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Review Article

Genetic regulation of enteric nervous system development in zebrafish

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The enteric nervous system (ENS) is a complex series of interconnected neurons and glia that reside within and along the entire length of the gastrointestinal tract. ENS functions are vital to gut homeostasis and digestion, including local control of peristalsis, water balance, and intestinal cell barrier function. How the ENS develops during embryological development is a topic of great concern, as defects in ENS development can result in various diseases, the most common being Hirschsprung disease, in which variable regions of the infant gut lack ENS, with the distal colon most affected. Deciphering how the ENS forms from its progenitor cells, enteric neural crest cells, is an active area of research across various animal models. The vertebrate animal model, zebrafish, has been increasingly leveraged to understand early ENS formation, and over the past 20 years has contributed to our knowledge of the genetic regulation that underlies enteric development. In this review, I summarize our knowledge regarding the genetic regulation of zebrafish enteric neuronal development, and based on the most current literature, present a gene regulatory network inferred to underlie its construction. I also provide perspectives on areas for future zebrafish ENS research.

Introduction to the enteric nervous system

The peripheral nervous system (PNS) encompasses all neurons and glia that reside outside of the brain and spinal cord and largely regulates unconscious events — such as the fight or flight response and digestive functions [1]. The largest branch of the PNS is the enteric nervous system (ENS). Also known as the 'gut brain,' the ENS is an autonomous network of neurons and glia located within the walls of the entire gut. The ENS mediates peristalsis, local digestive functions, and water balance in the gut, among many other functions [2]. However, although the human ENS contains more than 600 million neurons — rivaling the numbers of those found within the spinal cord — far less is known about its formation [2].

The vertebrate ENS primarily originates from neural crest cells (NCCs) [3–6]. NCCs are multipotent embryonic stem cells that migrate throughout the embryo, eventually contributing to numerous cell types in the vertebrate body [7,8]. NCCs are born along the anterior–posterior extent of the neural tube, and are subdivided according to their spatial level of origin — namely the 'cranial' from anterior, followed by the 'vagal', 'trunk', and 'sacral' populations in the most posterior [9]. Following emigration from the neural tube, vagal NCCs that approach the foregut are hereafter referred to as enteric NCCs (ENCCs). ENCCs are competent to become either enteric neurons or enteric glia [3,4]. ENCCs then migrate into and along the outer walls of the incipient gut tube and progress caudally along its length until reaching their final destinations, where they spatially position along the gut circumference. During their journey, ENCCs mainly migrate together in a 'follow the leader' chain pattern, such that chains of cells navigate the landscape of the growing gut tube [10–12]. Incredibly,

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during their migration ENCCs robustly expand in numbers sufficient to keep up with the growing gut tissue [13] and give rise to various enteric neuronal subtypes, as well as glia, which can be classified by molecular means [2,14].

Defects in ENS development and function manifest as various congenital and adult-onset gastrointestinal diseases. Such diseases include; Hirschsprung disease (HSCR), Esophageal Achalasia, Chronic Constipation, and Gastroesophageal Reflux Disease [15], among many others. In particular, HSCR is a birth defect characterized by the absence of ENS along variable lengths of the infant's gut, with colonic aganglionosis being the most common form, occurring every 1 in 5000 births [16,17]. The principal treatment for HSCR is invasive surgical resection of the aganglionic gut segment, highlighting the need for alternative therapies that may perhaps be based upon a more comprehensive understanding of ENS genesis *in vivo*.

To date, the field has identified evolutionarily conserved genetic and cellular signaling factors required for ENS development. Largely in the mouse model, progress has been made in identifying transcription factors required for ENS formation; these include Sox10, Phox2b, and Pax3 [18–20]. Among the most studied cellular signaling pathways during ENCC development, it is known that receptors, Rearranged during Transfection proto-oncogene (Ret) and Gdnf family member receptor alpha1 (Gfra1), and their ligand, glial-derived neurotrophic factor (GDNF), are instrumental for ENCC infiltration, proliferation, and survival in the gut [21–23]. Indeed, it has been appreciated that the loss of combinations of various intrinsic and extrinsic factors results in severe ENS defects such as HSCR, suggesting that complex phenomena underlie the formation of ENS [17].

For over 20 years now, the zebrafish, *Danio rerio*, has been leveraged as a relevant model to understand vertebrate ENS development, form, and function [24,25]. Zebrafish ENS development is under the control of genetic circuits, such as Sox10 [26], Phox2b [27], and the Ret-GDNF pathway [23,28–30], demonstrating the conservation of genetic pathways giving rise to the ENS, and thus the utility of this model for studying ENS development. In a nutshell, zebrafish offer many outstanding characteristics to study ENS development, including: **1**. Large clutches of transparent, externally developing embryos and larvae; **2**. Homologous organs and genes with high conservation to mammals; **3**. A large number of fluorescently tagged transgenic lines for live cell and whole-gut imaging; **4**. High amenability to transgenic, drug, and genetic approaches [24,25].

In this review, I summarize knowledge regarding the genetic and signaling level regulation of enteric neuronal development in zebrafish, and offer some brief perspectives about possible future directions in the field.

Zebrafish ENS developmental stages and major events

Following emigration from the neural tube, zebrafish vagal NCCs localize in ventrolateral domains posterior to the otic vesicles (Figure 1A). By 36 hours post fertilization (hpf), ENCCs then begin to migrate as two single chains medially toward and along the foregut entrance [28,31,32] (Figure 1B). Zebrafish ENCCs express a defining combination of marker genes that encode for various transcription factors and receptors; including *sox10*, *foxd3*, *hoxb5b*, *phox2bb*, *ret*, and *gfra1a/b* [23,27,28,33–37]. Once resident along the gut, ENCC chains migrate caudally within gut mesenchyme until they reach their final destinations, with the wavefront (a.k.a. vanguard cells) reaching the hindgut's distal region ~66–72 hpf [30,31,38]. Along the gut tube, mesenchymal tissues express chemoattractant-encoding genes, such as *gdnfa* [28], while intestinal tissues express additional signaling factor-encoding genes, such as *shha* [39].

Once reaching their destinations along the gut, which can extend from the foregut to the hindgut, ENCCs spatially pattern along the gut circumference, and begin to differentiate into enteric neurons [31,35] (Figure 1C), or glia [40,41], between 54 hpf and 7 days post fertilization (dpf) during the larval stage. Differentiating neurons express enteric neuronal subtype markers, such as *vipb*, *nos1*, *chata*, and *pbx3b* [36,41–43], while cells with differentiating glial identity express the markers *her4*, *cx43*, and *s100b* [40,41]. The zebrafish ENS continues neurogenesis after 7 dpf [40,43]; however, the ENS already contains an identifiable neuropil structure by 7 dpf [44]. The mature zebrafish ENS exists in one diffuse layer of neurons and glia, called the myenteric plexus, which is sandwiched between circular and longitudinal muscle layers of the gut [45]. While the zebrafish gut [46] and ENS represent simplified versions of the amniote counterparts — where zebrafish ENS is not arranged in ganglia and solely contains a myenteric plexus yet lack a submucosal plexus, the larger functions of the zebrafish ENS are conserved [25].

Spectacularly, research in the zebrafish ENS field has been especially fruitful for not only confirming the conservation of gene function across vertebrates — it has also functionally brought to light novel candidates that are required for ENS development. To date, we know of at least 65 genes (Table 1) [47] that are required for, and/or are functionally important for, zebrafish ENS development. While I do not discuss all the factors listed

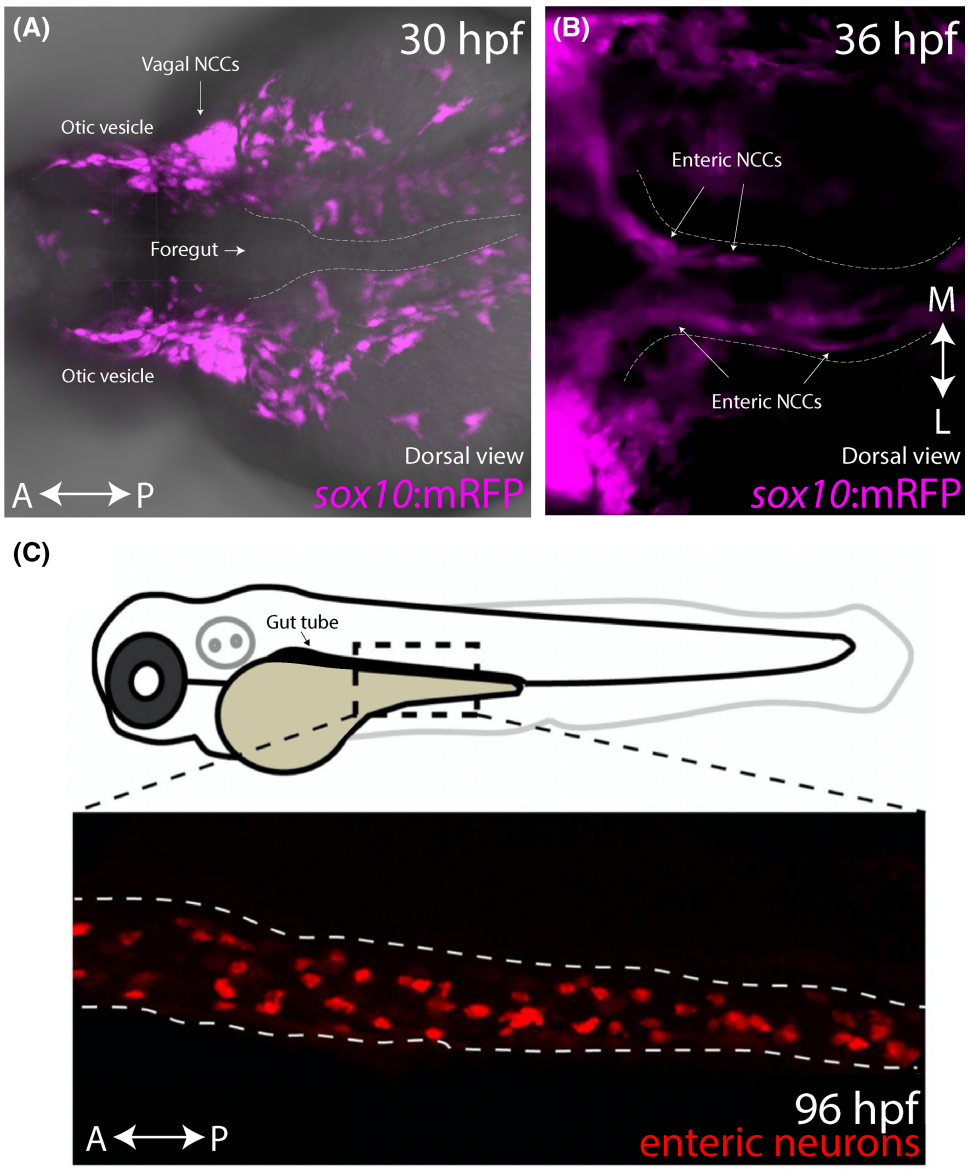

Figure 1. Early development of the zebrafish ENS from vagal and enteric neural crest cells.

A dorsal view of a transgenic zebrafish embryo at 30 hpf (**A**), or at 36 hpf (**B**) is shown, where NCCs are revealed by expression of *sox10*:mRFP [81] (shown here in magenta). (**A**) vagal NCCs reside posterior to the otic vesicles at 30 hpf (long arrow), while by 36 hpf (**B**) they emerge from the post-otic domain and thereafter are called enteric NCCs (arrows) as they enter and migrate along the foregut. (**C**) A cartoon schematic of a zebrafish larval fish at 96 hpf is shown, where the gut tube is outlined. The outlined image shows enteric neurons (red) along the gut tube. A, anterior; P, posterior; M, medial; L, lateral.

in Table 1, below I touch upon key gene regulatory network (GRN) takeaways from the data compiled and discuss what the results suggest regarding zebrafish enteric neuronal development.

A GRN crucial for enteric neuronal development is present in zebrafish

Not surprisingly, many genes encoding for transcription factors that are expressed in and required for the specification of NCC populations are functionally important for zebrafish ENS development. Specifically, genes

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Table 1. Genes with published functional roles in zebrafish ENS development Part 1 of 4

Gene name	Gene symbol	Mutation or condition	Phenotype(s)	Reference(s)
acyl-CoA synthetase short chain family member 2	*acss2*	MO-acss2	Reduction in ENS by larval stage	[59]
achaete-scute family bHLH transcription factor 1a	*ascl1a*	ascl1at$^{25215/t25215}$	ENCCs present; reduction in ENS by larval stage	[56]
AT hook containing transcription factor 1	*ahctf1*	ahctf1$^{ti262c/ti262c}$	ENCCs present; reduction in ENS by larval stage	[82]
ADP-ribosylation factor-like 6 interacting protein 1	*arl6ip1*	MO-arl6ip1	ENCCs reduced; reduction in ENS by larval stage	[83]
beta-secretase 2	*bace2*	MO-bace2	Mild reduction in ENS by larval stage	[84]
bone morphogenetic protein 2b	*bmp2b*	MO-bmp2b	ENCCs absent; complete loss of ENS	[61]
chromodomain helicase DNA binding protein 7	*chd7*	chd7$^{hsi3/hsi3}$; MO-chd7	Reduction in ENS by larval stage	[71,73,74]
chromodomain helicase DNA binding protein 8	*chd8*	CRISPR-chd8; MO-chd8	Mild reduction in ENS by larval stage	[85]
DENN/MADD domain containing 3a	*dennd3a*	CRISPR-dennd3a; MO-dennd3a	Reduction in ENS by larval stage	[77]
distal-less homeobox 2a	*dlx2a*	CRISPR-dlx2a	Reduction in ENS by larval stage	[86]
DNA (cytosine-5-)-methyltransferase 1	*dnmt1*	dnmt1$^{s904/s904}$	ENCCs present; reduction in ENS by larval stage	[68]
Down syndrome cell adhesion molecule a	*dscama*	MO-dscama	Mild reduction in ENS by larval stage	[84]
Down syndrome cell adhesion molecule b	*dscamb*	MO-dscamb	Mild reduction in ENS by larval stage	[84]
elongator acetyltransferase complex subunit 1	*elp1*	MO-elp1	Reduction in ENS by larval stage	[77]
endothelin receptor type Bb	*ednrbb*	MO-ednrbb	Reduction in ENS by larval stage	[59]
enolase 3, (beta, muscle)	*eno3*	MO-eno3	Reduction in ENS by larval stage	[59]
fascin actin-bundling protein 1a	*fscn1a*	fscn1a$^{zd1011/zd1011}$	Mild reduction in ENS by larval stage	[87]
forkhead box D3	*foxd3*	foxd3$^{m188/m188}$; foxd3$^{zdf10/zdf10}$	ENCCs absent; complete loss of ENS	[34,50,51,54]
forkhead box J3	*foxj3*	CRISPR-foxj3	Reduction in ENS by larval stage	[86]
gdnf family receptor alpha 1a	*gfra1a*	MO-gfra1a	Reduction in ENS by larval stage	[23]
gdnf family receptor alpha 1b	*gfra1b*	MO-gfra1b	Reduction in ENS by larval stage	[23]
glial cell derived neurotrophic factor a	*gdnfa*	MO-gdnfa	Reduction in ENS by larval stage	[28]
gutless wonder	*glw*	glw$^{b871/b871}$	Reduction in ENS by larval stage	[88]
gutwrencher	*gwr*	gwr$^{b1088/b1088}$	Reduction in ENS by larval stage	[88,89]

Continued

Table 1. Genes with published functional roles in zebrafish ENS development Part 2 of 4

Gene name	Gene symbol	Mutation or condition	Phenotype(s)	Reference(s)
hairy-related 9	*her9*	her9$^{uky2/uky2}$	Reduction in ENS glia by larval stage	[90]
heart and neural crest derivatives expressed 2	*hand2*	MO-hand2	Reduction in ENS by larval stage	[39]
histone deacetylase 1	*hdac1*	hdac1^{b382}	Reduction in ENS by larval stage	[91]
homeobox B5b	*hoxb5b*	*vp16-hoxb5b* mRNA	Expanded ENCCs; reduction in ENS by larval stage	[37]
Indian hedgehog signaling molecule a	*ihha*	MO-ihha	Reduction in ENS by larval stage	[92]
jumonji and AT-rich interaction domain containing 2a	*jarid2a*	CRISPR-jarid2a	Reduction in ENS by larval stage	[86]
kinesin family binding protein	*kifbp*	kifbp$^{st23/st23}$	Abnormal enteric neuron axonal connections; ENS maturation defect by larval stage	[93]
mab-21-like 2	*mab21l2*	mab21l2$^{au10/au10}$; MO-mab21l2	Reduction in ENS by larval stage	[92]
mediator complex subunit 24	*med24*	med24$^{w24/w24}$; MO-med24	Reduction in ENCCs; reduced migration of ENCCs; reduction in ENS by larval stage	[38,94]
Mitogen-activated proteiin kinase 8	*mapk8*	CRISPR-mapk8	Reduction in ENS by larval stage	[60]
mitogen-activated protein kinase 10	*mapk10*	mapk10$^{fci200/fci200}$; MO-mapk10	Reduction in ENS by larval stage	[29]
MYCN proto-oncogene, bHLH transcription factor	*mycn*	CRISPR-mycn	Reduction in ENS by larval stage	[86]
myeloid ecotropic viral integration site 3	*meis3*	MO-meis3	ENCCs reduced; reduced migration of ENCCs; reduction in ENS by larval stage	[32]
neuregulin 1	*nrg1*	MO-nrg1	ENCCs reduced; reduction in ENS by larval stage	[95]
nicalin	*ncln*	CRISPR-ncln; MO-ncln	Reduction in ENS by larval stage	[77]
nucleoporin 98 and 96 precursor	*nup98*	CRISPR-nup98; MO-nup98	Reduction in ENS by larval stage	[77]
PAF1 homolog, Paf1/RNA polymerase II complex component	*paf1*	MO-paf1	ENCCs absent; complete loss of ENS	[96]
paired box 3a	*pax3a*	MO-pax3a	ENCCs absent; complete loss of ENS	[49]
paired like homeobox 2A	*phox2a*	CRISPR-phox2a	Reduction in ENS by larval stage	[86]
paired like homeobox 2Bb	*phox2bb*	MO-phox2bb	Reduction in ENS by larval stage	[27]
polymerase (RNA) III (DNA directed) polypeptide B	*polr3b*	polr3b$^{m74/m74}$	Reduction in ENS by larval stage; ENS maturation defect by larval stage	[97]

Continued

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Table 1. Genes with published functional roles in zebrafish ENS development Part 3 of 4

Gene name	Gene symbol	Mutation or condition	Phenotype(s)	Reference(s)
protein tyrosine phosphatase non-receptor type 11a	*ptpn11a*	MO-ptpn11a	Reduction in ENS by larval stage	[98]
RAD21 cohesin complex component a	*rad21a*	MO-rad21a	Reduction in ENS by larval stage	[99]
Ras association and DIL domains	*radil*	MO-radil	ENCCs absent; complete loss of ENS	[100]
ret proto-oncogene receptor tyrosine kinase	*ret*	$ret^{hu2846/hu2846}$; $ret^{wmr1/wmr1}$; MO-ret	Reduction in ENCCs; reduced migration of ENCCs; reduction in ENS by larval stage; loss of ENS by larval stage; Loss of ENS function	[23,29,30,33,57–59,101]
ring finger protein 2	*rnf2*	CRISPR-rnf2	ENCCs reduced; reduction in ENS during by larval stage	[75]
sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	*sema3c*	MO-sema3c	Reduction in ENS by larval stage	[58]
sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D	*sema3d*	MO-sema3d	Reduction in ENS by larval stage	[58]
SH3 and PX domains 2Aa	*sh3pxd2aa*	MO-sh3pxd2aa	Reduction in ENS by larval stage	[59]
smoothened	*smo*	smo, unspecified mutation	ENCCs nearly absent	[39]
SRY-box transcription factor 10	*sox10*	$sox10^{t3/t3}$; $sox10^{m618/m618}$; $sox10^{tw2/tw2}$; $sox10^{tw11/tw11}$; MO-sox10	ENCCs nearly absent; complete loss of ENS	[26,52,53,102]
SRY-box transcription factor 32	*sox32*	MO-sox32; $sox32^{ta56/ta56}$	ENCCs absent; complete loss of ENS	[39]
tetratricopeptide repeat domain 8	*ttc8*	MO-ttc8	ENCCs reduced; reduction in ENS during by larval stage	[103]
thymus, brain and testes associated	*tbata*	CRISPR-tbata; MO-tbata	Reduction in ENS by larval stage	[77]
transcription factor AP-2 alpha	*tfap2a*	$tfap2a^{m610/m610}$; $tfap2a^{ts213/ts213}$	ENCCs absent; complete loss of ENS	[50,51]
transcription factor AP-2 beta	*tfap2b*	$tfap2b^{re32/re32}$	Reduction in ENS by larval stage	[104]
T-box transcription factor 2b	*tbx2b*	CRISPR-tbx2b	Reduction in ENS by larval stage	[60]
ubiquitin protein ligase E3 component n-recognin 4	*ubr4*	MO-ubr4	Reduction in ENS by larval stage	[59]
ubiquitin recognition factor in ER associated degradation 1	*ufd1l*	CRISPR-ufd1l	Mild reduction in ENS by larval stage	[60]
ubiquitin-like with PHD and ring finger domains 1	*uhrf1*	$uhrf1^{b1115/b1115}$	ENCCs present; reduction in ENS during larval stage	[68,88]

Continued

Table 1. Genes with published functional roles in zebrafish ENS development Part 4 of 4

Gene name	Gene symbol	Mutation or condition	Phenotype(s)	Reference(s)
zinc finger E-box binding homeobox 2a	*zeb2a*	MO-zeb2a	ENCCs absent; complete loss of ENS	[55]

A table summarizing genes involved in zebrafish ENS development, in alphabetical order. The table depicts: gene name, gene symbol, mutation or condition, phenotype(s), and reference(s). ENCCs, enteric neural crest cells; ENS, enteric nervous system. Under the mutation or condition column, stable mutant lines are denoted with a line designation, while perturbation conditions are listed as performed, i.e. MO: morpholino, CRISPR: F0 crispant data.

within the GRN of zebrafish NCC [48] are crucial for the subsequent development of ENCCs. For example, zebrafish with reduced *pax3a* levels [49], or harboring mutations in *tfap2a* [50,51], *sox10* [26,27,52,53], *foxd3* [50,51,54], and *zeb2a* [55] (formerly known as *sip1a*), all present with near complete ENCCs loss and total aganglionosis in larvae.

Across vertebrates, a core enteric GRN has been constructed from data largely using the mouse and chicken, which centers — the genes *Sox10*, *Pax3/7*, *Phox2b*, *GDNF*, *Ret*, *Hand2*, and *Ascl1a* [9]. Similarly, the execution of zebrafish enteric neuronal development relies upon an enteric neuron GRN (Figure 2). The zebrafish enteric neuron GRN represents transcription factors, chromatin-associated proteins, and signaling molecules, which together are expressed in vagal NCCs, ENCCs, microenvironmental tissues along ENCC pre-gut entry migratory paths, and/or in the developing gut tissues.

In zebrafish, morpholino-knockdown of *pax3a* [49], or loss of function mutation in *foxd3* [54], decreases the expression of *sox10* in migratory vagal NCC, later resulting in the near complete loss of ENCCs, and therefore, total aganglionosis in larvae. As evident in data from a zebrafish *sox10* mutant line (formerly known as *colorless*) [27], *sox10* is required for the expression of *phox2bb* and *ret* in ENCCs en route to and along the gut. While a paltry number of ENCCs reach the foregut in *sox10* mutants, the ENCCs do not further colonize the gut, and total aganglionosis occurs. In *foxd3* mutants [54], *hand2* expression along the gut is abolished, highlighting *foxd3*'s upstream role within the GRN. Furthermore, mutation and/or morpholino-knockdown of *ascl1a* [56], *hand2* [39], or *phox2bb* [27], results in compromised ENS development; although ENCCs are initially present, there is a reduction in enteric neuron numbers by the larval stage, likely due to defects in proliferation, migration, survival and/or differentiation of ENCCs.

The Ret signaling pathway, a key part of the enteric GRN, is necessary for zebrafish ENS development. Reduction in *ret* expression and/or function, via morpholino-knockdown or mutations, elicits enteric neuron loss, manifesting as colonic, and/or total aganglionosis [23,29,30,33,57–59]. In particular, dose-dependent disruption of *ret* in zebrafish, via heterozygous and homozygous mutant analyses [29,30], leads to colonic aganglionosis or total aganglionosis, respectively, demonstrating that even partial disruption of *ret* causes severe ENS defects. *mapk10*, encoding for the intracellular kinase Mapk10, has been shown to genetically interact with *ret*. Heterozygous disruption of *ret* combined with homozygous mutations in *mapk10* increases the severity of gut aganglionosis [29], therefore, putting forth Mapk10 as a timely player that may function to influence ENS development synergistically with Ret. Moreover, similarly to *mapk10*, various other genes [58,60] have been discovered to genetically interact with *ret*, such as *sema3c/d* [58], expanding the Ret-associated network. Finally, morpholino-knockdown of the Ret pathway-associated genes, *gdnfa* [28], encoding for ligand GDNF, and *gfra1a/b* [23], encoding for co-receptors Gfra1a/b, causes drastic reductions in enteric neuron numbers along the larval gut.

Further building the zebrafish enteric GRN, various other studies have been able to link signaling level inputs to intracellular signaling effectors that drive ENS development. These recent studies have begun to pave the way for more thoroughly fleshing out the signaling networks underlying the complexity of ENS development.

One example includes the linkage of the bone morphogenetic protein pathway to downstream ENS regulators. *bmp2b* is expressed along the developing gut path of ENCCs during ENS development in zebrafish [61]. Morpholino-knockdown of *bmp2b* leads to total aganglionosis due to ENCC loss, as well as disruptions in enteric GRN factor expression. Specifically, *bmp2b* reduction leads to the abolishment of *gdnfa* expression in the gut tube, therefore, placing *bmp2b* within the zebrafish enteric GRN.

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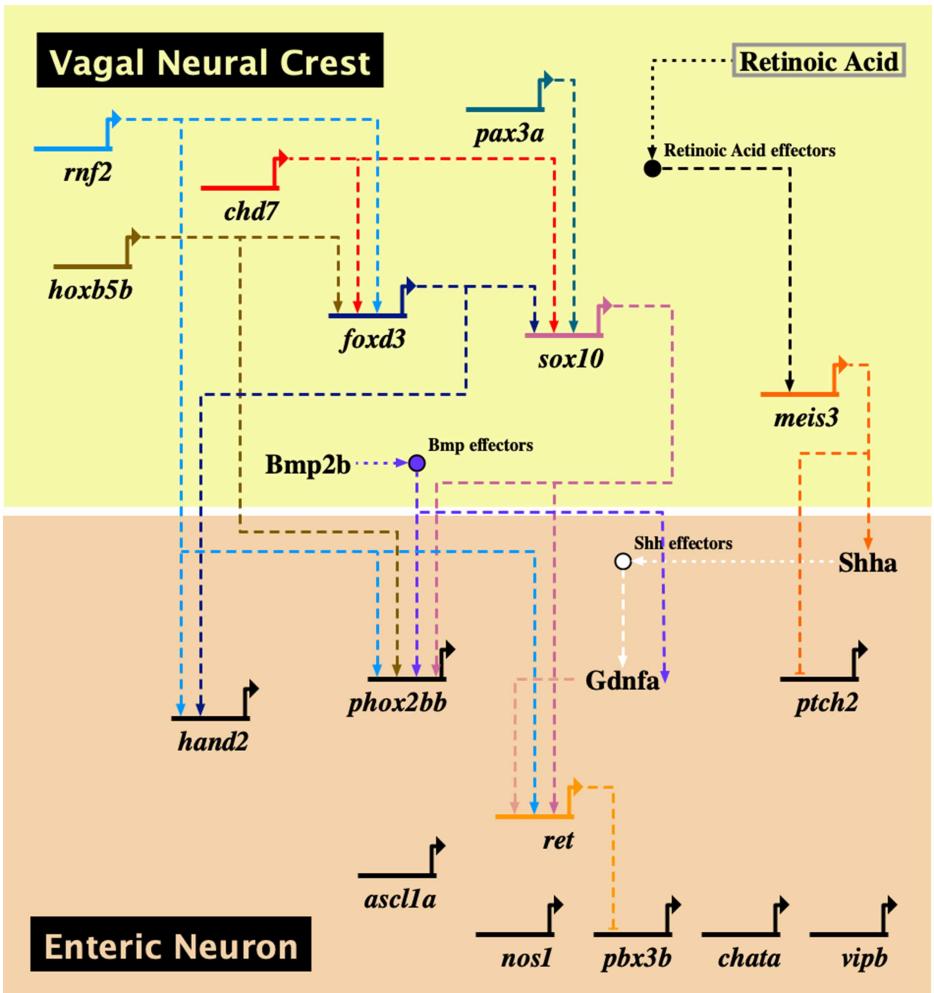

Figure 2. The zebrafish enteric neuron gene regulatory network.

The zebrafish enteric neuron GRN is constructed from many studies that examined how the loss or gain of gene function affected the expression of other NCC and enteric-associated genes, usually via *in situ* hybridization assays. The GRN takes into account how extrinsic factors, such as signaling molecules (i.e. retinoic acid), are known to influence GRN factor expression. Interactions between GRN nodes that have not yet been directly verified are depicted by dashed lines, with sharp arrows denoting enhancement or up-regulation of gene expression, while blunt arrows indicate inhibition.

Another example is illustrated by the association between the Sonic Hedgehog (Shh) pathway and zebrafish ENS. *shha*, encoding for the ligand Shha, is expressed in gut endoderm during pre-gut entry phases ~30 hpf, and along the intestine during colonization phases between 48 and 60 hpf, while *ptch2* (formerly known as *ptc1*), encoding for the Shh receptor and repressor Patched 2, is expressed within gut mesenchyme and in a subset of ENCCs between 36 and 42 hpf [39]. A zebrafish *shha* mutant line (formerly known as *sonic you*), presents with total aganglionosis due to failure of ENCC colonization of the gut [39]. In addition, treatment with the Shh pathway inhibitor Cyclopamine phenocopies the *shha* mutant ENCC colonization phenotype, and depletes *gdnfa* expression along the gut, indicating that intestinal Shh signaling is upstream of *gdnfa* expression during zebrafish ENS development.

Further connecting signaling and intracellular inputs, Meis3 has been identified as an important factor during early zebrafish ENS development. *meis3*, which encodes for the co-transcriptional regulator Meis3, is expressed within ENCCs, and along ENCC migratory paths, during pre-gut entry and early colonization phases of ENS development [32,36]. Reduction in Meis3 via morpholino stalls ENCC migration along the gut and causes colonic aganglionosis. Meis3 knockdown also results in a near complete loss of intestinal *shha*

expression, while also eliciting an expansion in *ptch2* expression in the adjacent gut mesenchyme — suggesting that Meis3 either directly or indirectly up-regulates *shha*, while it represses *ptch2* in the gut [32]. On the other hand, depletion of retinoic acid (RA) production via pharmacological inhibition of Aldh1a2 restricts *meis3* expression, resulting in ENCC failure to colonize the gut, and total aganglionosis [62].

Recently, a connection between Ret signaling and enteric neuronal subtype expression has been brought to light. Zebrafish larvae heterozygous for a *ret* mutation [30] display colonic aganglionosis, due to defects in proliferation and migration along the gut. However, the *ret* mutant enteric neurons prematurely differentiate, whereby they express aberrantly elevated levels of *pbx3b*, encoding for Pbx3b, a transcription factor associated with excitatory neuron differentiation in mammalian ENS [63]. In congruence, immunohistochemical detection against choline acetyltransferase, an indicator of acetylcholine presence, was elevated within *ret* mutant enteric neurons, when compared with sibling controls [30]. Thus, while it is not yet clear whether Ret indirectly or directly regulates the expression of *pbx3b*, the results suggest *pbx3b* is downstream of the Ret pathway.

Epigenetic regulation of zebrafish ENS development

Epigenetics involves understanding how cell states and/or gene expression are altered without changes in the DNA sequence of a cell. While epigenetic regulation of gene expression is known to occur in NCC populations [64,65], largely through changes in chromatin state, we know relatively little about this in terms of the zebrafish ENS. Nonetheless, several studies have begun to shed light on how epigenetic factors regulate zebrafish ENS development and enteric GRN factor expression.

One epigenetic modification is DNA methylation. DNA methylation is associated with the suppression of gene expression and is mediated by DNA methyltransferases (Dnmt). *De novo* DNA methylation is created by Dnmt3a/b, while maintenance of DNA methylation marks is enabled by Dnmt1 [66]. The Ubiquitin-like protein containing PHD and RING finger domains 1 (Uhrf1) is responsible for recruiting Dnmts to unmethylated DNA [67].

While zebrafish carrying homozygous loss-of-function mutations in *uhrf1* or *dnmt1* display no apparent change in ENCC production during pre-gut entry and early gut migratory stages, later during larval stages mutants present with strong, yet variable intestinal hypoganglionosis and colonic aganglionosis phenotypes [68]. *uhrf1* and *dnmt1* mutant ENS phenotypes are likely due to diminished ENCC proliferation, migration, or survival; however, it is not yet clear what downstream ENS-related gene expression is altered in these zebrafish mutants. Interestingly, ENS phenotypes detected within double mutants for *uhrf1* and *dnmt1* are not more severe than those of the single mutants [68], suggesting Uhrf1 and Dnmt1 co-operate, and that DNA methylation as a whole is required for proper ENS development.

Besides DNA methylation, other epigenetic influences are essential for zebrafish ENS formation. The enzyme Chromodomain helicase DNA-binding protein (CHD) 7 is a chromatin remodeler [69]. CHD7 can also act as a co-transcriptional regulator at promoters and enhancer regions, either as a context-dependent repressor or activator, to affect gene expression [70]. Loss of *chd7* via morpholino-knockdown in zebrafish results in widespread phenotypes, mimicking the human congenital condition known as CHARGE syndrome [71], which affects many tissue and organ systems, including the eye, craniofacial tissues [72]. Zebrafish *chd7* loss of function embryos suffer from altered NCC specification, exhibiting decreased expression of NCC specifiers *foxd3* and *sox10* within cranial and vagal domains, as well as reduced *crestin*$^{+}$ ENCC en route to and along the foregut [71]. Not surprisingly, *chd7* loss of function embryos display complete ENS loss by the larval stage [71,73], and disrupted gut motility [74].

Recently one factor associated with chromatin modification, Ring finger protein 2 (Rnf2), has been discovered to impact zebrafish ENS formation [75]. Modifying histones to control chromatin state is another prominent way regulators exert epigenetic control over gene expression. The mammalian Polycomb repressive complex 1 contains the ubiquitin E3 ligase RING1, as either RING1A or RING1B, catalyzing the ubiquitination of Lys119 of histone H2A, largely a repressive mark [76]. Zebrafish mutants for *rnf2*, with predicted homology to *Ring1b*, present colonic aganglionosis, intestinal hypoganglionosis, and a reduction in gut tube ENCCs [75]. Examination during early NCC specification stages in *rnf2* mutants revealed that the zebrafish NCC specifiers *foxd3* and *sox9b* were differentially reduced in expression amongst cranial, vagal, and trunk NCC domains, where *sox9b* exhibited a strong loss of expression in cranial NCC, while *foxd3* was globally restricted. During gut pre-entry stages, the ENCC expression of *phox2bb*, *ret*, and *hand2* was reduced [75], suggesting that Rnf2 regulates these developmental ENS genes.

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Knockdown of candidate genes identified from large-scale HSCR-patient datasets display zebrafish ENS defects

Within the past several years, zebrafish have been leveraged to determine if candidate HSCR genes identified from large-scale studies are required for ENS development. Specifically, several large studies analyzed HSCR-patient sequencing datasets and prioritized candidates for knockdown using the zebrafish model. These recent studies have identified many conserved, novel genes not known to have previous roles in ENS development. The identified genes include; *dennd3*, *ncln*, *nup98*, and *tbata* [77]; *ufd1l*, *tbx2b*, *slc18a1*, and *mapk8* [60]; and *acss2*, *sh3pxd2aa*, *eno3*, and *ubr4* [59] (Table 1). With this exciting field of zebrafish ENS research still relatively new, it will be interesting to see how the identified candidate genes functionally affect specific aspects of enteric development.

Summary

Over the past 20 years, there have been an increasing number of studies that utilize zebrafish to delineate the genetic networks, signaling landscapes, and epigenetic influences that underpin early ENS formation. To date, these studies have illuminated that at least 65 genes are essential for zebrafish ENS development (Table 1). As such, it is important to provide a timely overview of the GRN underlying zebrafish enteric neuron development, which I have synthesized from numerous studies and depicted using the GRN Biotapestry model [78] (Figure 2). Additionally, many recent transcriptomic studies have expanded our knowledge of the genes expressed within developing zebrafish ENS cells [36,40–42,79,80]. Looking to the future, it will be essential to validate connections between gene nodes within the GRN and incorporate how other newly discovered factors affect ENS ontogenesis.

Perspectives

- Zebrafish have been used as a robust and relevant model to understand vertebrate ENS development for over 20 years. To date, the field has discovered 65 genes necessary for proper ENS development.
- Genetic, epigenetic, and signaling inputs direct the early development of enteric neurons from vagal and ENCCs during ENS development. Validation of GRN links will be critical to resolve the zebrafish enteric GRN.
- Many novel candidate genes have been identified from HSCR-patient data sets as important for ENS development. Moving forward, it will be important to decipher how the newly identified genes regulate specific aspects of enteric development, how they interact with enteric GRN modules, and for purposes of furthering HSCR research, it will be important to validate findings in zebrafish with mammalian and human models of enteric development.

Competing Interests

The author declares that there are no competing interests associated with this manuscript.

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Abbreviations

dpf, days post fertilization; ENCC, enteric neural crest cell; ENS, enteric nervous system; GDNF, glial-derived neurotrophic factor; GRN, gene regulatory network; hpf, hours post fertilization; NCC, neural crest cell; PNS, peripheral nervous system; RA, retinoic acid.

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