescence over time and cell division rate. A. Shows amount of green fluorescence relative to final levels of green fluorescence in control embryos. Control embryos see a faster increase in green fluorescence levels than in *med24* embryos indicating a higher level of *-8.3bphox2b* enhancer activity (p=.002). B. Shows amount of red fluorescence relative to initial red fluorescence levels. Control embryos see a faster decrease in red fluorescence indicating a higher level of ENPC division (p=.001) . C. Shows number of divisions per cell during ENPC migration. Division rate is significantly higher in control embryos as opposed to *med24* embryos (p=.03, p=.04). The division rate is also higher in the first two cells of the migrating chain in control embryos but is not significantly higher in *med24* embryos (p=.01, p=.23).

Chapter 3: Differentiation Control of ENPCs and Other Neural Crest Derived Populations by EDNRB

(Adapted from Harrison C, S Wang, IT Shepherd. EDNRB1a and EDNRB1b Have Overlapping Function In Enteric Nervous System Development. Submitted.

Introduction

To begin to understand how differentiation is controlled during ENS development, we characterized the role of, *endothelin receptor B* (*EDNRB*), mutations in which can cause HSCR. *EDNRB* normally functions to maintain ENPCs in an undifferentiated state while they are migrating along the gut (154, 155). As previously mentioned, only around 50% of the defective genes in familial cases and 20% of the defective genes in sporadic cases have been identified in HSCR patients (8-10). Previous studies in zebrafish have shown that nearly all known HSCR disease genes have an evolutionarily conserved function in ENS development and gene expression knockdown of the zebrafish orthologue results in intestinal aganglionosis (156-158). Knockdown of the zebrafish EDNRB gene did not have an ENS phenotype, which is unusual, and had been unexplained (159). Here we describe our studies that identified a second zebrafish EDNRB orthologue and show that knockdown of both orthologues causes aganglionosis in zebrafish.

Endothelin receptor B (Ednrb) is important in ENS development and mutations in this gene can cause HSCR. *EDNRB* was identified as a candidate gene potentially having a role in HSCR when a conserved point mutation was identified in a specific HSCR pedigree (160). Since then a number of other mutations in *EDNRB* have been identified in HSCR patients, showing its importance in proper ENS development (161-165). EDNRB is a member of endothelin receptor family of proteins that are G protein-coupled heptahelical receptors with seven transmembrane domains (166). EDNRB signals through interaction with its ligand endothelin-3 (ET-3). ET-3 is a member of the

endothelin family, a group of proteins that were initially identified as vasoconstricting peptides (167).

The function of EDNRB in ENS development is conserved in mice. Mice with mutations in EDNRB and ET-3 show defects in ENS development as well as defects in other neural crest derived tissues. A spontaneous mouse mutant, the *piebald* mutant mouse, arose due to a deletion of the *Ednrb* gene. *piebald* mice have the megacolon phenotype associated with HSCR, along with defects in coat color due to melanocyte defects (another neural crest derived cell type) (168). *In vivo* studies of *Ednrb* mutants have shown that the initial migration of ENPCs from the neural crest to the gut is normal, however the migration along the gut is delayed (169, 170). One possible cause of this migration delay is that EDNRB is required to keep ENPCs in an undifferentiated proliferative state (154, 155).

In zebrafish an *ednrb* mutant called *rose* has abnormal pigmentation caused by defects in melanocyte development (159, 171). These defects are caused by a mutation in the zebrafish orthologue of *EDNRB*, *ednrb1a* (159). While the *rose* mutants have pigmentation defects similar to those seen in EDNRB mutants in other species they do not have the corresponding ENS aganglionic phenotype (159). The reason for this lack of an ENS phenotype in zebrafish is unclear. In this paper, we identify a second *ednrb* orthologue, *ednrb1b*, in zebrafish and show that the presence of two divergent *ednrb* paralogues is due to the genome duplication that occurred in a common teleost ancestor (172). We describe the expression pattern of these two orthologues through 120 hpf and

demonstrate that both paralogues are expressed in the developing zebrafish gut and have functional overlap during both ENS and melanocyte development.

Results

ednrb1a and ednrb1b Are Evolutionarily Conserved

A search of the Zv9 release of the Zebrafish genome revealed a second, previously uncharacterized *ednrb* orthologue. We hypothesized that this gene, *ednrb1b,* was a paralogue of the previously identified *ednrb1* that is defective in the rose mutant, which lacks an ENS phenotype. Functional redundancy between these two paralogues perhaps ensured sufficient gene function in the gut of *rose* mutants. Sequence comparison of the paralogues showed that both have a similar genomic structure of seven exons and 65.5% DNA sequence conservation (Fig 3.1). These two zebrafish paralogues are also similar to their orthologues in mice and humans; the order and number of *EDNRB* exons is the same in these mammals and zebrafish. In addition, there is 68-71% similarity within these exons (Fig 3.1) and the protein transmembrane domain structure is conserved among species (Fig 3.2). Furthermore, syntenic shows that several of the genes in the vicinity of the mammalian *EDNRB* genes are also found near the zebrafish orthologues (Fig 3.3). Further genomic analysis of several other fish species revealed that they also have two copies of the *ednrb* gene. Phylogenetic analysis revealed that these copies were much more similar within the teleost lineage than they were to their mammalian counterparts (Fig 3.4).

As our interest is EDNRB function in zebrafish neural crest derivatives during development, we focused on determining *ednrb* paralogue expression in both pigments cells and the zebrafish intestine. The expression pattern seen for *ednrb1a* is consistent with the pattern seen in previous studies (159, 173). We see expression of *ednrb1a* in pigment cells from 24 through 96 hpf (Fig 5A-D). In addition we see expression of *ednrb1a* in the pectoral fin at 48 hpf as well as low levels of expression in the hindbrain at all observed times (Fig 3.5A-D). *ednrb1b* expression, while similar to *ednrb1a*, has some differences in its expression pattern. *ednrb1b* shows stronger expression in the hindbrain throughout the observed times (Fig. 3.5E-H) and is expressed in the pectoral fin but at 72 hpf. Unlike *ednrb1a*, *ednrb1b* only shows expression in pigment cells at 24 and 48 hpf (Fig. 3.5E-F) in addition both *ednrb* genes are expressed in the neural crest region of the hindbrain from where melanocytes migrate (Fig. 3.5). Importantly, *ednrb1a* and *ednrb1b* are both expressed in the gut during the time when the zebrafish ENS is developing. At 48 hpf, both paralogues are expressed in the esophagus and anterior portion of the gut tube (Fig. 3.5a, 3.5d). At 72 hpf, after the completion of enteric neuron precursor (ENPC) migration along the gut, both paralogues are expressed throughout the gut (Fig. 3.5b, 3.5e). At 96 hpf there is continued expression in the gut, but the amount of expression has decreased especially for the *ednrb1b* paralogue (Fig. 3.5c, 3.5f).

Functional Analysis of EDNRB1a and EDNRB1b in Zebrafish Development

We determined the effect of knocking down expression of the two zebrafish *ednrb* paralogues, both individually and concurrently, by injecting morpholinos against each gene. Morphologically *ednrb1b* morphants were fairly normal while *ednrb1a* morphants

had a slightly curved body axis. Double morphants, in addition to the ENS and melanocyte defects to be discussed later had curved tails and body axis as well as enlarged hearts. Mismatched control morpholinos were used to control for off target effects and showed no abnormal phenotype. Our functional analysis of these morphants focused on ENS and melanocyte development. In *ednrb1a* morphants, we saw melanocyte defects in which there is significantly less pigmentation than that seen in wild type embryos of the same age (Fig. 3.6A-D). In contrast, *ednrb1b* morphants embryos resembled their wild type counterparts (Fig. 3.6E-F). However when we looked at *ednrb1a* and *1b* double morphants, the melanocyte defects seen in the *ednrb1a* morphants were enhanced (Fig. 3.6G-H).

We next looked at expression of the neuronal marker HuC/Elavl3 at 120 hpf in morphants embryos to analyze whether or not there were any issues with ENS development. Neither *ednrb1a* nor *ednrb1b* single morphants showed any defects in neuron numbers or distribution. *ednrb1a* and *1b* double morphants however did show a significant reduction in the number of enteric neurons leading to aganglionosis at the distal end of the gut when compared to wild type embryos (Fig. 3.7) The total % reduction was 69.5% (+/- 9%) when compared to wild type controls ($p<.001$).

In *Ednrb* mutant mice defects in ENPC migration have been shown to be responsible for the aganglionosis phenotype seen in that species. We wanted to determine if migration was similarly perturbed in our zebrafish *ednrb* double morphants (169, 170). We first examined the expression of the neural crest marker *crestin, which* is expressed by ENPCs

as they migrate to the gut from the vagal premigratory neural crest (174). There was no difference in the expression of *crestin* between wild type and double morphant embryos (Fig. 3.8). We then examined expression of the ENPC marker *phox2b* (175, 176). Double morphant embryos had a significant delay in the migration of ENPCs along the gut and at 72 hpf ENPCs failed to colonize the distal end of the gut (Fig 3.9). The total % reduction was 43.5% (+/- 13.4%) as compared to controls ($p<.001$).

Discussion

In this study we have identified a second *ednrb* orthologue in zebrafish, *ednrb1b*. Furthermore, we have shown that there is overlapping function between these two *ednrb* paralogues in zebrafish with both *ednrb1a* and *ednrb1b* being required for normal ENS and melanocyte development. This study shows that Ednrb function is vital for zebrafish ENS development just as it is in mammals, though the presence of a second functional orthologue adds a layer of complexity to the analysis of Ednrb function in zebrafish.

Through our bioinformatics analysis we were able to identify *ednrb1b* as a clear paralogue of *ednrb1a*. Both paralogues have 7 exons in identical order and a moderately high level of sequence conservation. The similar genomic organization of the two paralogues suggests that they arose as a result of the teleost genome duplication event (172). Both genes share a high level of conservation with their mammalian counterparts both in sequence and the organization of their exons. This conservation carried over to the protein structure as the transmembrane domains that are characteristic of EDNRB genes were highly conserved among zebrafish and mammals. This sequence and protein

conservation suggests that the Ednrb genes in zebrafish may have a similar functional role during embryonic development as their mammalian orthologues.

Not only was the organization of the *ednrb* loci similar within the genes themselves but syntenic analysis also showed a high level of similarity in the genes surrounding the *ednrb* genes in zebrafish with those seen in other species. While the order of these genes is varied due a potential genome rearrangement after the teleost duplication event, the conserved nature of genes in this region serves to further emphasize the similarities for *EDNRB* among species. When comparing the genes surrounding the two zebrafish paralogues, it is apparent that *ednrb1b* has more in common with its mammalian counterparts than *ednrb1a*, suggesting that despite its later discovery that *ednrb1b* may be the ancestral copy of the gene. Phylogenetic analysis lends further support to the idea that *ednrb1b* is the ancestral copy of the gene as it is more closely related to the *EDNRB* genes in other species. This analysis also highlighted the existence of two copies of the *ednrb* gene in other fish species due to the teleost genome duplication event.

Previous studies have shown that an *ednrb1a* mutant (*rose*) has melanocyte defects but no ENS defects (159). Through *in situ* hybridization, we were able to confirm the expression of both *ednrb* paralogues in the zebrafish developing gut during the period of ENS development. *ednrb* expression begins anteriorly when the ENPCs are in that section of the gut and expands as the ENPCs continue their migration along the gut. Once the ENPCs reach the posterior end of the gut tube *ednrb* expression becomes more restricted as the precursors continue to mature and differentiate into neurons. The lack of

ednrb1b expression later in pigment cell development along with Ednrb1a's function in these cells may explain the lack of an observed melanocyte defect in *ednrb1b* morphants. The increase in melanocyte defects seen in double mutants may be explained by Ednrb1b being able to co-opt some of the function of Ednrb1a early in melanocyte development in the neural crest. However once these cells leave the neural crest and *ednrb1b* is no longer expressed, the lack of *ednrb1a* causes the melanocyte defects seen in *rose* mutants. It would be interesting to see whether forced expression of *ednrb1b* in melanocytes later in development could rescue the pigmentation defects seen in *ednrb1a* morphants. The expression of both transcripts in neural crest and neural crest derived cells is consistent with the expression pattern seen in other species, further indicating the role of *ednrb* is consistent in zebrafish with the role in other species (170, 177)

We utilized morpholinos antisense oligonucleotides for *ednrb1a* and *ednrb1b* to investigate their functional role in zebrafish development. Examination of melanocyte development in *ednrb* morphants revealed that there were pigmentation defects in *ednrb1a* morphants similar to those that had been previously described in ednrb1a *rose* mutants (159, 171). There was no observable difference in melanocyte development in *ednrb1b* morphants. However by visual assessment, double morphants had a more severe pigmentation phenotype than those seen in *ednrb1a* single morphants. This indicates that while Ednrb1a has a critical role in zebrafish melanocyte development, Ednrb1b may have a limited role in normal melanocyte development though not as critical as that of Ednrb1a.

Our results using HuC/ Elavl3 antibody examining the effects of *ednrb* paralogue knock-down on the development of mature enteric neurons in zebrafish were also in line with those observed in *rose* mutants. We saw no reduction in enteric neuron number in *ednrb1a* morphants. We also saw a similar result in our *ednrb1b* morphants. However in *ednrb1a* and *1b* double morphants there was a significant reduction in the number of enteric neurons in the distal part of the gut.

To determine the point during ENS development that knockdown of *ednrb* paralogues led to aganglionosis we examined the migration of the ENS precursors ~~too and~~ along the gut. We observed that this ENS phenotype in the double morphants is due to a failure of the ENPCs to migrate the full length of the developing gut tube. While there is no initial delay in migration of ENS neural crest precursors to the gut, as shown by *crestin in situ* hybridization, migration through the gut, as seen in *Tg(-8.3bphox2b:Kaede)* transgenic zebrafish, is significantly delayed. This is consistent with studies in mice where EDNRB function has been shown to be important for promoting survival and development of ENPCs in the developing gut (154, 155). In addition the *Tg(-8.3bphox2b:Kaede)* confirmed that the ENPCs are spaced further apart in morphants. The surviving ENPCs might have low proliferative potential that resulted in a greater space between differentiating neurons. This might lead to the failure of ENPCs to populate the entire length of the gut, as there are insufficient numbers of precursors. While the results in our morphants were clear and the phenotype we see are similar to those found in *rose* mutants and *EDNRB* mutants in other species, it will be important in future studies to

introduce a true *ednrb1b* mutant into the *rose* background to better analyze the contributions of each *ednrb* gene to ENS and melanocyte development.

In summary we have identified a second *ednrb* orthologue in zebrafish Ednrb1b. The mRNA expression pattern of these two paralogues is similar with an expression pattern consistent with their presumed roles in specific neural crest derivative development. Ednrb1b appears to be functionally redundant to the previously identified zebrafish Ednrb1a orthologue with respect to the role of EDNRB in ENS development but is only partially redundant in zebrafish melanocyte development. These results further support our view that the zebrafish is an excellent model to study vertebrate ENS development and Hirschprung's Disease given the conserved functional role of the of all known HSCR genes in zebrafish ENS development so far examined.

Methods

Bioinformatics Analysis

A BLAST search was performed first to identify the *ednrb* orthologues in zebrafish. FASTA software was then used to analyze the percent of identity between zebrafish EDNRB genes and those of mouse and human. Genome structure analysis was completed using GenBank and the Zebrafish Genome browser. The CLUSTALW program was used to align amino acid sequences from the zebrafish, mouse and human orthologues. Drawtree and Drawprogram were used to conduct a phylogenetic analysis.

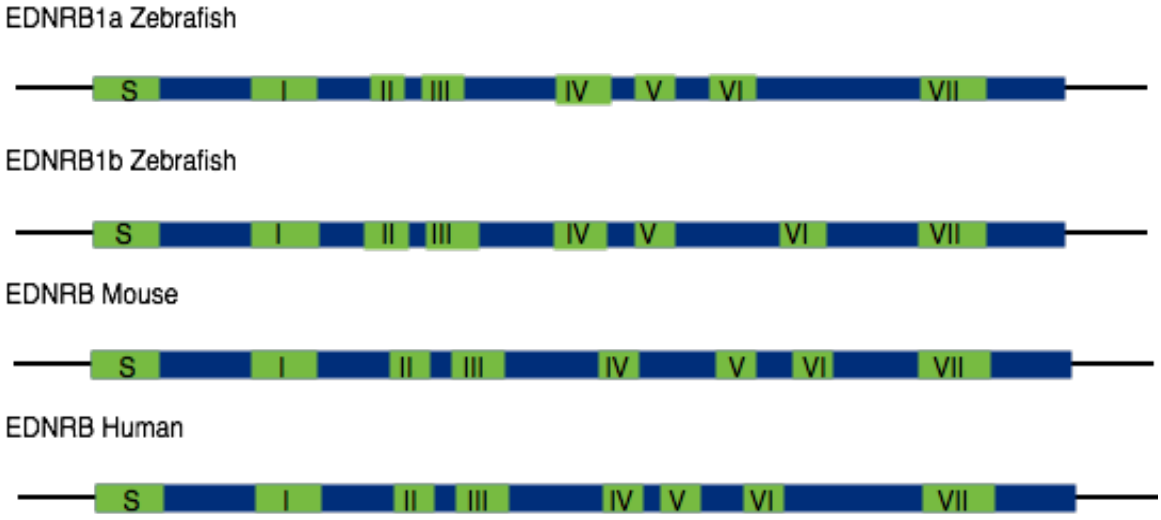
Whole-mount In situ Hybridization

Methods for riboprobe synthesis and *in situ* hybridization of embryos have been previously described (178). Digoxigenin-labeled riboprobes were prepared from templates linearized with *Xho*I using Sp6 RNA polymerase for both *ednrb1a* and *ednrb1b*.

Morpholino Injection and Morphant Analysis

The 25-mer morpholino antisense oligonucleotides were synthesized by Gene Tools, LLC. Translation and splice blocking morpholinos were initially used for *ednrb1a* (translation- AGCCAGAAGCTGAAAAACAGGTACT, splice-CTCATTCAGTAATACTCTGACCTGT targeting the splice junction at the end of exon 2) and 2 splice blocking morpholinos were used for *ednrb1b* due to the lack of an annotated 5' translation start site (splice blocking-TGATGCTATATTGCTCTCACCTGTC targeting the splice junction at the end of exon 4, splice- ACGAGGTGTAGAAACGCACCTGTTT targeting the splice junction at the end of exon 4). One nl of EDNRB and control morpholinos were injected into one-cell embryos at a concentration of 100 μM. Embryos were raised to the appropriate age at 28.5° C. Embryos were antibody stained for the neuronal marker HuC/Elavl3 with the antibody HuCD (Invitrogen). Embryos were also *in situ* hybridized with a previously characterized *crestin* riboprobe (179). *Tg(-8.3bphox2b:Kaede)* were also injected with morpholinos and then ENPCs were observed during development.

Figure 3.1



Comparison		I	II	III	IV	V	VI	VII	Overall
EDNRB1a Zebrafish	EDNRB1b Zebrafish	66.3%	77.7%	65.4%	67.4%	66.4%	73.1%	40.2%	65.6%
EDNRB1a Zebrafish	EDNRB Mouse	69.9%	71.7%	68.8%	76.7%	70.1%	74.8%	59.3%	70.0%
	EDNRB Human	68.3%	74.0%	68.3%	77.3%	73.1%	76.4%	60.7%	70.9%
EDNRB1b Zebrafish	EDNRB Mouse	69.2%	75.9%	63.7%	68.6%	66.4%	77.4%	50.4%	68.1%
	EDNRB Human	69.7%	72.6%	65.2%	64.8%	70.1%	77.2%	44.4%	68.4%

Figure 3.1 Analysis of *EDNRB* orthologue homology. Shown is a comparison of the mouse and human *EDNRB* genes as well as both zebrafish *ednrb* paralogues. The seven exons are arranged similarly in all species. All exons show a relatively high percentage of homolgy and the overall homolgy is similar between both zebrafish genes and their human and mouse counterparts.

Figure 3.2

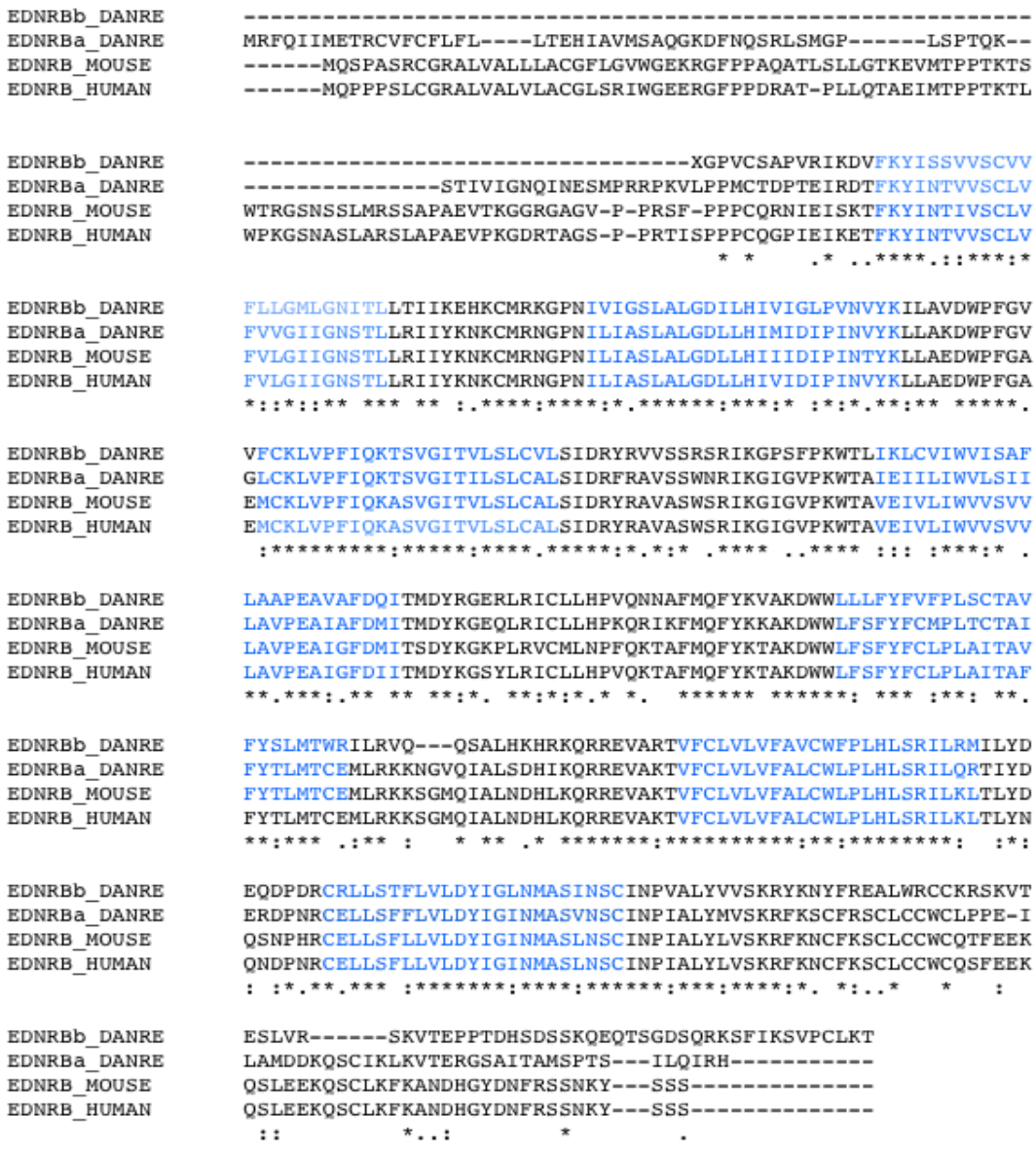


Figure 3.2 Amino acid sequence comparison of EDNRB. There is a high level of sequence conservation, as denoted by the asterisks, between EDNRB proteins in mouse, humans, and both zebrafish genes. The conservation is especially strong within the transmembrane domains of the protein (blue). The 5' region of the Ednrb1b protein sequence has not been annotated yet.

Figure 3.3

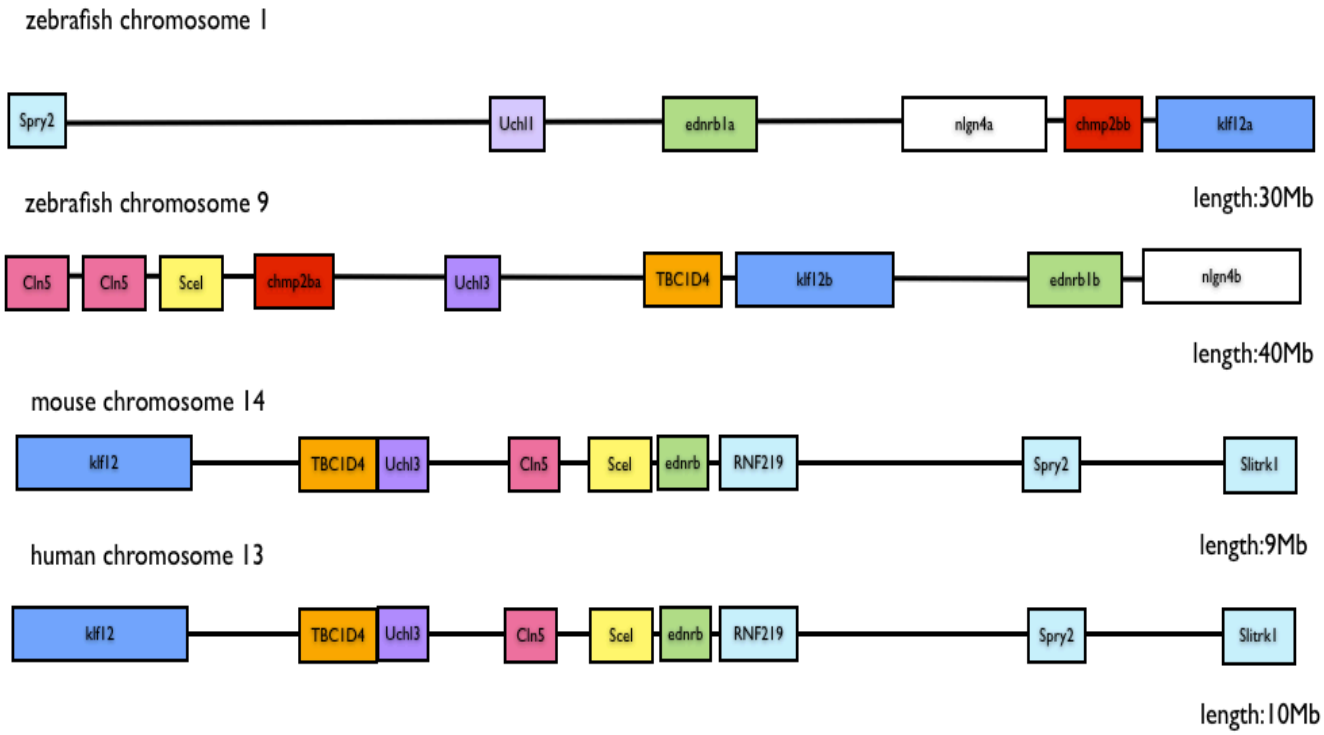

zebrafish chromosome 1
Spry2
Uchl1
ednrb1a
nlgn4a
chmp2bb
klf12a
length:30Mb
zebrafish chromosome 9
Cln5
Cln5
Scel
chmp2ba
Uchl3
TBC1D4
klf12b
ednrb1b
nlgn4b
length:40Mb
mouse chromosome 14
klf12
TBC1D4
Uchl3
Cln5
Scel
ednrb
RNF219
Spry2
Slitrk1
length:9Mb
human chromosome 13
klf12
TBC1D4
Uchl3
Cln5
Scel
ednrb
RNF219
Spry2
Slitrk1
length:10Mb

Figure 3.3 Syntenic analysis of the EDNRB gene region. Shown are the genomic locations for the *EDNRB* genes in mice, humans, and zebrafish. Many of the genes in this genomic region are similar among species. The genomic region of the zebrafish *ednrb1b* gene is more similar to the mouse and human counterparts than it is to the *ednrb1a* locus.

Figure 3.4

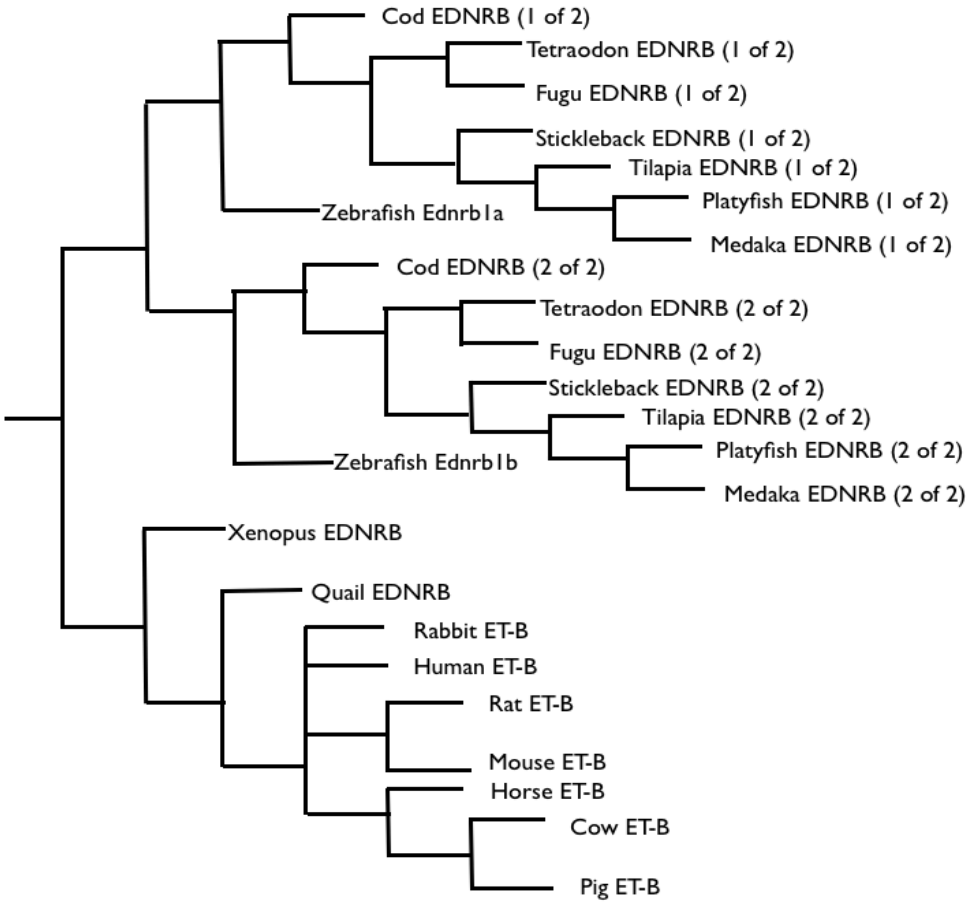

Cod EDNRB (1 of 2)
Tetraodon EDNRB (1 of 2)
Fugu EDNRB (1 of 2)
Stickleback EDNRB (1 of 2)
Tilapia EDNRB (1 of 2)
Platyfish EDNRB (1 of 2)
Zebrafish Ednrb1a
Medaka EDNRB (1 of 2)
Cod EDNRB (2 of 2)
Tetraodon EDNRB (2 of 2)
Fugu EDNRB (2 of 2)
Stickleback EDNRB (2 of 2)
Tilapia EDNRB (2 of 2)
Platyfish EDNRB (2 of 2)
Zebrafish Ednrb1b
Medaka EDNRB (2 of 2)
Xenopus EDNRB
Quail EDNRB
Rabbit ET-B
Human ET-B
Rat ET-B
Mouse ET-B
Horse ET-B
Cow ET-B
Pig ET-B

Figure 3.4 Phylogenic analysis of the *ednrb* gene. The zebrafish copies of the gene are much more similar to the *ednrb* genes found in other fish species. The teleost genetic duplication is apparent as all fish species contained two copies of the gene.

Figure 3.5

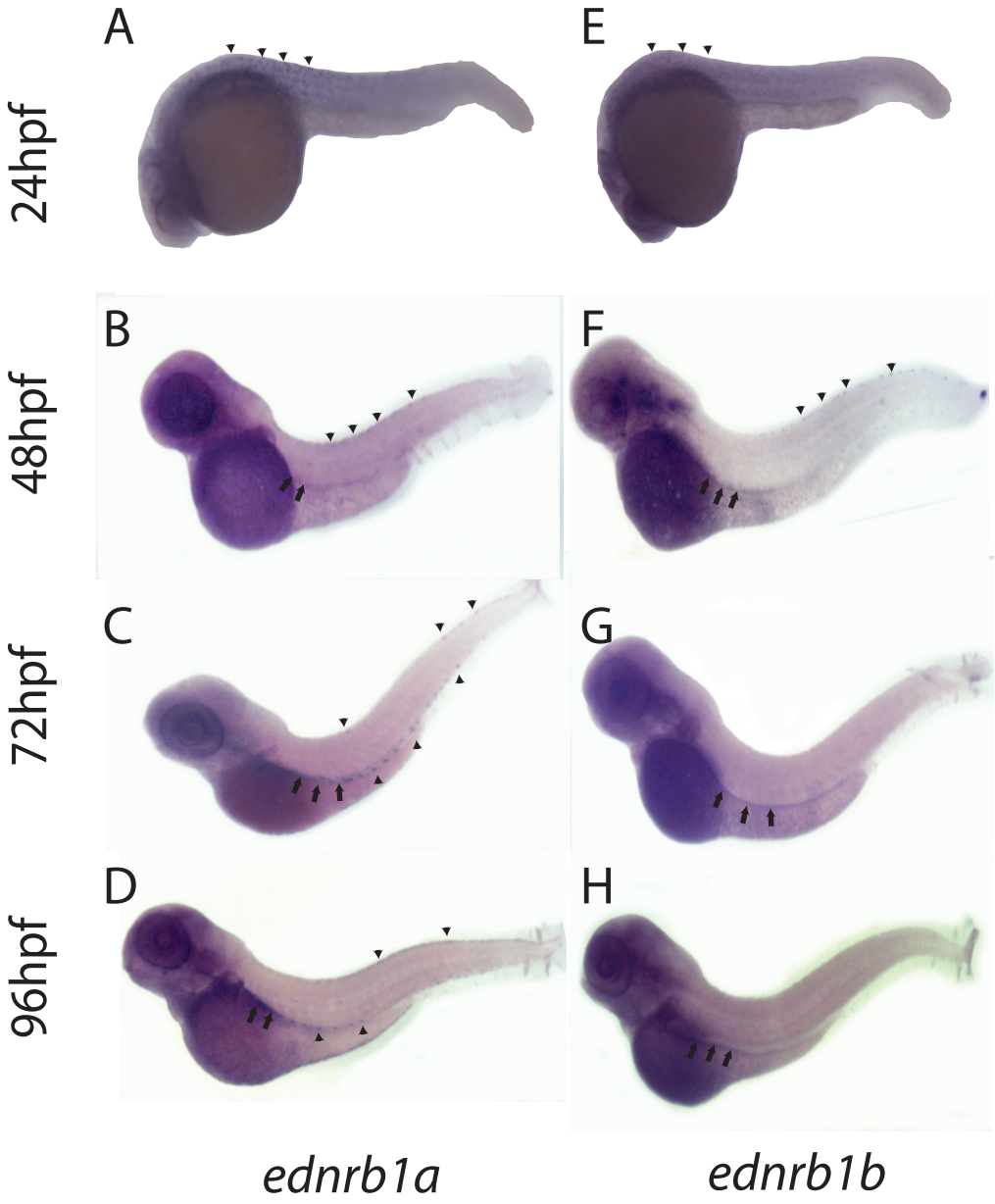


Figure 3.5 *In situ* hybridization of *ednrb1a* and *ednrb1b*. Expression pattern of the zebrafish EDNRB genes from 24 to 96 hpf. Arrows indicate gut expression and arrowheads indicate melanocyte expression. *ednrb1a* shows melanocyte expression throughout the timepoints observed, with expression decreasing at 96 hpf (A-D). *ednrb1b* shows melanocyte expression only at 24 and 48 hpf with expression disappearing at 72 hpf (E-H). *ednrb1a* shows expression in the anterior portion of the gut at 48 hpf and expression throughout at 72 hpf (A-B). Gut expression of *ednrb1a* decreases at 96 hpf. *ednrb1b* expression is present in the anterior gut at 48 hpf with expression throughout at 72 hpf (D-E). Expression continues in the gut at a lower level at 96 hpf (F).

Figure 3.6

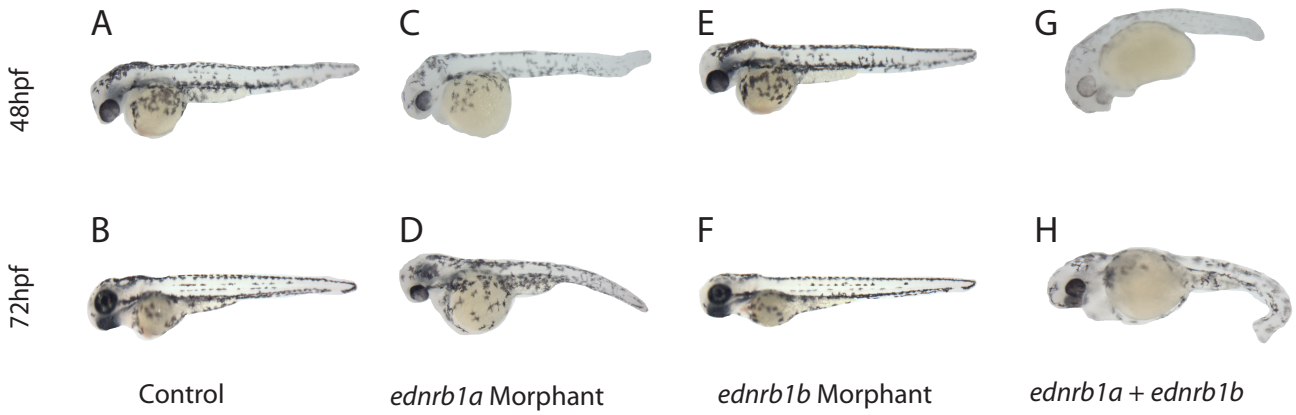

A
C
E
G
48hpf
B
D
F
H
72hpf
Control
ednrb1a Morphant
ednrb1b Morphant
ednrb1a + *ednrb1b*

Figure 3.6 Melanocyte development of control and *ednrb* morphant embryos. Embryos were injected with control, *ednrb1a*, or *ednrb1b* morpholinos and then observed for proper melanocyte development. *ednrb1a* morphants had lighter colored less defined melanocytes compared to controls (A-D). *ednrb1b* morphants had no noticeable melanocyte defect (E-F). *ednrb1a* and *ednrb1b* double morphants had a more extreme melanocyte defect than that seen in *ednrb1a* single morphants (C-D,G-H)

Figure 3.7

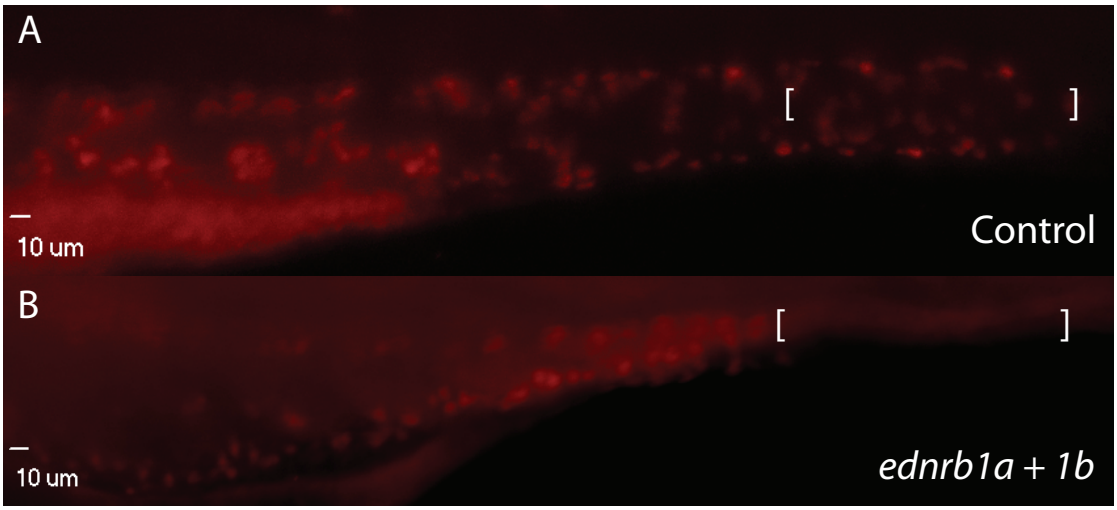


Figure 3.7 HuC/Elavl3 antibody stain of control and *ednrb* double morphants. *ednrb1a* and *ednrb1b* double morphants had a reduction in the number of ENS neurons as shown by HuC/Elavl3 antibody stain. The area of aganglionosis in mutants is indicated by the brackets in B, which differs from the equivalent bracketed region in wild type embryos (A). *ednrb1a* and *ednrb1b* single morphants had no ENS phenotype (data not shown).

Figure 3.8

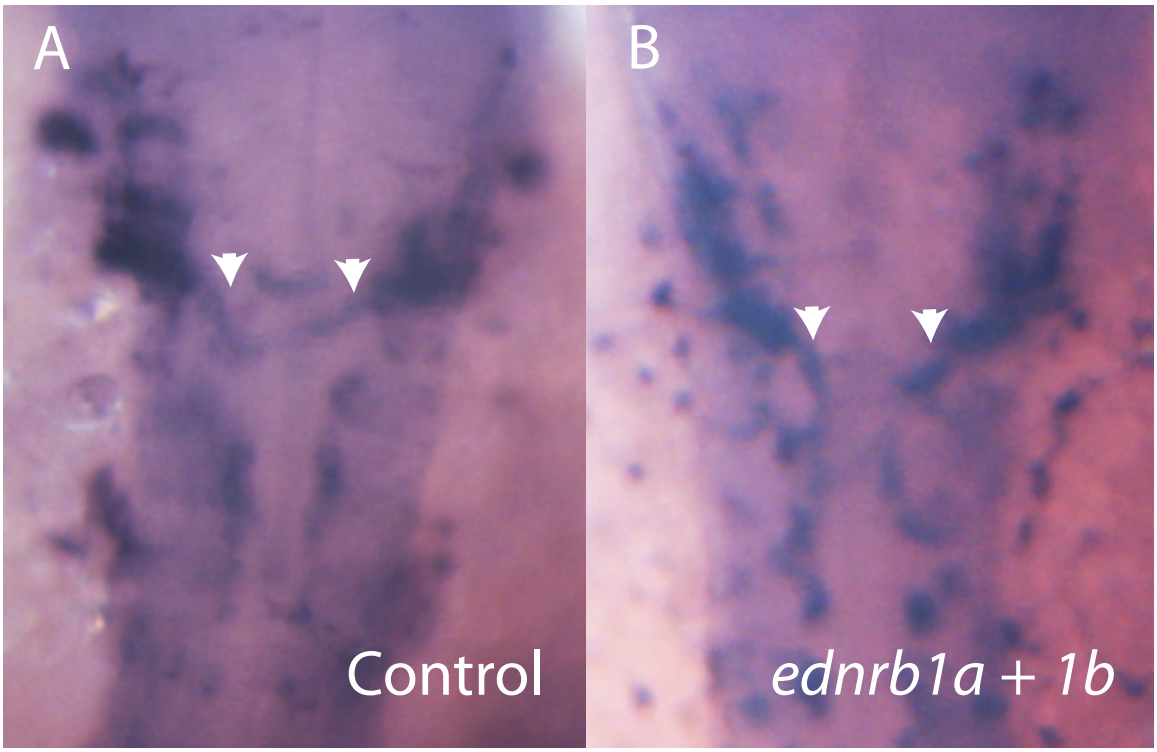


Figure 3.8 *crestin in situ* hybridization of control and *ednrb* double morphant embryos. The expression pattern of *crestin*, as indicated by the arrowheads, is normal in *ednrb1a* and *ednrb1b* double morphants.

Figure 3.9

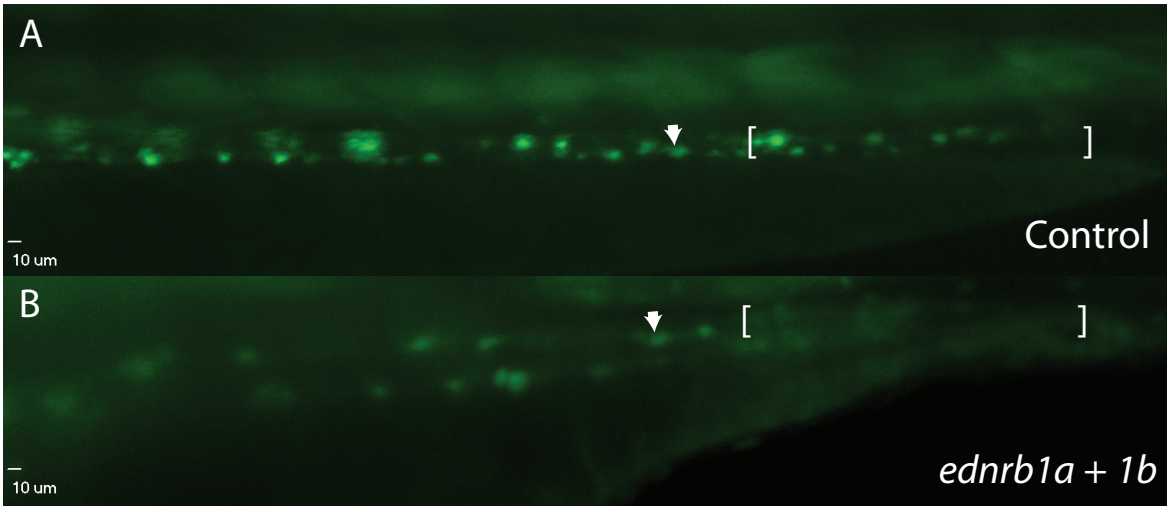


Figure 3.9 72 hpf *Tg(-8.3bphox2b:kaede)* control and *ednrb* double morphant embryos. *ednrb1a* and *ednrb1b* double morphant embryos have significant delay in their migration as shown by phox2b expressed in developing ENPCs. ENPCs should have migrated to the end of the gut at 72 hpf but this is not the case in double morphants (indicated by brackets, compare to the equivalent bracketed region in controls). Arrowheads indicate representative ENPCs. The end of the gut tube is the right boundary of the image.

Chapter 4: The Function of ID2a in ENS Subtype Differentiation

(Adapted from Harrison C and IT Shepherd. SIP1a and SMAD Interact to Influence ID2a Function In Enteric Nervous System Development. In preparation.)

Introduction

While a major error in ENS neuronal differentiation, like that seen in EDNRB mutants, can lead to significant intestinal aganglionosis subtler defects in ENS neuronal differentiation will potentially lead to much milder phenotypes. There are 17 different types of neurons in the gut and each has its own set of neurotransmitters that allow their specific role in ENS function (1, 180, 181). A change in the position or differentiation of any ENS neuron or neurons could explain some of the gastrointestinal (GI) disorders that currently cannot be diagnosed (113, 182-185).

To achieve the proper ratio of ENS subtypes in the gut requires careful regulation of the differentiation of enteric neurons (120, 186, 187). Enteric neurons are first specified in the neural crest and must migrate to, and then along, the gut to populate it. A small population of initial enteric neuron precursor cells (ENPC) generates the entire ENS, so it is important that they maintain an undifferentiated proliferative state for the appropriate length of time. Major errors in differentiation can lead to aganglionosis of the gut as seen in the disorder Hirschprung's Disease (HSCR) (155, 188, 189). For example 3-7% of HSCR cases have errors in the EDNRB, a gene involved in the prevention of premature differentiation (154, 155, 190). Errors in differentiation can also lead to a more subtype specific phenotype as *Ascl1*-/- mice have a particular reduction in serotonergic neurons (107).

One gene that has been shown to have a key role in ENPC differentiation is *Bmp*, potentially in a dose-dependent manner (191-194). BMP appears to be involved in both

general neural differentiation and in the differentiation of different neuronal subtypes, depending on when they appear during ENS development (195, 196). BMP has also been shown to promote the differentiation of NPY, nNOS, and TH expressing cells (196, 197). This suggests that BMP could have multiple roles during ENPC differentiation and that these roles may be temporally dependent (120, 196).

SIP1 (Smad Interacting Protein 1) is a protein that interacts with the BMP pathway (198). It is expressed in the gut, and may have a role in ENS development (199). SIP1 binds to the SMAD complex at the promoter region of genes and represses gene expression (198, 200). Patients with Mowat-Wilson syndrome, who also exhibit HSCR phenotypes, frequently have mutations in the SIP1 gene (13, 201, 202). Zebrafish, because of the teleost genome duplication event, have two copies of the *SIP1* gene, *sip1a and sip1b*. Disruption of *sip1a* and/or *sip1b* with morpholinos leads to a lack of ENPCs in the developing gut (199). While *sip1b* morphants have an ENS phenotype only *sip1a* is expressed in the gut during ENS development indicating that *sip1b* functions while ENPCs are still in the neural crest while *sip1a* has additional roles after ENPCs leave the neural crest (199).

My goal was to understand how the Bmp pathway and Sip1a interact to influence ENPC differentiation, so I identified an inhibitor of DNA-binding 2a (Id2a) as a candidate that might mediate a response in ENPCs; Id2a is a member of the inhibitor of DNA-binding (ID) family. The ID gene family is compromised of four different members in mammals, while there are five in zebrafish due to gene duplication (203). The *Id* gene family is

known to influence neuronal differentiation by preventing the expression of several pro-neural genes (204-206). They have also been implicated in neural crest development (207). *Bmp* expression also seems to influence *Id* expression in a bi-modal manor, as it can both be an enhancer or repressor of *Id* expression (205, 208).

In this study we investigated how Bmp differentially regulates *id2a* gene expression via recruitment of Sip1a to the id2a gene promoter. We show that at high levels of Bmp, Sip1a and the Smad complex interact at the *id2a* promoter to repress *id2a* expression. This in turn allows ENPCs that had previously been inhibited by Id2a, to differentiate at the appropriate time during ENS development. We also show that proper *id2a* expression is important for the maintenance of a pool of nitrergic precursors within the migrating ENPCs in zebrafish.

Results

BMP and ID2a Expression

I examined the expression pattern of *id2a* in the developing zebrafish at 48, 72, and 96 hpf using a digoxigenin-labelled *in situ* probe (Fig 4.1). *id2a* is expressed in the intestine at all stages examined. In addition, we saw *id2a* expression in the hindbrain and other neural crest cells at all time points with expression becoming more restricted over time (Fig 4.1). *id2a* expression is present in the anterior gut and migrating ENPCs at 48 hpf (Fig 4.1A-B). This expression in ENPCs continues in the 72 hpf embryos and expression in the anterior gut begins to be lost (Fig 4.1C-D). At 96 hpf there continues to be low-

level expression in the gut, but we see a decrease in the amount of expression in the ENPCs (Fig 4.1E-F).

We then looked at expression of *bmp2* and *bmp4* in the developmental period leading up to the initial differentiation of the ENS at 72 hpf in zebrafish. Both *bmp2* and *bmp4* are expressed in the hindbrain at all times observed (Fig 4.2). There is little to no expression in the developing guts at 48 hpf when ENPCs are still migrating through the gut (Fig 4.2A-B, G-H). Expression of both genes appears in the gut at 60 hpf, when ENPCs have colonized the length of the gut, with especially strong expression of *bmp4* (Fig 4.2C-D, I-J). At 72 hpf when the first wave of differentiation is occurring we see more robust expression of *bmp4* along the length of the gut with a weaker expression of *bmp2* (Fig 4.2E-F, K-L).

id2a Promoter Region

We analyzed the DNA sequence of the *ID2* promoter to identify possible conserved sites of regulatory control. When we aligned the sequences of zebrafish with mice and humans we found a highly conserved region upstream of the *ID2* gene (Fig 4.3). Within this conserved regions we identified a putative cyclic AMP response element (TGACGTCAC, in purple), BMP response element (TGGCGCC, in red), and SMAD binding elements (GTCTG, in blue). This suggests a significant evolutionarily conserved role for BMP signaling influencing *ID2* gene expression across species. The orientation and structure of these 3 elements is consistent in all three species examined. In addition

SIP1 binding elements (CACCT[G], in green) are present and conserved in all three species suggesting a role for SIP1 in modulating *ID2* gene expression.

Interaction of Sip1a and p-SMAD at the ID2a Promoter

To determine if Sip1a and p-SMAD directly bind to each other and to the previously described regulatory element of the *id2a* promoter we first carried out co-immunoprecipitation experiments with SIP1a and phospho-SMAD to show an interaction between the two proteins. Previous studies in mice had shown that SIP1 protein directly interacts p-SMAD protein as well as to CREB (198, 200). Using an antibody against the Sip1aa protein we were able to pull down p-Smad (Fig 4.4). We were also able to pull down Sip1a with an antibody against p-Smad (data not shown). Since Sip1a is maternally expressed and is highly abundant in early embryos we were able to use 8 hpf embryos in our pull down experiments. We were also able to pull down the complex in the guts of 72 hpf embryos showing that the two proteins interact in the developing gut during stages relevant to ENPC differentiation.

To show that the Sip1a-p-Smad complex was interacting at the *id2a* promoter region we carried out chromosome immunoprecipitation experiments for the region. We again utilized our Sip1a and p-Smad antibodies to pull down the promoter region of *id2a*. We were able to detect a 500 base pair region around the conserved Bmp region when we pulled down with both Sip1a and p-Smad antibodies in 8 hpf embryos and 72 hpf guts ($p<.0001$ for 8 hpf Sip1a and 72 hpf Sip1a and p-Smad, $p=.0281$ for 8 hpf Smad) (Fig 4.5). Utilizing Q-PCR we were able to amplify both the *id2a* promoter region containing

the Sip1a/p-Smad binding loci as well as a similar binding region in the brachyury homolog promoter, a region that had already been shown to have Sip1/Smad interaction (Fig 4.5) (209). We saw little to no amplification using primers for a separate region of the *id2a* promoter (Fig 4.5).

Quantitative Real Time PCR of ID2a Expression

To confirm the influence of Bmp on *id2a* gene expression, we carried out QRTPCR experiments in which we analyzed *id2a* expression levels in embryos with different amounts of *bmp* expression. We injected one-cell embryos with *bmp-4* and *follstatin*-like (a Bmp antagonist) and then collected embryos at 8 hpf. When we examined *id2a* expression at this stage we saw a significant decrease in expression levels when embryos were injected with *bmp-4* ($p<.001$) (Fig 4.6). This indicated an increased repression of *id2a* expression correlated with increased *bmp* expression levels . Furthermore we saw a corresponding increase in expression levels of *id2a* in *follstatin-like* injected embryos however this was not at a statistically-significant level ($p=.222$) (Fig. 4.6).

Subtype Specification in ID2a Morphant Embryos

Having confirmed the influence of Bmp signaling on *id2a* expression we wanted to determine the effect of loss of *id2a* expression on ENPC differentiation. We injected zebrafish embryos with a previously characterized *id2a* splice blocking morpholino and then determined the effect on ENS neuronal differentiation (210). Comparing the total number of enteric neurons at 120 hpf in *id2a* morphants to controls while there was no aganglionosis there was a significant reduction in numbers of neurons present ($p<.001$)

(Fig. 4.7A-B, 4.8). When we further examined these *id2a* morphants embryos to determine if there was any change in the number / proportion of specific subtypes of enteric neurons in the ENS at 120 hpf, we found that while the number of serotonergic enteric neurons was statistically unchanged ($p=.45$) the number of nitrergic enteric neurons was significantly reduced in the *id2a* morphants as compared to controls ($p<.001$) (Fig 4.7C-F, 4.8).

Discussion

Tightly controlled timing of ENPC differentiation is vital for the proper formation and function of the ENS. Premature differentiation of ENPC can lead to aganglionosis or improper connection of the neural circuitry of the gut, depending on what stage during development the error in differentiation occurs (115-118, 154, 155). While this process is vital for normal ENS development it is not clear how different signaling pathways influence ENPC differentiation. Here, we have shown that Bmp regulates *id2a* expression and influences, proliferation of nitrergic neurons. This Bmp action might occur by maintaining a pool of ENPCs in an undifferentiated state until the correct developmental timing when nNOS neurons would normally differentiate.

Perhaps the purpose of *id2a* expression during gut differentiation is to maintain a subset of ENPCs in an undifferentiated state. At 48 hpf, when ENPCs are migrating through the anterior portion of the gut, we see strong anterior expression of *id2a* (Fig. 4. 1). This expression in ENPCs continues through 72 hpf but has significantly decreased at 96 hpf (Fig. 4.1). This correlates with the onset of nNOS marker expression that occurs when

nitrergic neurons first appear in the zebrafish ENS around 72 hpf (120). The expression of *bmp4* also correlates well with nNOS neuronal differentiation, as it is first observed after migration is complete at 60 hpf and gets stronger during the first wave of differentiation at 72 hpf (Fig 4.2).

I found that the *ID2* promoter of various vertebrate species all have similar transcription binding sites that are spaced similarly, suggesting that control of *ID2* expression is highly conserved (Fig 4.3). This indicates that *ID2* probably plays a conserved role during ENS development in other species, including humans. Furthermore both the presence of BMP related regulatory elements in the *id2a* promoter and the *id2a* morphant phenotype confirms previous studies showing that BMP has a role during ENS differentiation (195, 196).

I have also shown that the conserved transcription factor binding sites (described above) are functionally relevant to both the expression of *id2a* as well as to regulation of ENPC differentiation in zebrafish. I have shown that both p-Smad and Sip1a interact with the *id2a* promoter region in the developing gut and influence *id2a* expression. This interaction was shown by both ChIP and Q-PCR and my data show that these two transcription factors interact both during ENS development (72 hpf), as well as earlier during embryogenesis (8 hpf) because sip1a is maternally loaded. Being able to identify the interaction early in embryogenesis allows us to observe the effect of injecting *bmp* and *bmp* antagonist RNA on *id2a* transcriptional control in these early embryos. Apparently because both Smad and Sip1a interact at multiple other promoters, I did not

see a change in their binding to the *id2a* promoter after *bmp* and *follstatin-l* RNA injections.

Despite the fact that I was not able to directly demonstrate p-SMAD or SIP1a binding to the ida2 promoter, *bmp* and *bmp* antagonist experiments offered a second, albeit less direct, way to show that these transcription factors played a critical role during ENS differentiation. Increasing *bmp* expression leads to a subsequent decrease in *id2a* expression due to the interaction of p-Smad with Sip1a. At high levels of Bmp, p-SMAD interacts with SIP1a more extensively, which leads to a repression of *id2a* consistent with how Sip1a represses transcription from other genes. This leads us to propose a model where low levels of Bmp or no Bmp allows *id2a* expression in ENPCs, causing repression of the pro-neural genes so that ENPCs are kept in an undifferentiated, highly proliferative state (Fig 4.9). However, at higher concentrations of Bmp, p-Smad interacts with Sip1a at the *id2a* promoter, repressing *id2a* expression and increasing pro-neural gene activity to drive differentiation of the ENPCs (Fig 4.9).

We propose that repression of ENPC differentiation by Id2a maintains a pool of nitrergic neuronal precursors. It is unclear, however, if Id2a functions in this pool of ENPCs by blocking the general expression of pan-neuronal genes or only of ones specifically expressed in nitrergic neuronal subtypes. Perhaps ENPCs respond to other environmental cues that determine the timing and pattern of ENPC differentiation. Alternatively, ENPCs might differentiate in a cell autonomous manner (211), but if this were true, it would mean that any secreted regulatory factors would have to function in an autocrine manner.

One possibility is that Id2a inhibits all pro-neural genes in a random subset of ENPCs until they receive a signal from the gut environment to differentiate into the nitrergic subtype. This would indicate that there was a specific environmental cue expressed at the time of nitrergic neuronal differentiation. Another possibility is that certain ENPCs are fated to become nitrergic and these cells express *id2a* to inhibit nitrergic specific pro-neural genes. This would indicate that there is some factor involved in promoting the expression of *id2a* in only a specific subset of ENPCs at some point in ENPC development. A third possibility is that some combination of these two models applies and, while *id2a* is expressed in a specific subset of cells, pro-nitrergic must be expressed at the right time and place to be effective in promoting proper differentiation. Studies looking into when ENPCs are specified to differentiate into a specific subtype could help elucidate the model for control of nitrergic neuronal differentiation. In addition, because Id2a is necessary so late in ENS development, morphant embryos may not show the whole picture of what Id2a is doing during development. Developing an *id2a* mutant that has a temporal, as well as spatial, control of *id2a* gene expression would allow for determining the consequence of perturbing signaling at various stages of ENS development. It would also allow us to verify the morphant phenotype we observe and to potentially see additional ENS phenotype that are missed in morphants.

In conclusion, BMP was known to influence ENPC differentiation, but it was not known how this signaling pathway influenced the differentiation of so many different neuronal subtypes (197). We have demonstrated one way in which BMP can influence the formation of a specific subtype of neuron. While Bmp and Id2a are involved in the

differentiation of nitric oxide-expressing neurons in the ENS, it is still unclear whether Bmp influences the specification and differentiation of other neuronal subtypes in zebrafish and other species. Previous studies have shown that BMP influences early and late arising neurons differently and that other regulatory genes might behave in a similar way as *id2a* in other subtypes of ENS neurons (196). The ID gene family has 5 different genes in zebrafish and we also know that *id3* is expressed in the developing zebrafish intestine during early stages of ENS development (data not shown).

Differentiation is a complicated and essential part of ENS development. By better understanding the differentiation of the ENS we can potentially better understand the etiology of many other GI disorders that are current poorly understood.

Methods

Whole-mount in situ hybridization

id2a, bmp2, and bmp4 expression were identified in *in situ* hybridization experiments using a DIG labeled *id2a* probe (F-GGTCCTTCCGGAAAAGTAGC R-CAGTCCTTCATGTGCCAAAA), *bmp2* probe (F-GCAGAGCAAACACGATACGA, R-AACAGGCTTTGGGAATGTTG), and *bmp4* probe (F-ATCAGGAGATGGTGGTCGAG, R-TTTGCGAATTGCATTTGTGT). *In situ* hybridization procedure carried out as described previously (178, 212). The riboprobes were synthesized from templates linearized and transcribed with SP6 polymerase (mMESSAGE mMACHINE®, Life Technologies)

ID2 Promoter Region

ID2 promoter region was compared using Clustal Omega (213). Putative binding regions for SIP, SMAD, and CREB were identified through previously published data (209, 214-216).

Co-IP

Co-IP was carried out utilizing Life Technologies™ co-immunoprecipitation kit. One hundred 8 hpf embryos were isolated for experiments. One hundred 72 hpf embryo guts were dissected and used for experiments. Antibodies used were Zeb2 (Sip1a) antibody middle region (Aviva Systems Biology), Anti-phospho-SMAD1 (PSER465) (Sigma Aldrich™), and Anti-Rabbit IgG Alkaline Phosphatase Conjugate (Promega). Blotted membranes were stained with NBT/BCIP solution (Roche).

ChIP

ChIP was carried out utilizing Sigma-Aldrich™ Imprint® Chromatin Immunoprecipitation kit. One hundred 8 hpf embryos were isolated for the experiment. Antibodies used were same as in Co-IP experiments. Samples were sonicated for 200 seconds (4 sec on/10 sec off) to yield fragments of approximately 500 bp. Fragments were amplified in Q-PCR using primers for the Smad/Sip1a region of the *id2a* promoter (F-CGAGCTGAACTCCGATCTCT, R-CGGAATGCACCTCATTATCA), SMAD/SIP1a region of brachyury (F-CTTCTAATCCGTCTGCGTGTT, R-GCACCGGTTGTTTACTGGAT), and non-Smad/Sip1a binding region of the *id2a* promoter (F-TGCCAAAATAATGCCACTGA, R-CCTTCAGCTTCCACGGATAA).

QRTPCR

QRTPCR reactions were carried out using Qiagen Quantifast™ SYBR® Green RT-PCR Kit. Embryos were injected with 1 nl of a 50ng/µl of *bmp4* or *follstatin-l* RNA. Injected and control embryos were collected at 8 hpf and RNA was extracted, 50ng/µl of experimental and control RNA was used for each reaction. RNA samples were amplified with ID2a promoters (F-GCAGAACAAAAACGTGAGCA R-GTCAGGGGTGTTCTGGATGT). Results were analyzed with Bio-Rad CFX Manager Softwar.

Neuronal and Subtype Identification

One-cell embryos were injected with 1 nl of a previously characterized 100µM *id2a* morpholino (SBMO, GCCTTCATGTTGACAGCAGGATTTC) or control morpholino then raised at 28.5° C (210). Embryos were raised until 120 hpf and then antibody stained with a HuCD antibody (Invitrogen), 5-HT antibody (Sigma-Aldrich™) or *in situ* hybridized with a nNOS DIG-labeled probe (217). Positive cells were counted from the swim bladder to the end of the gut tube.

Figure 4.1

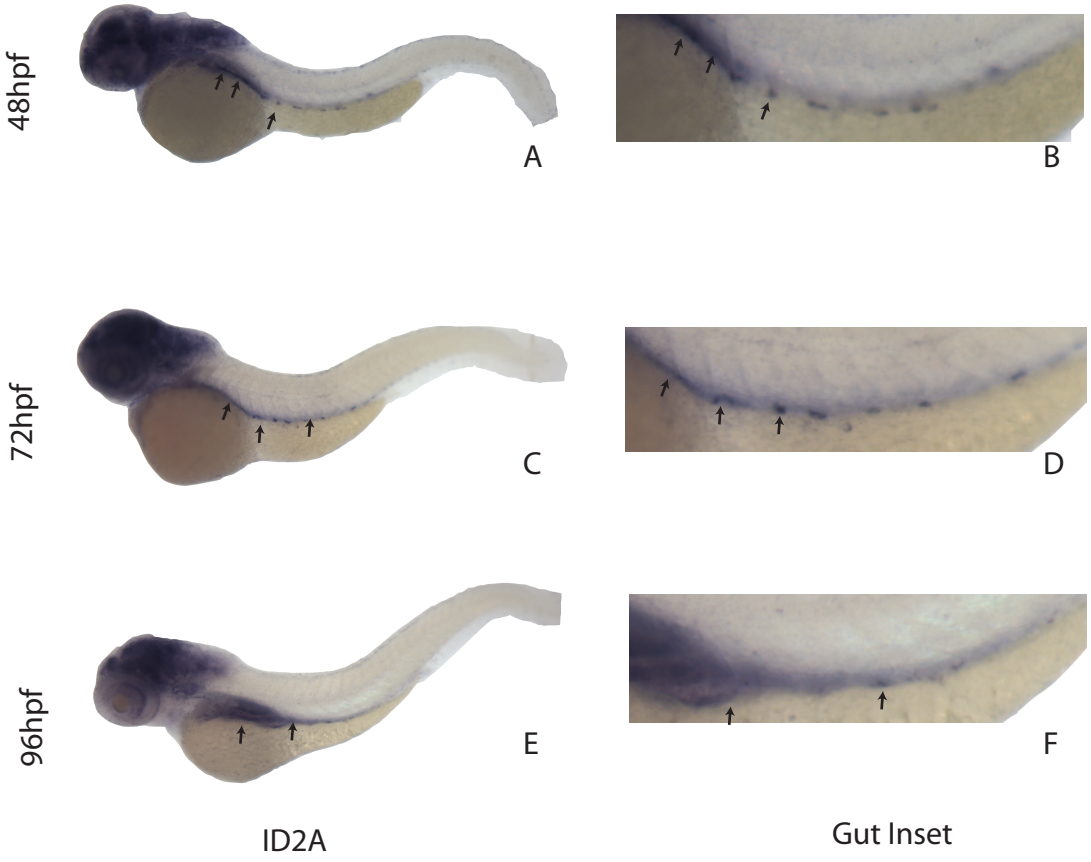


Figure 4.1 Id2a expression from 48-96 hpf. This Figure shows *id2a* expression in whole embryos as well as expression in the gut (inset, arrows indicate representative ENS expression). *id2a* expression is present in the head and hindbrain through all time points with expression becoming more restricted over time. *id2a* is strongly expressed in the anterior portion of the gut and in the ENPCs at 48 hpf (A-B). *id2a* continues to be expressed in in the gut and ENPCs at 72 hpf (C-D). While *id2a* is still expressed in the gut the expression in ENPCs disappears at 96 hpf (E-F).

Fig 4.2

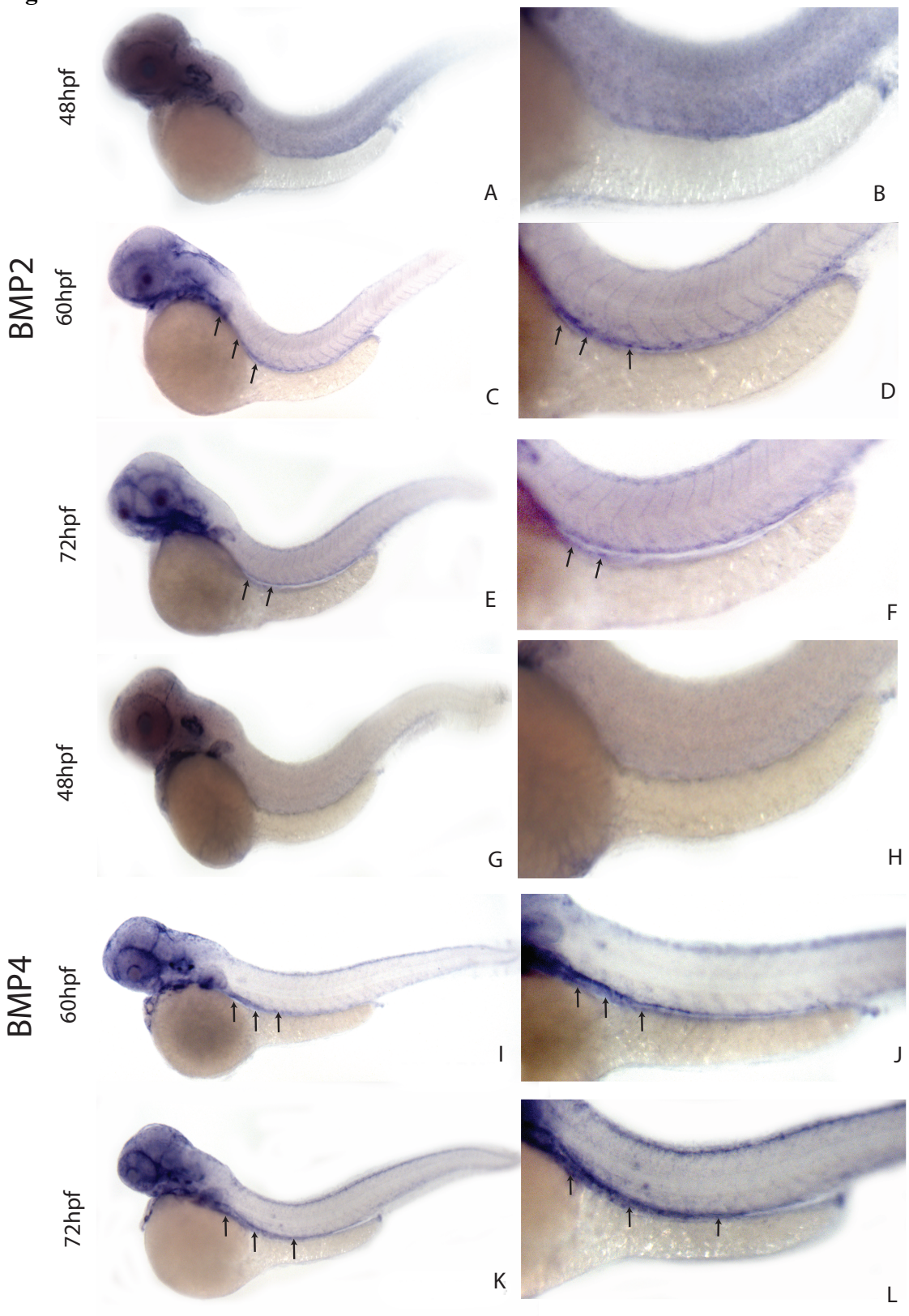
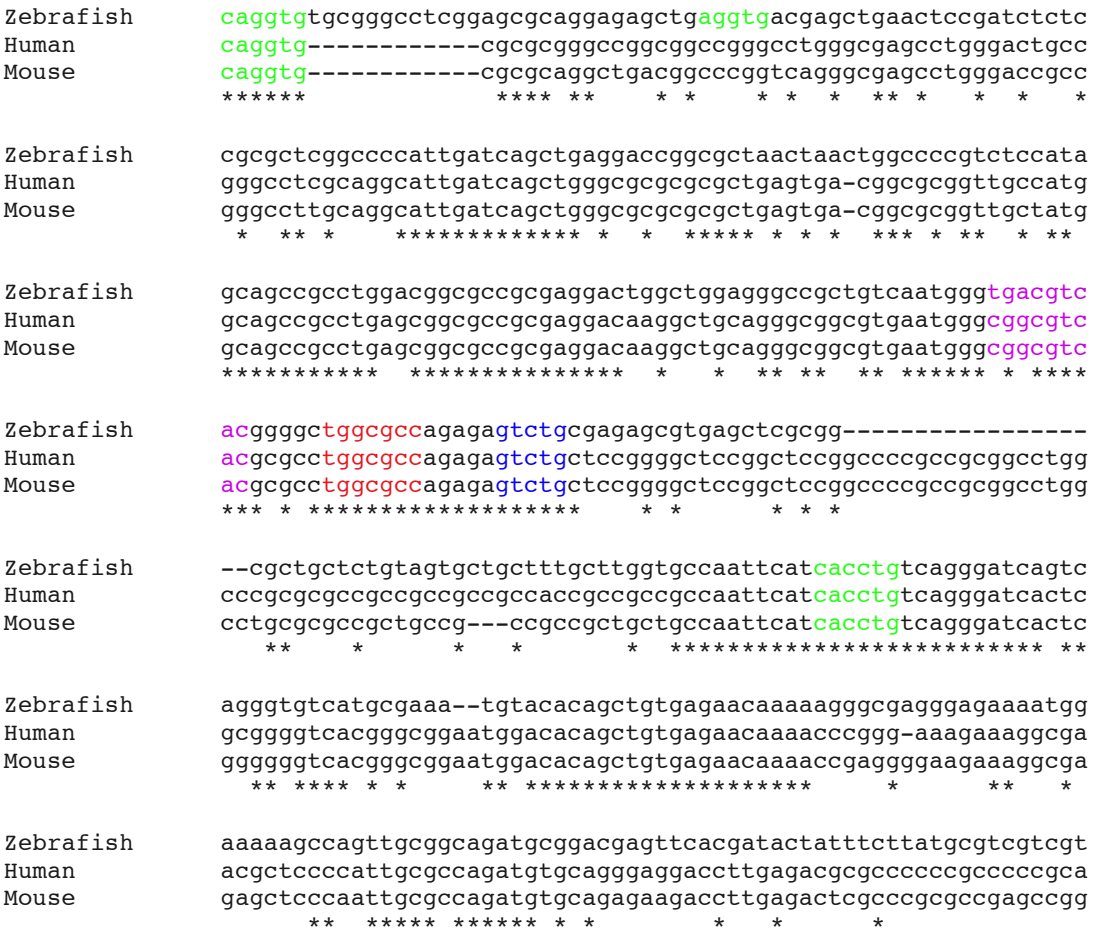

BMP2
BMP4
48hpf
60hpf
72hpf
48hpf
60hpf
72hpf
A
B
C
D
E
F
G
H
I
J
K
L

Fig 4.2 Expression pattern of *bmp2* and *bmp4*. *In situ* hybridization showing expression of *bmp2* (A-F) and *bmp4* (G-L) at 48, 60, and 72 hpf. Insets show up close view of the developing gut (B,D,F,H,J,L). Gut expression (as indicated by arrows) is seen at 60 and 72 hpf in both genes. Gut expression gets stronger and more robust between 60 and 72 hpf in *bmp4* (G-L).

Figure 4.3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Human caggtg------------cgcgcgggccggcggccgggcctgggcgagcctgggactgcc
Mouse caggtg------------cgcgcaggctgacggcccggtcagggcgagcctgggaccgcc
Zebrafish cgcgctcggccccattgatcagctgaggaccggcgctaactaactggccccgtctccata
Human gggcctcgcaggcattgatcagctgggcgcgcgcgctgagtga-cggcgcggttgccatg
Mouse gggccttgcaggcattgatcagctgggcgcgcgcgctgagtga-cggcgcggttgctatg
Zebrafish gcagccgcctggacggcgccgcgaggactggctggagggccgctgtcaatgggtgacgtc
Human gcagccgcctgagcggcgccgcgaggacaaggctgcagggcggcgtgaatgggcggcgtc
Mouse gcagccgcctgagcggcgccgcgaggacaaggctgcagggcggcgtgaatgggcggcgtc
Zebrafish acggggctggcgccagagagtctgcgagagcgtgagctcgcgg----------
Human acgcgcctggcgccagagagtctgctccggggctccggctccggccccgccgcggcctgg
Mouse acgcgcctggcgccagagagtctgctccggggctccggctccggccccgccgcggcctgg
Zebrafish --cgctgctctgtagtgctgctttgcttggtgccaattcatcacctgtcagggatcagtc
Human cccgcgcgccgccgccgccgccaccgccgccgccaattcatcacctgtcagggatcactc
Mouse cctgcgcgccgctgccg---ccgccgctgctgccaattcatcacctgtcagggatcactc
Zebrafish agggtgtcatgcgaaa--tgtacacagctgtgagaacaaaaagggcgagggagaaaatgg
Human gcggggtcacgggcggaatggacacagctgtgagaacaaaacccggg-aaagaaaggcga
Mouse gggggtcacgggcggaatggacacagctgtgagaacaaaaccgaggggaagaaaggcga
Zebrafish aaaaagccagttgcggcagatgcggacgagttcacgatactatttcttatgcgtcgtcgt
Human acgctccccattgcgccagatgtgcagggaggaccttgagacgcgcccccgccccgca
Mouse gagctcccaattgcgccagatgtgcagagaagaccttgagactcgcccgcgccgagccgg

Figure 4.3 Alignment of genomic sequence of *ID2* promoter. There are large regions of the *ID2* promoter that are highly conserved between humans, mice, and zebrafish genomes. Identical bases found in all three species are indicated by asterisks. Green = Sip1a binding motif (CACCT[G]). Pink = CAMP response element (TGACGTCAC). Red = BMP response element (TGGCGCC). Blue = SMAD binding element (GTCTG). The various binding elements and the regions around them have a high degree of sequence identity.

Figure 4.4

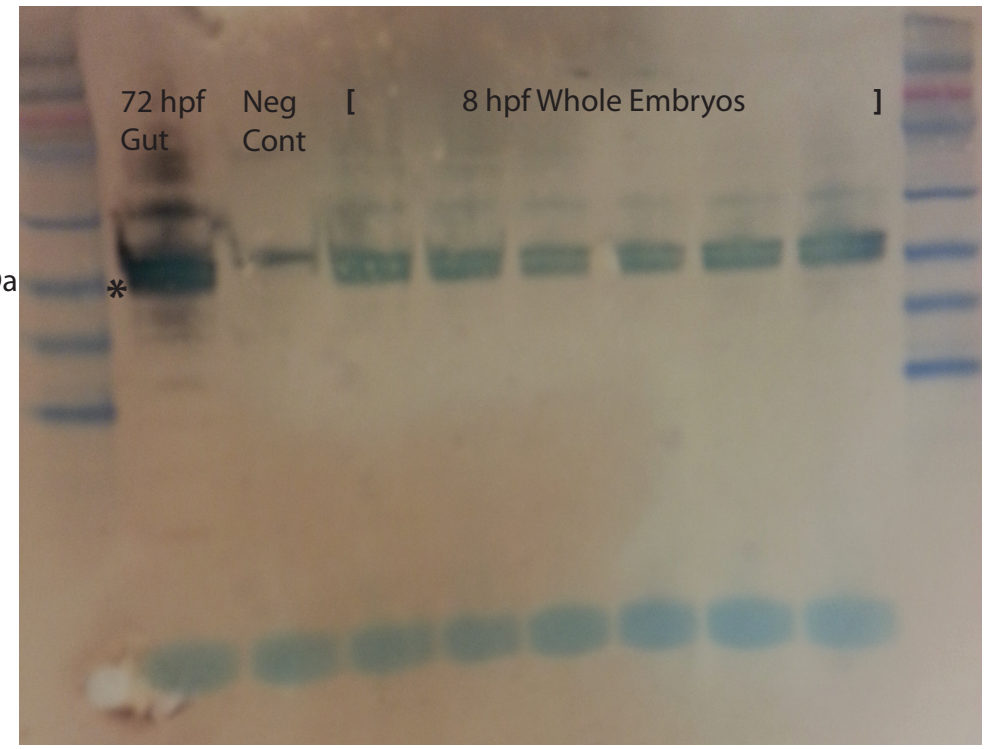


Figure 4.4 Co-immunoprecipitation of Sip1a and p-Smad. p-Smad was pulled down with an anti-Sip1a antibody in both 72 hpf guts and 8 hpf whole embryos. P-Smad has a molecular weight of 55 kDa, which lines up exactly with the protein ladder (Indicated by the asterisk). There is a protein artifact picked up by the beads that explains the band found slightly above 55 kDa.

Figure 4.5

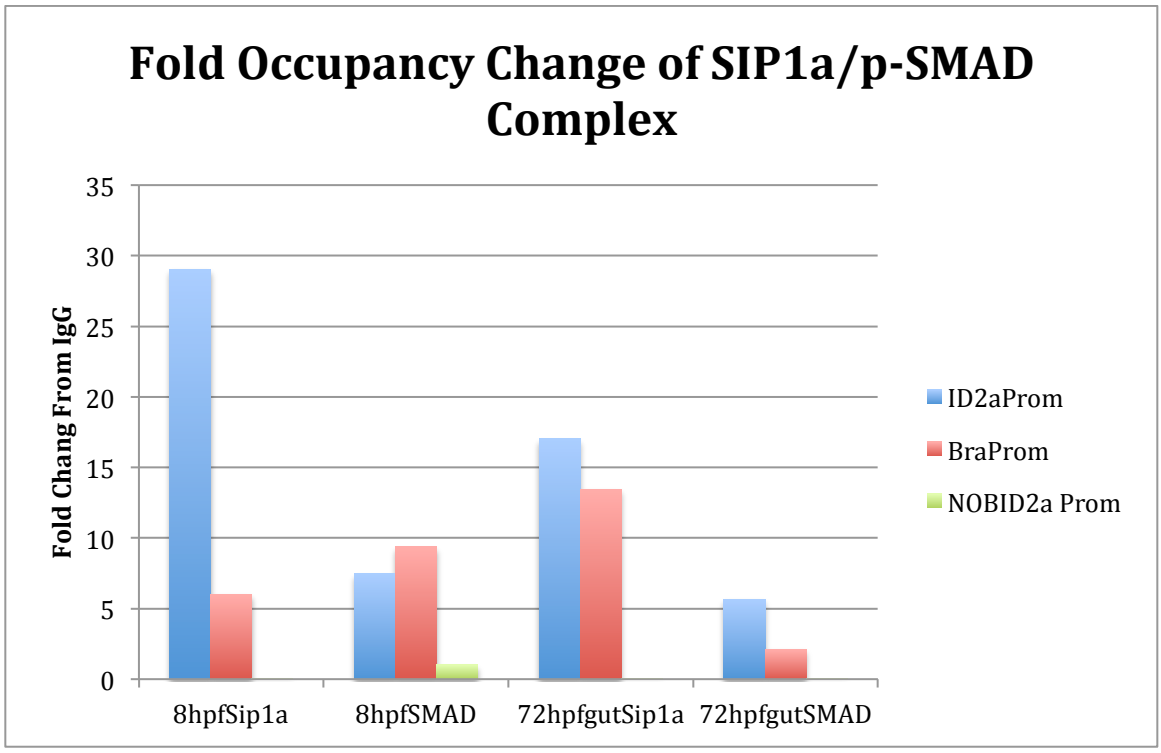

Fold Occupancy Change of SIP1a/p-SMAD Complex
Fold Chang From IgG
35
30
25
20
15
10
5
0
8hpfSip1a
8hpfSMAD
72hpfgutSip1a
72hpfgutSMAD
ID2aProm
BraProm
NOBID2a Prom

Figure 4.5 Chromosome immunoprecipitation experiment with *id2a* promoter region and Sip1a and p-Smad. The region of the *id2a* promoter containing the conserved Bmp response regions was pulled down with both p-Smad and Sip1a antibodies. Q-PCR was carried out with primers for the conserved Sip/Smad *id2a* binding region (ID2aProm), the brachyury promoter a positive control for Sip/Smad interaction (BraProm), and a non-Sip/Smad complex binding area of the *id2a* promoter as a negative control. Sip1a pull down is more effective due to Smad having a more prevalent role in other promoter interactions.

Figure 4.6

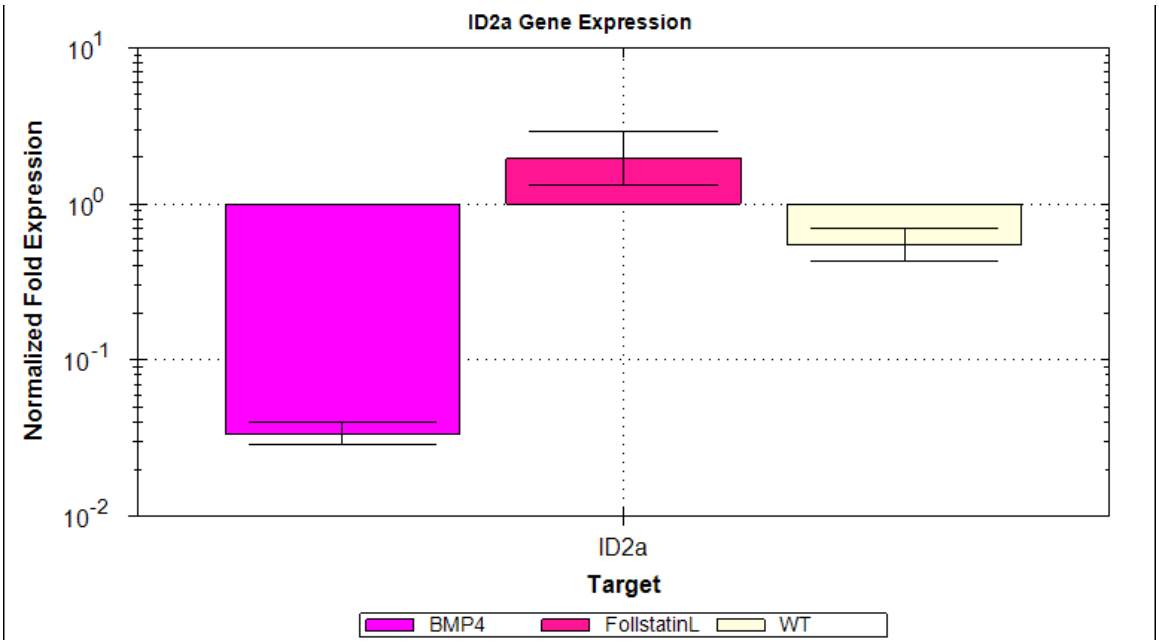

ID2a Gene Expression
Normalized Fold Expression
10^{1}
10^{0}
10^{-1}
10^{-2}
ID2a
Target
BMP4
FollstatinL
WT

Figure 4.6 Quantitative real-time PCR showing relative expression of BMP4 and follistatin L in 8 hpf embryos. One-cell embryos were injected with *bmp4* and *follstatin-like* and then collected at 8 hpf. *bmp4* injected embryos had a statistically significant almost 2-fold decrease in the amount of *id2a* gene expression. There was a slight increase in *id2a* expression in *follstatin-l* injected embryos but it was not statistically significant.

Fig 4.7

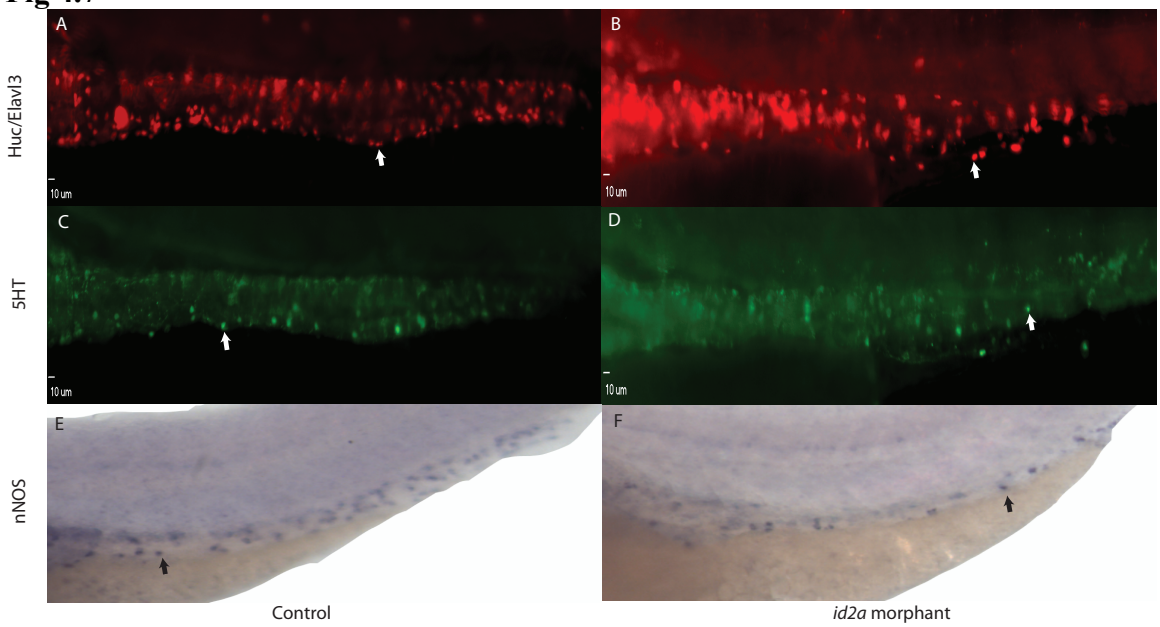

A
B
C
D
E
F
Huc/Elavl3
5HT
nNOS
10 um
10 um
10 um
10 um
Control
id2a morphant

Fig 4.7 Expression of the neuronal markers HuC/Elavl3, 5HT, and nNOS. Immunofluorescence reveals the presence of the general neuronal marker HuC/Elavl3 and the serotonergic neuron marker 5HT (A-D). *In situ* hybridization shows expression of the nitrergic marker nNOS (E-F). There is a decrease in total neuron numbers seen in *ida2* morphants as compared to control embryos (A-B). There is no change in the serotonergic neurons counts when ida2 morphants are compared to control embryos (C-D). There is a decrease in the number of nitrergic neurons in *id2a* morphants as compared to control embryos (E-F).

Figure 4.8

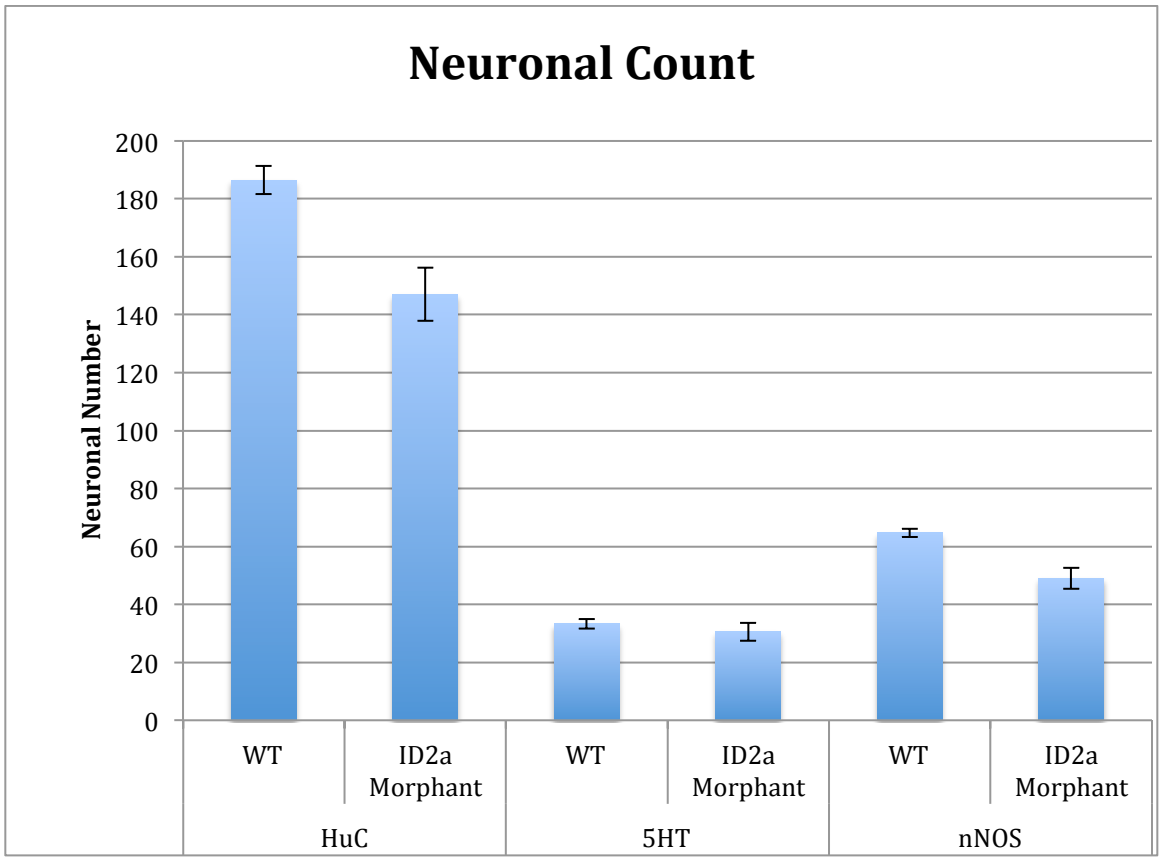

Neuronal Count
Neuronal Number
200
180
160
140
120
100
80
60
40
20
0
WT
ID2a Morphant
WT
ID2a Morphant
WT
ID2a Morphant
HuC
5HT
nNOS

Figure 4.8 Neuronal count of wild type and *id2a* morphant embryos. There is no statistical difference in overall neuron numbers or 5HT neuronal numbers between *id2a* morphants and control embryos. There is a statistically significant decrease in the amount of nNOS neurons in *id2a* morphants.

Figure 4.9

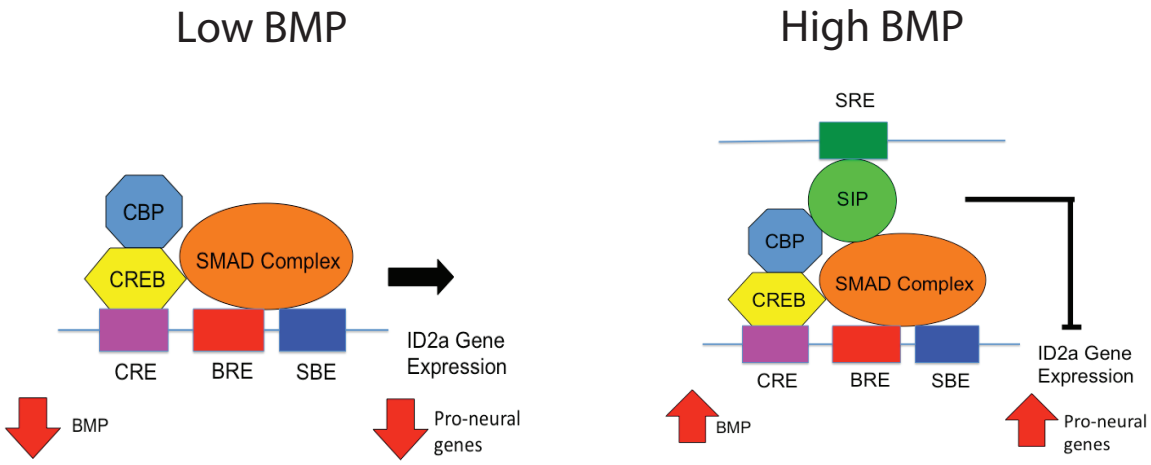

Low BMP
High BMP
CBP
CREB
SMAD Complex
CRE
BRE
SBE
ID2a Gene Expression
BMP
Pro-neural genes
SRE
SIP
CBP
CREB
SMAD Complex
CRE
BRE
SBE
ID2a Gene Expression
BMP
Pro-neural genes

Figure 4.9 Model of *id2a* promoter control. At low levels of Bmp, the Smad complex interacts at the conserved Bmp region of the *id2a* promoter and helps enhance *id2a* gene expression leading to a repression of pro-neural gene function. At high levels of Bmp, Sip binds to the Smad complex and represses *id2a* gene expression leading to an increase in pro-neural gene function. CRE = CREB Response Element. BRE = BMP Response Element. SBE = SMAD Binding Element. SRE = SIP Response Element

Chapter 5: BMP and a Link To HSCR

Introduction

In the previous chapters we have shown that the known HSCR gene EDNRB has a conserved function during zebrafish ENS development. We have also shown a link between BMP and differentiation of zebrafish ENS neuronal subtypes. This finding is consistent with previous studies in other model systems that have similarly suggested a role for BMP signaling in ENS development (101,103, 189,191). However a direct link between BMP signaling and human ENS disorders has not been shown, though SIP1 mutations are associated with Mowat Wilson Syndrome, where patients sometimes exhibit HSCR (201, 202). In this chapter, we describe studies of zebrafish genes that are orthologous to HSCR candidate genes identified in collaboration with Prof. Robert Hofstra (Erasmus University Rotterdam, The Netherlands). The Hofstra lab has identified a novel susceptibility locus at 4q31.3-q32.3 in HSCR patients (218). No previously known HSCR susceptibility genes mapped to this region, which includes 57 genes.

I focused on two of the several genes that are within the interval that the Hofstra lab has defined as involved in new HSCR disease because they potentially linked to my prior work. The first one chosen for further study was Mab21l2, which is a downstream antagonist of BMP activity. It interacts directly with the SMAD1 protein to repress function of the SMAD complex and the effects of BMP activation (219). In humans, the *MAB21l2* gene is located on chromosome 4 within the *LRBA* gene (Fig 5.1). *LRBA* is involved in the immune system as its expression is induced in B cells and macrophages when they are in the presence of bacterial lipopolysaccharides (220, 221). While there is not a mutation in *MAB21l2* in the HSCR patients studied by the Hofstra laboratory, there

is one in *LRBA* (218). Here we show that in zebrafish *mab21l2* is expressed in the gut and neural crest and that zebrafish *mab21l2* morphants have a significant aganglionosis phenotype while zebrafish *lrba* has a less ENS related expression and *lrba* morphants have little to no ENS phenotype. Sequencing of the ORF of the MAB21L2 gene in this HSCR patient revealed no mutations. However the *MAB21L2-LRBA* genome structure is conserved among species and *LRBA* introns may have enhancer activity (222). This led us to hypothesize that key parts of the *LRBA* gene locus have enhancer function in *MAB21l2* expression and the mutation in the observed HSCR patients disrupts this activity.

Results

Identification of Zebrafish lrba Orthologue

The zebrafish orthologue for *lrba* was identified in an Ensmbl gene search and showed strong sequence similarity, as well as genome organization, to its human orthologue (82% sequence identity) (Fig 5.1). As in the human orthologue the zebrafish *mab21l2* gene is located within the *lrba* gene (Fig 5.1)

Expression Patterns of mab21l2 and LRBA

Whole-mount *in situ* hybridization (WISH) studies revealed that *mab21l2* is strongly expressed in zebrafish embryos from 24-96 hpf (Fig 5.2). It has particularly strong expression in the hindbrain and cranial neural crest especially at 48 hpf (Fig 5.2). This cranial neural crest expression can be most clearly seen in the pharyngeal arches (Fig 5.2). This pattern of expression is consistent with previously reported *mab21l2*

expression. Significantly for this study we observed that *mab21l2* is expressed in the gut mesoderm from 48 hpf onward, which had not been previously reported (Fig 5.2B-D).

By contrast, the pattern of zebrafish *lrba* expression, is much more restricted. *lrba* is expressed along the yolk sack boundary and weakly in the hindbrain at 24 hpf (Fig. 5.3A). At 48 hpf there is very weak expression in the hindbrain but no apparent expression elsewhere in the embryo (Fig 5.3B). The weak expression in the hindbrain continues from 72-96 hpf and at 72 hpf expression appears in the intestinal bulb and continues at 96 hpf (Fig 5.3C-D).

Functional Analysis of mab21l2 and lrba in ENS Development

To determine the functional significance of these two genes we obtained morpholinos to both to test whether perturbation of either gene's function could influence ENS development and differentiation of ENPCs. We used a previously validated TBMO for mab21l2 and designed two different SBMOs for *lrba* (223).

When examined at 120 hpf *mab21l2* morphants show a significant decrease in the number of enteric neurons when compared to control embryos (Fig 5.4A-B). *mab21l2* morphants also displayed other morphological phenotypes identical to those previously published (223). By contrast *lrba* morphants showed a shortened body axis and subtle gut morphogenesis problems but no significant reduction in enteric neuron number when compared to control embryos.

Discussion

I examined the zebrafish orthologues of two potential HSCR candidate genes, LRBA and MAB21L2 located in the 4q31.3-q32.3 region. Zebrafish *mab21l2* has been previously identified however its role in ENS development has not been determined. Zebrafish *lrba* had not been previously cloned. Here, we describe the expression of these genes with respect to the developing GI tract and ENS. Subsequently we have determined the functional significance of these genes in ENS development using morpholinos. We show that *mab21l2* is expressed in regions of the developing embryo consistent with it playing a role in ENS development but, more significantly, we show that *mab21l2* morphants have a significant reduction in ENS neuronal number. By contrast *lrba* does not appear to have a significant role in zebrafish ENS development based both on its expression pattern and the lack of an obvious phenotype in morpholino knock down experiments.

While I found no evidence that Lrba plays a role during zebrafish ENS development, this gene does have mutations in HSCR patients so it remains possible that it contributes to disease development. Perhaps these mutations in the LRBA gene are regulatory elements that control MAB21L2 expression, given the orientation of the genes in the genome (Fig 5.1). Our Dutch collaborators are using a luciferase assay to test ŧ if the LRBA mutations alter or abolish enhancer activity (222); preliminary results from these studies are promising. More recent exome sequencing has revealed that these HSCR patients have mutations in other previously characterized genes implicated in HSCR. Perhaps the *LRBA* mutation in these patients works epistatically with these previously characterized genes to cause the HSCR phenotype (218). Furthermore, *LRBA* may have also have role

in irritable bowel disease (IBD) indicating potentially another role in ENS/gut function (224, 225).

Significantly our results clearly indicate that Mab21l2 has a role in ENS development and potentially may be a new HSCR linked gene. Furthermore this is a second gene in addition to SIP1 that suggests BMP signaling does play a significant role in ENS development and is linked to human ENS disorders. Finally, this study shows that the zebrafish model system can be used to quickly (and comparatively cheaply) test if new, potential HSCR linked genes play a role in ENS development; this approach is now being pursued by the Shepherd and Hofstra labs.

Methods

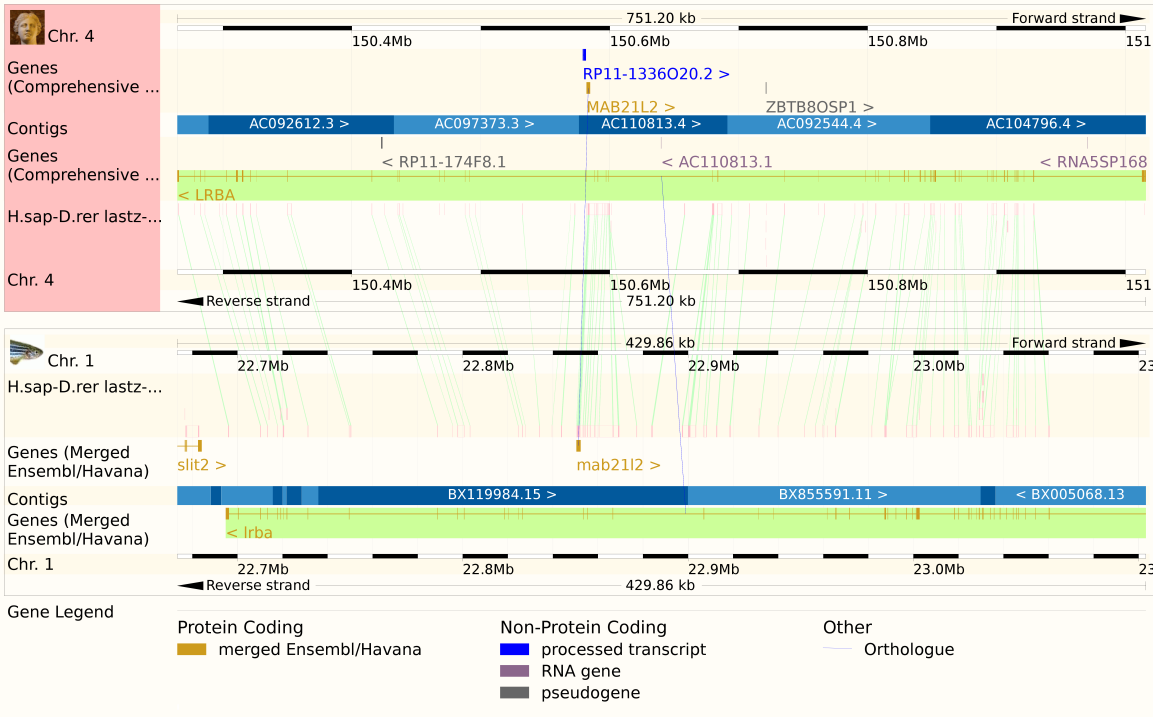
Cloning and In situ Hybridization

mab21l2 and *lrba* expression patterns were shown by whole-mount *in situ* hybridization as previously described (178). The two genes were amplified by RT-PCR with primers for *mab21l2* (F- ATTCGCTCCCGCTTTCAG, R-TCGTCCCAGTCAGTCTCCC) and *lrba* (F-CTTTTGACCAAAGGAATGGGTTACG , R-TCCAAGCATGACTTCTGCTTTCC) and subcloned in to TOP TA PCRII vector. Digoxigenin labeled antisense probes were generated using SP6 polymerases after linearizing the templates using restriction enzymes (mMESSAGE mMACHINE®, Life Technologies)

mab21l2 and lrba Morphant Analysis

A TBMO morpholino for mab21l2 (ACTGTAGACCGGAGTTTCGCAGTAC) was generated as per previously described (223). Two lrba SBMO morpholinos were designed, one to exon 13 (AGTTGGTTTAGTCTCTTACCGAGAC) and the other to exon 24 (ACTGCATACTAACCGAAGAAGAAGT). Effectiveness of these *lrba* SBMOs were confirmed by RT-PCR. *mab21l2* and *lrba* morphants were generated by injecting 100μM morpholinos into one-cell embryos. Morphants and controls embryo were allowed to develop to 120 hpf and were then fixed and antibody stained for ENS neurons using the HuC/D antibody (Invitrogen). Control embryos were injected with scrambled morpholinos.

Fig 5.1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Forward strand
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150.6Mb
150.8Mb
151
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ZBTB8OSP1 >
Contigs
AC092612.3 >
AC097373.3 >
AC110813.4 >
AC092544.4 >
AC104796.4 >
Genes (Comprehensive ...
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< AC110813.1
< RNA5SP168
< LRBA
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150.6Mb
150.8Mb
151
Reverse strand
751.20 kb
Chr. 1
429.86 kb
Forward strand
22.7Mb
22.8Mb
22.9Mb
23.0Mb
H.sap-D.rer lastz-...
Genes (Merged Ensembl/Havana)
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mab21l2 >
Contigs
BX119984.15 >
BX855591.11 >
< BX005068.13
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Chr. 1
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22.8Mb
22.9Mb
23.0Mb
Reverse strand
429.86 kb
Gene Legend
Protein Coding
merged Ensembl/Havana
Non-Protein Coding
processed transcript
RNA gene
pseudogene
Other
Orthologue

Figure 5.1 Alignment of region of *LRBA* and *MAB21L2* in humans and zebrafish. *LRBA* is a large gene whose area is encompassed by the green shaded boxes. Both human and zebrafish versions align fairly well as shown by the green lines and pink bars (indicating homology, 70% sequence identity). The *MAB21L2* gene is a smaller gene that is within the area of the *LRBA* gene in both species and shows strong homology between the human and zebrafish versions (82% sequence identity). Sequence analysis generated with Ensmbl database.

Figure 5.2

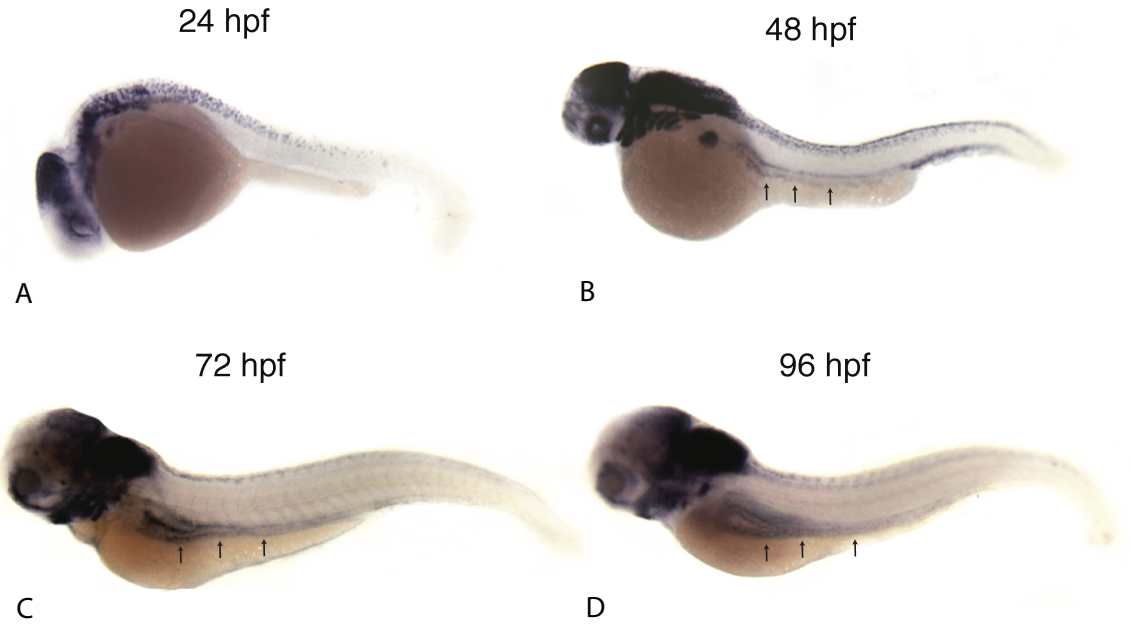

Figure 5.2 *mab21l2* expression from 24-96 hpf. *In situ* hybridization showing that *mab21l2* is expressed abundantly in zebrafish embryos at all time observed. Expression is especially present in the hindbrain, neural crest, and neural crest derived cells at all points. Expression is present in the pharyngeal arches at all time points observed. Gut expression becomes apparent at 48 hpf and continues through 96 hpf (B-D) (Representative gut expression indicated by arrows).

Figure 5.3

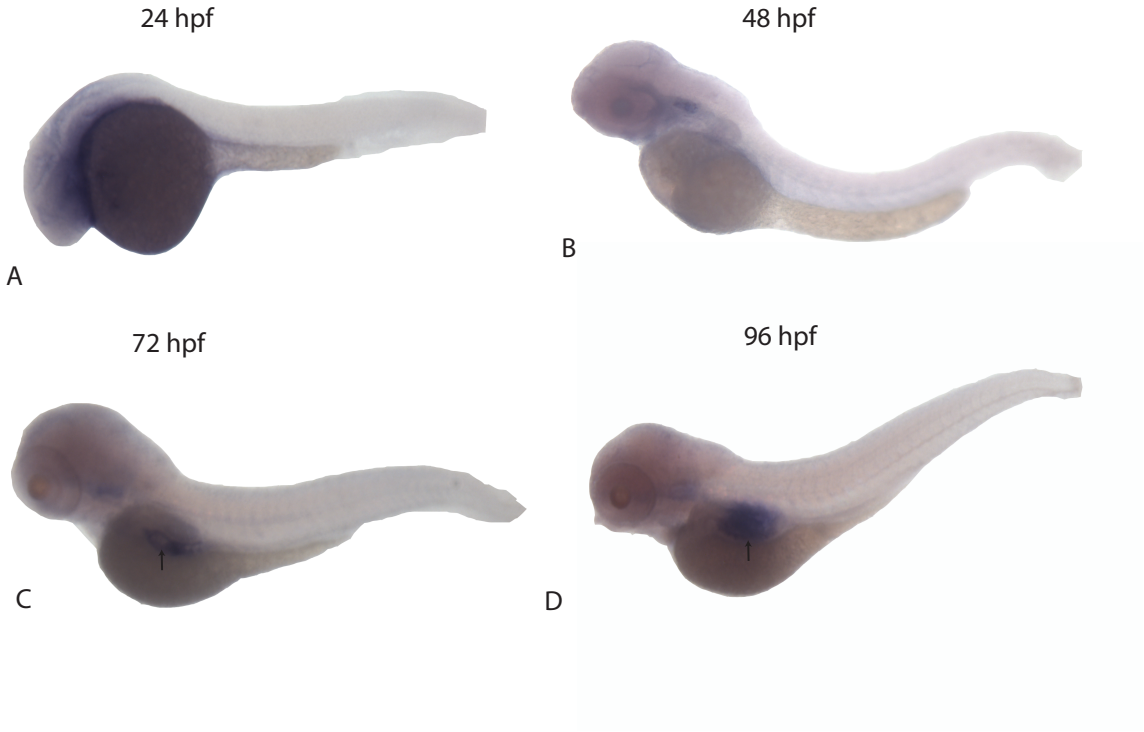

Figure 5.3 *lrba* expression pattern from 24-96 hpf. *In situ* hybridization showing that *lrba* has a very discrete expression pattern through all time points observed (Arrows indicate intestinal bulb expression). Expression is present along the yolk sack and weakly in the hindbrain at 24 hpf (A). Weak expression in the hindbrain continues throughout all times observed. Strong expression appears in the intestinal bulb from 72-96 hpf (C-D).

Figure 5.4

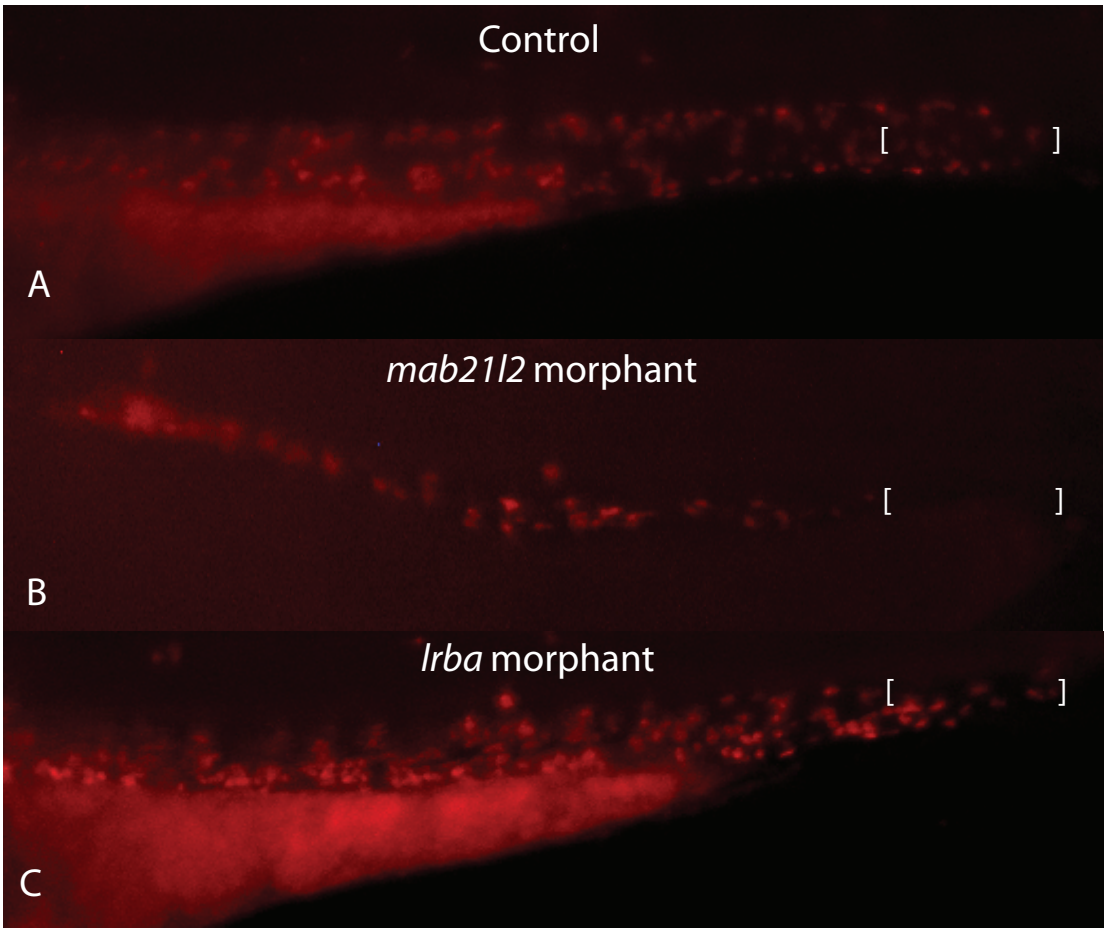

Figure 5.4 Enteric neuron presence in control, *mab21l2* morphant, and *lrba* morphant embryos. HuC/Elavl3 antibody stain shows differentiated neurons in control and morphant embryos at 120 hpf. *mab21l2* morphants show a distinct decrease in ENS neuron numbers and an aganglionosis in the distal part of the intestines compared to control embryos (A-B) (Brackets indicate aganglionic region in B and equivalent regions in A and C). *lrba* morphants do not display the same decrease in ENS neuronal number or aganglionosis in the distal intestine.

Chapter 6: Future Directions

ENS development is a complex process with multiple steps that must be coordinated in a tightly regulated manner. There must be a balance between proliferation and differentiation to obtain the right number of neurons in the right ratios to achieve a fully functioning gut. Errors that cause this balance can cause a range of outcomes from intestinal aganglionosis to less dramatic phenotypes that currently are undetectable by modern medicine, such as changes in the proportion of different neuron subtypes in the intestine. In the preceding chapters, we have demonstrated how just a few of the proteins involved in ENS differentiation can have a large effect on the development of ENPCs. As we obtain a better understanding of the molecular mechanisms of differentiation in the ENS, we can gain a better understanding of the GI disorders that afflict so many people around the world every year.

Given the complexity of ENS development, it is important that we understand not only specific steps but also where these steps fit into the entire developmental process. From neural crest specification and induction to the final location of the ENS, neurons and glia in the gut, the cells that give rise to the ENS, must be exposed to the right factors at the correct time to develop a fully functioning nervous system. All the aspects that we have discussed so far fit together to control ENPC differentiation as they migrate along to and along the gut (Fig 6.1).

As we saw in chapter one errors in the *med24* gene lead to the aganglionosis phenotype seen in *lessen* mutants (146). *med24* is not expressed in the ENPCs themselves but is expressed in the mesoderm of the developing gut where, as a member of the

transcriptional mediator complex, it plays a role in influencing the expression of several genes (146). In a microarray screen of *lessen* morphants compared to wild type embryos our lab found that *bmp-2* and *bmp-4* were upregulated in while *et-3* was downregulated (data not shown). This raises the possibility that *med24* may influence expression of these genes in the mesoderm.

Both Bmp and Et-3 then influence differentiation of ENPCs downstream by binding to their receptors on ENPCSs (Fig 6.1). Et-3 influences differentiation in a more straightforward way, as activating its receptor it blocks differentiation of ENPCs (96). Bmp signaling on the other hand is a much more complicated process that may involve alternate modes of influence. The specific way we studied the influence of Bmp was in relation to its involvement with *id2a* expression. Depending on the levels p-Smad either interacted with Sip1a to block *id2a* expression at high Bmp levels or didn't interact with Sip1a at low levels leading to *id2a* expression (Fig 6.1). Id2a subsequently prevent nNOS neuronal differentiation (Fig 6.1).

Bmp may also affect general neuronal differentiation and subtype differentiation through a Sip1a based method or other methods (Fig 6.1). Another wrinkle into this is the function of a Bmp pathway antagonist that may have a role in human HSCR patients (218, 219). Mab21l2 blocks Smad phosphorylization and thus may influence the effect of Bmp concentration on differentiation (Fig 6.1).

While there is a clear connection between all these different aspects of ENS development there are several unanswered questions and new opportunities provided by the results of this dissertation. Although the *Tg(-8.3bphox2b:kaede)* line was able to help us in our analysis of *med24* morphants, its true value might be its potential application in gene and drug discovery. Because of the dual fluorescent properties of the Kaede protein, it lends itself to easy initial analysis and screening for normal ENS development. Knocking out expression of specific genes and then observing the results in *Tg(-8.3bphox2b:kaede)* embryos will allow for rapid analysis of the effects of these genes on the migration and proliferative potential of ENPCs. The *Tg(-8.3bphox2b:kaede)* line can be used to quickly investigate the function of single genes in ENS development in a high throughput manner. Zebrafish are increasingly being used for high throughput small molecule screens to test the physiologic, cellular, and genetic effects of these molecules in an organism with a relatively high degree of similarity to humans (226, 227). These types of screens are frequently more informative than similar studies in mammalian cell culture (226, 227), and can be used to screen for drug effects in a wide range of diseases (228-232). By utilizing the *Tg(-8.3bphox2b:kaede)* line, we could easily test for small molecules and pathways that can inhibit ENPC migration and ENS formation. Alternatively, by injecting *Tg(-8.3bphox2b:kaede)* embryos with MED24 morpholinos or crossing these embryos with *lessen* mutants, we could quickly screen drugs for efficacy in an aganglionosis model. This high throughput method of screening drugs has huge potential in translational research dealing with ENS development.

While we have shown that there is overlap in the function of Ednrb1a and Ednrb1b, it is not clear molecularly how each one functions. Does each gene phosphorylate a specific set of proteins downstream of endothelin activation or is it some other modulator downstream that causes the difference seen? It also remains to be seen whether both genes act to influence pigmentation development only in the early embryo or in the development in juveniles (159). Creating a true *ednrb1b* mutant would allow for further exploration of these issues. By crossing the *rose* mutant line with an *ednrb1b* mutant line we would then be able to observe ENS development and see how it compares to our *ednrb* morphants and further validate our functional observations. It would also allow us to observe differences in juvenile pigmentation defects. In addition, *EDNRB* mutations are often found in concert with other mutations in HSCR patients and having a true *ednrb* mutant would allow us to see how different genes can modulate the effects of the two *ednrb* genes (233, 234).

It is still unclear whether or not Id2a maintains a pool of ENPCs by blocking general neural-promoting factors or ones specific to nNOS neurons. By comparing the expression patterns of *id2a* mutants or morphants with wild type embryos we could begin to look at which pro-neural genes are affected. It also is unclear at what point the initial specification of these nNOS neurons occurs. Does specification occur in the neural crest or later after the ENPCs have already started migrating? Is the specification random or are there certain precursors cells that are always specified and fated to become nNOS neurons? There are several ways we can begin to answer these questions. One approach to this question that would specifically investigate the role of ID2a function in specifying

nNOS enteric neurons, would be to create an ID2a mutant where its expression was under the control of a heat shock promoter. Using this mutant, we would be able to control when ID2a expression occurred. We could then knock down ID2a function at specific times in ENS development and narrow down the time period as to when it is important for these ENPCs to express ID2a. A second, more general method that will give us a view of fating of ENPC, is to utilize the brainbow system of fluorescent labeling (132). By utilizing the ENPC specific expression of the phox2b enhancer, we could drive the expression of an inducible Cre recombinase in ENPCs that, when activated in a *brainbow* reporter line at different points in ENS development, would lead to the expression of multiple, different fluorescent colors in the cells that make up the ENS. We can then follow development of these differentially labeled clones and see where the various colors end up later in ENS development. It would be easy to address whether the same regions of neural crest cells are always responsible for the same region in the ENS in multiple different embryos or is the process of populating the intestine with ENPCs much more random? We could then also use this transgenic line in combination with morphant analysis to see how the normal development of specific subpopulations of neurons may be perturbed when specific gene function is knocked down. Identifying when specification actually occurs is one of the most important efforts into further understanding neuronal subtype differentiation.

Even though different neuronal subtypes begin to appear at different times in ENS development, it may be possible that they are controlled through similar means. While we were able to show a specific role for BMP in ENS development and Id2a function, there

still is more to be learned. BMP clearly has roles in the development of other parts of the developing ENS outside of nNOS neurons (195, 196). Does BMP have other roles in development of neuronal subtypes or is it more of a general function? By identifying genes with a role in ENS development and conserved BMP-binding promoter regions, we can begin to elucidate BMP's further role in ENS differentiation and subtype specification.

The fifth chapter shows just how complicated studying ENS development in humans can be as many different genes could be involved in modulating the phenotype of HSCR patients. It also demonstrates the utility of using zebrafish as a model for the human condition, allowing us to easily test genes identified in patient samples for ENS phenotypes. We are currently working with the Hofstra lab to test additional candidate genes that are identified in HSCR patients for ENS defects in zebrafish.

The ENS is a complicated, but vitally important, part of life in vertebrates. The need for a precisely developed network of neurons to coordinate most of the functions of the gut makes ENS development a complex and fascinating issue. Differentiation is a key step in normal ENS development, as the variety and number of ENS neuron subtypes is significant. In this thesis, we have investigated several aspects of ENS differentiation, and our results have help fill important pieces of the puzzle. The more we know about ENS differentiation, the better we will be able to help the millions of people that suffer from GI disorders.

Figure 6.1

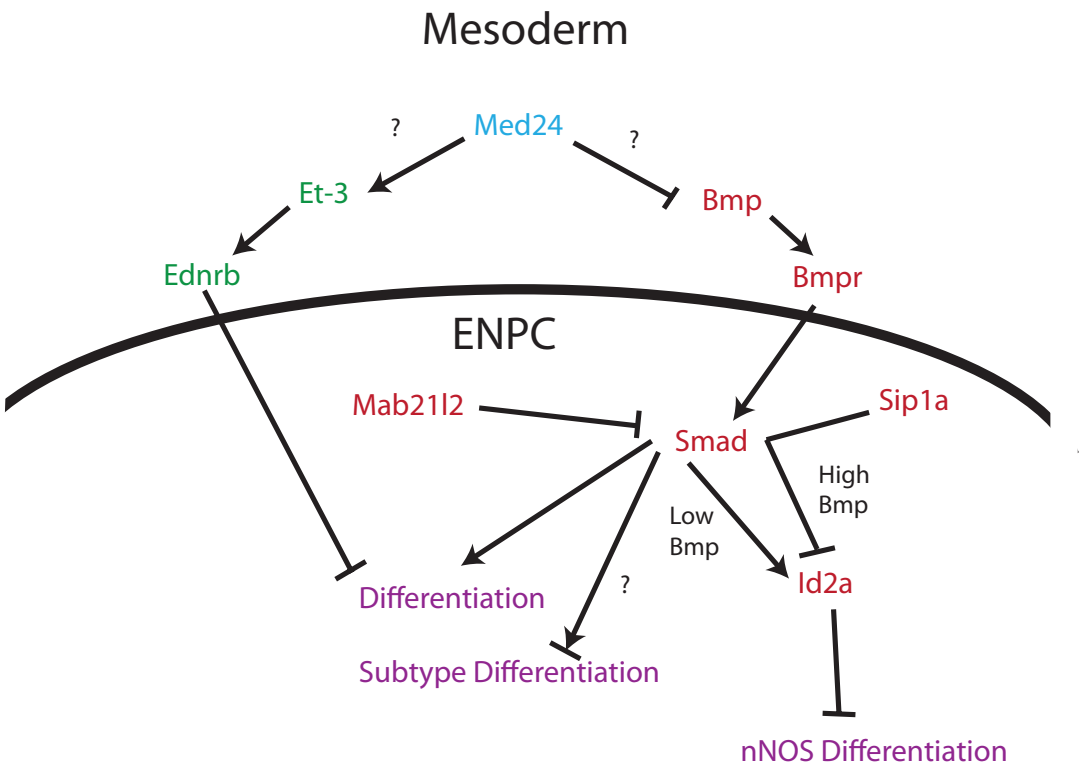
Mesoderm
?
Med24
?
Et-3
Bmp
Ednrb
Bmpr
ENPC
Mab21l2
Smad
Sip1a
High
Bmp
Low
Bmp
?
Id2a
Differentiation
Subtype Differentiation
nNOS Differentiation

Figure 6.1 Schematic of differentiation control in ENPCs. Arrows indicate activation/expression, blocks indicate blocking/repression. *med24* is expressed in the gut mesoderm where it may influence *bmp* and *et-3* expression. Et-3 subsequently activates Ednrb which blocks differentiation. Bmp activates Bmpr which in turn activates Smad which affects differentiation in several ways. Smad interacts with Sip1a at high levels to prevent *id2a* expression which itself blocks nNOS differentiation. Bmp signaling also blocks overall differentiation of ENPCs at high levels. Bmp signaling also affects neuronal subtype differentiation perhaps by both promoting differentiation and blocking differentiation.

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