

Transcriptional Regulation
of Zebrafish Enteric
Nervous System
Development

Thesis by

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The Caltech logo, featuring the word "Caltech" in a bold, orange, sans-serif font.

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The defense gives me a chance to look back and reflect on how I ended up where I am today. So I'd like to share a short personal story about my journey. Please bear with me for a few minutes.

I went to Cornell for undergrad—a beautiful school with amazing views in every season, except for its very long winter. At the time, I was deeply interested in both biology and physics, and I struggled for quite a while to choose between them.

I once shared this dilemma with a physics professor. He said, “If you really like physics that much, why don't you drop biology?” So I dropped physics.

But I still kept thinking about how to apply physics to biological problems. In my sophomore year, Kelly Liu became my advisor. She's a developmental biologist, and although my interests didn't quite align at the time, she was incredibly supportive. She told me, “I may not be able to solve your problem, but I know someone who can.”

So she introduced me to Gerald Feigenson—a chemist. And somehow, my biology-and-physics problem ended up being solved by a chemist.

Gerald is one of the founders of biophysics at Cornell, and he became a long-term mentor to me. He not only introduced me to biophysics, but also kept asking me an important question—not just whether I was working hard, but whether I was doing something I truly enjoyed.

I later joined a structural biology lab led by Joshua Chappie, where I was encouraged to explore freely. That experience meant a lot to me, and I don't think I would be here today without that support.

When I applied to graduate school, Caltech wasn't even on my list. But Gerald immediately pointed that out. He said, "Why not Caltech? I had a great time there—and honestly, I prefer it much more than MIT."

So I applied... and I ended up here.

Interestingly, I didn't end up doing biophysics. Instead, I joined a developmental biology lab—and had a lot of fun. I still remember the first time I met Marianne. She showed me a beautiful video of cells migrating in a chick embryo.

And I suddenly realized—I had seen something like this before, years ago, at a seminar. At the time, I didn't remember the science... but I did remember that the speaker was very good-looking.

So the lesson I learned is: you should really pay attention to every random seminar you walk into.

Jokes aside, I'd like to borrow a line from *Legally Blonde*:

"To everyone who has been present in my life, I'm standing here today because you helped me find my way."

I would like to express my deepest gratitude to the many people who have supported me along this journey. Pursuing a Ph.D. is not easy, and I could not have done it alone.

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Thank you, Mom and Dad. I hope that one day I can support your dreams, just as you have supported mine.

Last but not least, I want to thank myself—for continuing forward and never giving up. I still remember, as a child, dreaming excitedly about who I might become.

Life is an ongoing journey, but today I want to pause, look back, and say with confidence that I am becoming someone my five-year-old self would be proud of.

Thank you again for joining my defense, and I hope you have a wonderful afternoon.

ABSTRACT

The vertebrate enteric nervous system (ENS) is mainly derived from vagal neural crest cells, which enter the foregut as progenitors that migrate from rostral to caudal to populate the entire length of the gut. Here, we show that transcription factors *sox11a* and *sox11b*, zebrafish orthologs of the human SOX11 gene, are highly expressed in neural crest cells transitioning from progenitors to differentiating neuronal subtypes. Accordingly, CRISPR-Cas9 depletion shows that loss of *sox11* paralogs reduces the number of neurons that express the inhibitory motor neuron marker *adcyap1b* without affecting cell proliferation or death. Transcription factor footprinting analysis of open chromatin regions identified by ATAC-seq reveals Sox11 binding sites in the *adcyap1b* enhancer. Furthermore, mutational analysis shows these binding sites are required for mediating enhancer-driven reporter expression. Taken together, our results demonstrate an important and previously unrecognized role for *sox11a* and *sox11b* in neuronal subtype specification in the developing zebrafish ENS.

To further probe neuronal differentiation in the developing ENS, we examine its spatial structure using multiplex hybridization chain reaction in situ hybridization. Developing a pipeline that covers both the sample preparation and downstream imaging analysis, we are able to capture the temporal- and spatial- specific expression pattern of neuronal clusters in zebrafish ENS at multiple developmental stages. The results reveal spatial heterogeneity in neuronal subtypes and changes as a function of stage in neuronal composition.

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Y. H. took a leading role in the conception of the project, performing experiments with her collaborators, collecting and analyzing data, and writing the manuscript with her advisor M.E.B.

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NOMENCLATURE

Glia. A group of non-neuronal cell. Glia are crucial for supporting and protecting the environment of the nervous system .

Myenteric Plexus. A part of the enteric nervous system that resides in between the inner circular and longitudinal muscle layers in the gut.

Neural Crest Cell. A group of multipotent stem cells that delaminate from the neural tube during early development of vertebrates, and migrate throughout the body to give rise to multiple organs and tissues.

Rostro-Caudal Axis. Anatomical orientation referring to from the head to tail along the body axis.

Sacral Neural Crest Cell. A group of neural crest cells that delaminate from neural tubes posterior to somite 28, and contribute to the formation of sympathetic ganglia and enteric ganglia.

Submucosal Plexus. A part of the enteric nervous system that resides within the submucosal layer in between mucosa and the circular muscle layer.

Vagal Neural Crest Cell. A group of neural crest cells that delaminate from neural tubes between somites 1-7, and contribute to the formation of both enteric ganglia and cardiac ganglia.

Chapter 1

INTRODUCTION

**THE GUT IS INNERVATED BY THE ENTERIC NERVOUS SYSTEM (ENS),
AN INDEPENDENT SYSTEM OF THE CENTRAL NERVOUS SYSTEM**

My PhD work has focused on deciphering the gene regulation program underlying development of the vertebrate enteric nervous system (ENS), the largest and most complicated portion of the peripheral nervous system, which provides intrinsic innervation to the gastrointestinal (GI) tract. Due to its ability to sense and initiate reflex responses independent of central nervous system (CNS) regulation, the ENS has long been referred to as ‘the second brain’^{1,2}. As early as 1755, Albert Von Haller reported that intestines ‘preserved their peristaltic motion’ after being removed from the body³. The notion that ENS could function independently of the CNS was further supported by multiple experiments, demonstrating that local stimulation is sufficient to trigger neural responses in isolated intestines⁴, and such response results in an ascending excitation and a descending inhibition around the stimulus point⁵. The word ‘peristaltic reflex’ was coined to describe such propulsion that moves the content along the gut⁶. Understanding of the function of the ENS has expanded from regulating gut motility, absorption, and expulsion, to controlling epithelial secretion, blood flow, and interactions with microbiota and the local immune system².

The ENS of amniotes is a complicated neuronal network comprises two layers: the myenteric plexuses that sit between the longitudinal and circular muscle, and the submucosal plexuses that reside under the epithelium. Known neuronal subtypes in ENS include excitatory/inhibitory motor neurons, excitatory/inhibitory interneurons, sensory neurons, also called intrinsic primary afferent neurons (IPANs), secretomotor neurons, and

secretomotor/vasodilator neurons. Myenteric neurons mostly play roles in regulating gut motility, and submucosal neurons control epithelial secretion and blood flow regulation, whereas IPANs are usually pleiotropic. In the colon, IPANs participate in evoking the enteric neural reflexes upon release of serotonin (5-HT) from enterochromaffin (EC) cells located in the bowel mucosa. 5-HT not only triggers peristalsis with IPANs, but also stimulates vasodilator neurons or secretomotor neurons for neurogenic vasodilation of submucosal arterioles, and activating electrocyte secretion by crypt epithelial cells, respectively^{1,2,7,8}.

The neuronal circuitry underlying peristalsis has been largely resolved: mechanosensory and chemosensory neurons that reside in the myenteric plexus, usually IPANs, transmit information about the luminal content to interneurons. With ascending excitatory interneurons projecting to ascending excitatory motor neuron, and descending inhibitory interneurons projecting to descending inhibitory motor neuron, local propulsion is triggered¹. Spencer proposed that this occurs via a ‘neuromechanical loop’, where the physical consistency of the luminal content affects luminal distension that then activates polarized reflexes, which in turn regulates distension of the gut. Hence, the ‘neuromechanical loop’ is a dynamic loop that enables gut motility to accommodate different types of content⁹. While the neural circuit of peristalsis is mostly understood, the mechanism of another main gut movement, segmentation contraction, remains elusive. Different from polarized peristalsis, segmentation is the process that mixes food back and forth for better digestion and absorption. The transition between these two modes of neurogenic contractility is especially intriguing⁸.

THE ENS IS DERIVED FROM NEURAL CREST CELLS

The ENS of jawed vertebrates is entirely derived from neural crest cells, most of which arise from the caudal hindbrain¹⁰. Vagal neural crest cells migrate to and invade the foregut and then migrate along the entire rostrocaudal extent of the gut, differentiating into diverse neuronal subtypes and glial cells. In addition to the vagal neural crest cells, sacral neural crest

cells also contribute to ENS formation via different migration routes in amniotes. In chicken, vagal neural crest cells enter the foregut around embryonic day E3-3.5. In contrast, sacral neural crest cells first form the Nerve of Remak next to the hindgut, a structure unique to birds, and then initiate migration rostrally around E8 to populate the gut up to the umbilicus^{11,12}. In mouse, sacral neural crest cells first populate the pelvic ganglia adjacent to the hindgut, and then migrate to occupy the hindgut and caudal midgut. In addition to the rostrocaudal migration, vagal neural crest cells in mice have been shown to exhibit trans-mesenteric migration¹³. Although direct experimental evidence has not been found to confirm the contribution of sacral neural crest cells to human ENS, hPSC-derived sacral neural crest cells can rescue a mouse model of Hirschsprung's disease (see below), suggesting their importance in ENS formation¹⁴.

GENE REGULATORY NETWORK (GRN) UNDERLYING DYNAMIC ENS DEVELOPMENT

Studies of the ENS have focused on characterizing the morphology and electrophysiological activity of neurons, as well as signature neurotransmitters that are involved. However, much still needs to be resolved about how ENS circuits are established. This is an important area of research, as malformation and malfunction of ENS wiring can result in severe diseases, such as Hirschsprung's (HSCR) disease and irritable bowel syndrome (IBS). HSCR is characterized by a paucity of enteric neurons in the terminal regions of the gut, resulting in intestinal obstruction and massive distension that causes megacolon, which can be fatal to children without surgery¹⁵⁻¹⁷. Mutations of the gene *RET*, encoding a transmembrane tyrosinase kinase receptor, are found in many HSCR cases¹⁶. Depletion of *RET* in both mice and zebrafish causes massive loss of neurons throughout the gut^{18,19}. This is due to lack of function of the *RET-GDNF* pathway, which is crucial for survival for ENS progenitor cells, whose deficiency results in their early apoptosis²⁰.

Multiple molecular factors involved in progenitor specification and maintenance have been identified as regulators of ENS development, and appear to be conserved across vertebrates. For example, critical factors that regulate ENS development in mice, including the transcription factor *Sox10*, signaling receptor *Ret*, and the homeodomain transcription factor *Phox2b* (paired-like homeobox 2b), are essential for ENS development in both mice^{19,21–23} and zebrafish^{20,24–27}. *Sox10* is required to maintain the multipotency of progenitor cells and is also important for glial cells, pigment cells, and neuronal differentiation in the neural crest lineage. Both *Ret* and *Phox2b* are crucial for the enteric lineage choice. *Phox2b* is a marker for both neural crest-derived enteric precursors and ENS neurons. Mutations of all three factors result in severe intestinal aganglionosis^{18,19,21,28,28}. Roles of many other transcription factors have also been revealed, in processes ranging from regulation of neurogenesis to neuronal lineage specification. For example, knockdown of transcription factors like *Hand2* and *Hoxb5* causes loss of ENS neurons in both zebrafish^{29,30} and mice^{31,32}.

The recent application of single-cell/single-nuclei RNA sequencing offers unprecedented opportunity for a more comprehensive characterization of ENS neuronal cluster composition and development across vertebrates^{12,33–36}. Many neuronal clusters appear to be conserved throughout species examined to date, based on their neurotransmitter profile, e.g. expression of *Nos1* and *Vip* is characteristic of the inhibitory motor neuron cluster^{33,35}, and *Chat* and *Tac1* are observed in the excitatory motor neuron cluster^{12,33,36}. The neurotransmitter profile for IPANs and interneurons, however, are less similar across species. For example, *Nmu* has long been identified as a marker for IPANs in mouse and human^{33,36}, but it shows very specific co-expression pattern with *slc18a3a*, a cholinergic neuronal marker, in the developing zebrafish ENS³⁵. One potential explanation for such difference is the pleiotropy of most IPANs⁸. A recent study focused on building scRNA-seq meta-atlas of juvenile and adult mouse ENS revealed rarer subclusters that were hard to capture due to the limit of cell number within individual scRNA-seq. This suggests that the ENS may have a complicated neurotransmitter coding system³⁷.

Several transcription factors have been implicated from scRNA-seq as involved in neuronal development. *Pbx3* appears to function as a cell fate switch in the ENS in mouse since its depletion decreases the number of calbindin-expressing neurons while expanding *Vip*⁺/*Nos1*⁺/*Gal* (*galanin*)⁺ neurons³³. Also, recent findings in zebrafish from our lab suggest roles for *gata3*, *ebf1a*, and *satb2* in excitatory, inhibitory, and serotonergic ENS lineages, respectively³⁵. Although a well-characterized gene regulatory network (GRN) has been identified for neural crest cells³⁸, the assembly of a GRN of ENS development is in the early stage³⁹.

By examining gene expression profiles at multiple time points, scRNA-seq enables construction of ENS developmental trajectories, and identification of crucial transcription factors at branch points. Bifurcation development is characteristic of ENS development across vertebrates, where two prototypic states are first reached prior to final neuronal identity choice. *Etv1* (Branch A) and *Bnc2* (Branch B) were identified to define the early binary neurogenic branching in murine myenteric plexus development³³. Similarly, excitatory motor neurons branch off from other neuronal types in zebrafish ENS development³⁵. One interesting shared pattern between murine intestinal myenteric and submucosal development is the transient expression of *Nos1* in one prototypic states during the first branching event, which is eventually lost in the final neuronal clusters^{33,40,41}. This transient *Nos1*⁺ cluster indicates that the neurotransmitter coding may be stage-specific. Similarly, during zebrafish ENS development, *Nos1* was expressed around 2 days post fertilization (2dpf), being the first neurotransmitter observed^{35,42}. Whether those neurons initially expressing *Nos1* in the developing zebrafish ENS ultimately function as nitrergic neurons, or if there is a similar transient expression pattern in zebrafish is not yet known.

ENS DEVELOPMENT IS A DYNAMIC PROCESS

One main difference between ENS and CNS development is that ENS progenitor cells are migratory. Therefore, they lack positional identities, and their fate choices may be greatly affected by the surroundings, which is a continuously changing environment. Regional differences of ENS along the gut have been observed across vertebrates^{42,43}. Recent

transcriptomic profiling suggests that neuronal clusters showed regional- and layer- specific diversification in both mouse and zebrafish ENS^{35,36,40}. A recent study on the contribution of vagal and sacral neural crest cells to the chicken ENS offers interesting insights into intrinsic-lineage versus extrinsic-environmental effects on cell fate of ENS progenitors¹². By utilizing RIA retrovirus to separately assess the contribution of vagal versus sacral neural crest cells, it was found vagal neural crest cells that colonize the pre-umbilical region exhibits some differences in transcriptional profile from either vagal or sacral neural crest cells that colonize the post-umbilical region¹². A recent study developed a pipeline to capture spatiotemporal gene expression along the entire length of zebrafish embryonic gut, coupled with spatial genomic analysis (SGA)⁴⁴. Consistent with the possibility that environmental cues may play a role, the authors characterized genes of interest from two dimensions: differentially expressed genes (DEGs) between cell-type clusters, and spatially variable genes (SVGs) depending on the axial level.

Besides continuous environmental inputs that affect ENS development, many other factors also shape the ENS formation. Neural crest cells generate both neurons and glia. Enteric glia in adult mice have the ability to undergo neurogenesis⁴⁵. In zebrafish, glial cells labeled by the *Tg(her4.3:EGFP)* fishline but lacking canonical glial markers, such as *Plp1* and *SI00β*, underwent neurogenesis in a process regulated by Notch signaling, which was required for the maintenance of enteric neural crest progenitors during early ENS development^{46,47}. scRNA-seq suggests that zebrafish “enteric glial” cells retain neurogenic potential, with key neurogenic loci for ENS progenitors remaining in an open chromatin configuration. These cells employ the same transcriptional program as early ENS progenitors⁴⁸. The principle was supported by scRNA-seq with the chicken ENS, where coexpression of glial-specific markers and progenitors markers was observed in the glial cell cluster¹². Furthermore, gut-extrinsic Schwann cell precursors also contributed to the post-embryonic zebrafish ENS, a process regulated by the serotonergic signaling⁴⁹. Together, these experiments show that ENS development is a dynamic process that receives continuous influence from the surrounding environment.

SPATIAL DISTRIBUTION OF ENS CIRCUIT CONNECT ITS DEVELOPMENT TO ITS FUNCTION

The concept of functional circuit unit of ENS has long been proposed and validated^{8,9}, with loss of specific GRN factors always impeding gut motility⁵⁰. While advancement of bioinformatic tools has provided opportunities for a more detailed and comprehensive characterization of ENS composition, it remains unknown of how to relate the gene profile of neuronal clusters to their function and spatiotemporal position, that is, how is a GRN implemented at a physical level to accomplish its function?

To address this, researchers have used Ca^{2+} imaging, neuron tracing with viral vector injection, and volumetric reconstruction of synaptic contacts, to tackle the complexity of ENS neuron circuit at a physiological level and relate it to complexity of ENS function⁵¹. Results in the mouse large intestine showed that the neuronal wiring in the distal colon is more polarized and physiologically simpler compared to that in the proximal colon, whereas the latter exhibits mixing movement in addition to peristalsis, and has more complicated neuronal projections, suggesting that motility control is hard-wired in the ENS.

A lineage tracing study of neural crest-derived ENS progenitors in mice has shown that the ENS is composed of overlapping clonal units that expand from individual post-migratory ENS progenitors⁵². What's more, ENS neurons that are clonally related were identified within a columnar space that expands through submucosal layer and myenteric layer, and demonstrated coordinated neuronal activity, though it is not clear how synchrony is attained. Intriguingly, different luminal nutrients can trigger specific neuronal clusters in ENS with different neurotransmitter expression profile⁵³. Not only did this study suggest that the complicated neurotransmitter composition uncovered by transcriptomic data could be served for a more fine-tuned, nutrient-specific, ENS response, but it also record nutrient-induced ENS responses in vivo at a single neuron level. When examining the connection between ENS neurons, researchers found that the ENS neuronal signal was first passed to the

myenteric plexus for initial processing and integration, and then propagated back to the submucosal layer plexus, regardless of proximity of the submucosal layer to the lumen. Whether the wiring that connects the two plexus is related to the columnar structure founded on clonal expansion⁵² remains to be determined.

Combining gene expression profile with neuronal manipulation has shed light on the composition of ENS circuitry. Two type II classic cadherins, *Cdh6* and *Cdh8*, were identified as specific markers for IPANs in the mouse colons. Optogenetic activation of cells expressing *Cdh6* alone successfully triggered retrograde colonic migrating motor complexes (CMMCs), a propulsive high-amplitude contraction specific to the colon. Besides revealing the role of colonic IPANs in triggering CMMCs, the study also suggested that such function could be achieved via a previously unidentified IPAN-IPAN synaptic connection⁵⁴. A comprehensive resource article has integrated spatiotemporal transcriptomic data of mouse ENS with corresponding functional assays of each neuronal cluster³⁶. In addition, they also compared transcriptomic profiles of mouse ENS to that of adult human ENS. They found fewer neuronal clusters in human ENS, but with higher signaling diversity, suggesting a different wiring program between two species. They also emphasized the impact of viscerofugal connectivity to the sympathetic nervous system on regulating gut motility, which added another layer of complexity in interpreting ENS functional output.

UNDERSTANDING ENS FROM AN EVO-DEVO PERSPECTIVE

The ENS has long been described as ‘the second brain’ due to its ability to function independently of input from CNS². However, the gut is innervated across many phyla, even in cnidarians, like Hydra, whose ENS is a meshwork composed of hundreds of neurons within the wall of the gut tube^{55,56}. The general form of the ENS is conserved throughout evolution, and the cnidarian ENS possesses similar function, controlling peristalsis, mixing (segmentation) and expulsion, as the mammalian ENS⁵⁷. Moreover, crucial factors, such as serotonin, were found in not only vertebrates, but also in many invertebrate ENS, such as

hydra and insects^{58,59}. Together, it suggests that ENS could be ‘the first brain’, prior to the evolution of centralized nervous system.

There are numerous other examples of conservation of ENS across species. First, cell migration is a common feature of ENS development of both insects and vertebrates⁶⁰. Although sea lamprey lacks vagal neural crest cells, studies revealed that the trunk-derived neural crest cells contribute to the formation of its ENS⁶¹. In addition, neurotransmitter observed in mammal ENS, like *5-HT*, *Nos1*, *Vip*, and *Calbindin*, have been found to be expressed in lamprey ENS as well⁶². For invertebrate, though insects do not have neural crest cells, their ENS is derived from a group of delaminated and migratory cells that undergo an epithelial-to-mesenchymal transition^{60,63}. Such similarities of developmental process in the insect ENS could potentially explain why *Ret* is also crucial for stomatogastric (enteric) nervous system (SNS) development in *Drosophila*. Although GDNF ligands didn’t appear until the emergence of jawed fish, a TGF β family member, *Mav*, was discovered to cooperate with *Drosophila Ret*, and together function similarly as Ret-GDNF pathway in vertebrates ENS development. Moreover, similarities of gene regulation have been observed across species. A study on the ENS of *C. elegans* pharynx, resembling the foregut, showed that a homeobox gene, *ceh-34*, is crucial for all ENS neuron specification⁶⁴. The study further discussed the broad impact of homeobox genes in neuron specification in *Drosophila* and sea urchin ENS development. In addition, as previously discussed, *Phox2b*, another homeobox gene, is required for neurogenesis of all neurons in vertebrate ENS development.

ZEBRAFISH AS A GOOD MODEL FOR ENS DEVELOPMENT AND FUNCTION

My thesis work uses zebrafish as a model organism to understand ENS development. While overall ENS organization is similar between zebrafish and mammals, the zebrafish ENS has a simplified structure, composed of myenteric neurons but lacking a submucosal layer that presents in amniotes. Also, enteric neurons of zebrafish are only observed between the circular and longitudinal smooth muscle layers but not within the adjacent connective tissue⁶⁵.

The zebrafish ENS is entirely derived from vagal neural crest cells. In zebrafish embryos, vagal neural crest cells delaminate from the neural tube at 16 hours post fertilization (hpf) and enter the foregut around 32 hpf. They then migrate along the rostrocaudal extent of the gut, in two parallel streams along the left and right sides, and reach the most distal regions by the end of 3 days postfertilization (dpf)^{18,25,66}. Sequential differentiation into various types of neurons occurs during their rostrocaudal migration. Like amniotes, zebrafish enteric neural crest cells differentiate into various types of neurons, ranging from serotonergic, cholinergic, and dopaminergic to vasoactive intestinal peptide (VIP), substance P (SP), and nitric oxide synthase (NOS)-containing neurons³⁵. Some factors that regulate enteric neural crest differentiation into these diverse neuronal subtypes have been uncovered. Similar to mouse and chicken, a GRN of zebrafish ENS development has been established³⁹. While many genes show conservative function across vertebrates, such as *Sox10*, *Pax3/7*, *Phox2b*, *GDNF*, *Ret*, *Hand2*, and *Ascl1a*, transcription factors that regulate specification of specific neuronal subtypes remain to be discovered.

In the first project, to better understand how ENS development is regulated in zebrafish, we conducted a screening of multiple transcription factors identified by scRNA-seq with CRISPR-Cas9 mediated knock-out, and identified a novel transcription factor, *Sox11*, in affecting the specification of the inhibitory motor neuron cluster⁶⁷. Although the expression of *Sox11* was observed in both mouse and chicken ENS scRNA-seq data^{12,33}, its role in ENS development had not been explored. The high fecundity and high accessibility to genetic manipulation make zebrafish an excellent model system for exploring candidate factors for ENS development. Our data regarding *Sox11* function in the zebrafish ENS suggested that its function may be conserved in other species.

In the second project, to obtain a more comprehensive understanding of the change in ENS structure along development, we developed a pipeline for imaging zebrafish ENS and its downstream data-processing. We applied this pipeline to visualize the neuronal diversity of both embryonic and adult zebrafish ENS by creating neuronal atlas at both stages. Benefiting from the relatively small size of zebrafish, we were able to capture the complexity of ENS

and further analyze the regional differences along the entire length of the gut, which is currently not feasible in other model organisms, like mouse and chicken. The relative simplicity of the gut structure, and the clarity for imaging make zebrafish an excellent model system for visualizing ENS development⁶⁸. Taken together, my research illustrates the advantage of zebrafish in both constructing GRN and capturing complex spatial information for ENS development.

SOX11 GENES AFFECT NEURONAL DIFFERENTIATION IN THE DEVELOPING ZEBRAFISH ENTERIC NERVOUS SYSTEM

Background

To glean information about additional transcription factors involved in the differentiation of ENS neuronal subtypes, we have delved deeply into a comprehensive single-cell RNA-seq dataset of 25,000 zebrafish ENS precursors and differentiated neurons from 2 to 6 dpf⁸⁵. Focusing on factors that are highly enriched in precursors and neuroblasts from this single-cell RNA-seq data set of the forming zebrafish ENS, here we identified *sox11a* and *sox11b* as highly upregulated during early neurogenesis, consistent with a potential role in neuronal differentiation. *sox11a* and *sox11b* are zebrafish orthologs of the human *SOX11* gene that belongs to the SRY-related high-mobility group (HMG) box (SOX) transcription factors family. The *sox11a* and *sox11b* paralogs share 75% protein sequence identity^{69,70}. Here, we show that *sox11a/b* are expressed in both progenitors and developing inhibitory motor neurons of the ENS. Moreover, CRISPR-Cas9 mediated F0 knock-out crispants reveal a critical role for *sox11a/b* in regulating *adcyap1b* expression, the zebrafish ortholog of peptide PACAP(pituitary adenylate cyclase-activating polypeptide), by directly regulating its enhancer. These results further our knowledge of the transcription factors involved in specification and neuronal differentiation in the developing ENS and identify *sox11a/b* as a key regulator of the inhibitory motor neuron lineage.

Results

Single-Cell RNA Sequencing Reveals *sox11a* and *sox11b* Transcripts in ENS Neuronal Progenitors and a Neuronal Cluster.

To identify transcription factors with possible roles in ENS development in zebrafish, we took advantage of a single cell (sc) RNA-seq of thousands of FAC-sorted *phox2bb*⁺ cells, expressed in both precursors and differentiated ENS neurons, from the dissected gut of zebrafish embryos from 2 to 6 dpf³⁵. The UMAP from the scRNA-seq data reveals a combination of enteric progenitor cells and differentiated neurons (Fig. 1A). Enteric progenitors are identified based on the coexpression of pan-neural crest marker *sox10* and *phox2bb*, whereas postmitotic neurons are identified by expression of *elavl3* and *phox2bb* but not *sox10*. In total, 24 cell clusters were identified based on the gene expression profile, covering the transition process from enteric progenitors to mature neurons that further subdivided into three main subtypes, inhibitory motor neurons, excitatory motor neurons, and serotonergic neurons, based on neuronal marker expression (SI Appendix, Fig. S1)^{2,35}.

Focusing on transcription factors with a possible role in regulating neurogenesis and/or neuronal differentiation, we found that zebrafish orthologs of the human SOX11, *sox11a*, and *sox11b*, were highly expressed in cell clusters that appear to represent cells transitioning from progenitors to mature neurons at around 3 and 4 dpf (Fig. 1B). In addition to overlapping expression at the transition region, *sox11a* was also expressed in the cluster 13 of the inhibitory motor neuron clusters (cluster 0, 6, 11, 13, 16) at 6 dpf, suggesting a possible role in ENS cell fate decisions (Fig. 1B and SI Appendix, Fig. S1). Moreover, in the developing ENS of chicken embryos at E10¹², *Sox11* expression was also found in both progenitor and neuronal clusters, suggesting a possibly evolutionarily conserved role (Fig. 1C and D).

sox11a and *sox11b* Are Expressed During Early Neuronal Differentiation in ENS.

To examine the expression pattern of *sox11a/b* in ENS, we performed in situ hybridization chain reaction (HCR)⁷¹ with probes to *sox11a* and *sox11b* mRNA on isolated zebrafish guts as a function of time. We noted that *sox11a* and *sox11b* were expressed transiently in the ENS at ~3 and 4 dpf (Fig. 2A). At 3 dpf, both *sox11a* and *sox11b* transcripts overlapped with the antibody staining of Phox2b and the proliferation marker, pH3 (Fig. 2B), indicating that they marked the progenitor population. Interestingly, the expression of both transcription factors decreased by 5 dpf. While *sox11a* expression remained detectable at low levels, *sox11b* was undetectable by 5 dpf (Fig. 2A).

To probe the relationship between *sox11a/b* and specific neuronal subtypes, multiplex HCR enabled examination of the coexpression of *sox11a* and *sox11b* with other neuronal markers. The scRNA-seq analysis of zebrafish developing ENS revealed three neuropeptide markers, *adcyap1b*, *nos1*, and *vip*, in inhibitory motor neuron clusters. While *adcyap1b* was expressed in all five clusters, *nos1* was expressed in cluster 0, 6, 11, 16, and *vip* was expressed in cluster 11, 13, 16 (SI Appendix, Fig. S1). Since *sox11a* expression was only found in cluster 13 at 6 dpf, we stained guts with both *vip* and *nos1* to examine their cell type-specific expression. At 3 dpf, when the ENS is mainly populated by progenitor cells, *sox11a* and *sox11b* were coexpressed with both *nos1* and *vip* (Fig. 2C). However, at 5 dpf, when many cells in the ENS have already differentiated into mature neurons, the expression of *sox11a* overlapped only with *vip* (Fig. 2D). This correlated with the inhibitory motor neuron cluster observed from our scRNA-seq data and indicated that *sox11a* and *sox11b* were expressed differentially among inhibitory

motor neuron clusters only at a later stage. In addition, no overlapping expression of *sox11a* and *nmu*, a neuronal marker of excitatory motor neurons³⁵, was found at 5 dpf (Fig. 2D). Taken together, the HCR analysis suggests specific expression of *sox11a* and *sox11b* in both precursors and a subset of inhibitory motor neurons. In addition to ENS expression, *sox11a* and *sox11b* are also expressed in the brain as seen with whole mount staining (SI Appendix, Fig. S2).

Loss of *sox11a* and *sox11b* Results in a Significant Reduction in Inhibitory Motor Neurons and Gut Motility.

To interrogate the function of *sox11a* and *sox11b* in ENS development, we used CRISPR-Cas9 mediated gene knockout. Given their overlapping expression, we concomitantly knocked out both *sox11a* and *sox11b* by injecting six guide RNAs (gRNAs), three for each paralog⁷². Sibling embryos were injected with gRNAs to the tyrosinase gene as controls. Nanopore sequencing validated the CRISPR efficiency for each gRNA (SI Appendix, Fig. S3 and Dataset S1)⁷². At 4.5 and 6.5 dpf, we dissected the gut from both the experimental and control groups. In these crispants, we observed no noticeable morphological differences between the two groups, with the exception of a slight delay in overall larvae development, as indicated by slow formation of the swim bladder (SI Appendix, Fig. S4). The phenotype of *sox11a/b* knock-out fish was examined using various ENS markers. During ENS development, the downregulation of *sox10* is coupled with the upregulation of *elavl3* as an indicator of neuronal maturation. Therefore, to examine the neuronal maturation process, we used antibodies to Phox2b (for neuronal

precursors and all ENS neurons), HuC/D (for mature neurons), together with HCR probes to *sox10* (to mark ENS precursors) in 4.5 and 6.5 dpf gut. This enabled investigation of progressive gut development at an early and later stage.

By examining the expression of each marker in both the experimental and control groups followed by cell counting, we quantitatively characterized the effect of *sox11a/b* double knockout on overall neurogenesis during ENS development. At 4.5 dpf, we noted a reduction in both HuC/D and Phox2b expression in the experimental group. There was a significant decrease in the hindgut region for both markers (mean of the relative count of experimental group/control group: Phox2b in hindgut = 0.72/1, with $p = 0.019$; HuC/D in hindgut = 0.62/1, with $p = 0.010$; $N_{sox11a/b} = 11$, $N_{tyr} = 14$, replicates = 3) (Fig. 3A and C), while no obvious difference was observed in the *sox10* signal (SI Appendix, Fig. S5A). This suggests that the decreasing number of mature neurons was not due to ENS cells being arrested at the progenitor cell stage. Instead, the reduction in cells expressing HuC/D and Phox2b in the hindgut region suggests a possible delay in ENS development in the double-knockout embryos.

Interestingly, at 6.5 dpf, we noted no significant difference in the numbers of Phox2b and HuC/D expressing cells ($N_{sox11a/b} = 7$, $N_{tyr} = 7$, replicates = 3) (Fig. 3B and D), suggesting that *sox11a/b* knockout specifically affected neuronal differentiation, rather than altering migration of vagal neural crest cells into the hindgut. By staining with antibodies to Caspase 3 and pH3, we found that the knockout of *sox11a/b* did not alter cell apoptosis or proliferation (Fig. 3D and SI Appendix, Fig. S5B and C).

Next, we performed HCR for enteric neuron subtype markers, including *adcyap1b*, *nos1*, and *vip* for inhibitory motor neurons, *nmu* and *slc18a3a* for excitatory motor neurons, and *tph1b* for serotonergic neuron (Fig. 2 and SI Appendix, Fig. S1). At 4.5 dpf, we noted a significant reduction in *adcyap1b* expression throughout the length of the gut (mean of the relative count of experimental group/control group: *adcyap1b* in foregut = 0.69/1, with $p = 0.017$, midgut = 0.58/1, with $p = 6.5E-4$, hindgut = 0.43/1, with $p = 7.8E-5$; $N_{sox11a/b} = 11$, $N_{tyr} = 14$, replicates = 3) (Fig. 3A and C). This suggests that the effect was not due to a general delay in development but rather specifically due to the loss of *sox11* paralogs.

At 6.5 dpf, we found that only inhibitory motor neuron markers were reduced (Fig. 3 B and D). Quantitative analysis revealed a significant reduction of neurons expressing *adcyap1b*, *nos1*, and *vip*, with no apparent differences in other neuronal markers (Fig. 3D). Interestingly, we noted some region-specific differences, with significant loss of *nos1* expression in the foregut and midgut regions, and *vip* expression in the midgut and hindgut regions, while *adcyap1b* was reduced throughout the gut (mean of the relative count of experimental group/control group: *nos1* in foregut = 0.81/1, with $p = 0.039$, midgut = 0.81/1, with $p = 0.038$; *vip* in midgut = 0.68/1, with $p = 2.52E-3$, hindgut = 0.45/1, with $p = 1.84E-4$; *adcyap1b* in foregut = 0.29/1, with $p = 8.71E-7$, midgut = 0.58/1, with $p = 1.47E-4$, hindgut = 0.66/1, with $p = 1.72E-3$; $N_{sox11a/b} = 8$, $N_{tyr} = 12$, replicates = 3). Taken together, the expression level of neuronal markers belonging to the inhibitory motor neuron clusters showed the most apparent phenotype ($p < 0.001$), suggesting that *sox11a/b* have a specific effect on inhibitory motor neuron differentiation,

particularly on *adcyap1b* expression. The effects of loss of *sox11a/b* appeared to begin during neuronal differentiation and last until a fully functional ENS should have formed.

To determine whether *sox11a* and *sox11b* function independently or redundantly, we separately performed loss of function of *sox11a* or *sox11b*, using three guide RNAs (gRNAs) per paralog. As controls, embryos from the same pair of parents were injected with gRNAs to the tyrosinase gene. The guts of F0 crispants larvae were dissected at 6.5 dpf and stained for *adcyap1b*, *vip*, and *nos1*, three neuronal markers for the inhibitory motor neuron cluster (SI Appendix, Fig. S6). In *sox11b* single knockout, we noted a significant difference in *adcyap1b* expression throughout the gut. In contrast, no significant difference was found in *sox11a* single knockout compared with the control. The finding that *sox11b* single knockout results in a less severe phenotype than the double knockout (SI Appendix, Fig. S6B) suggests that *sox11a/b* may function redundantly or compensate for one another, with *sox11b* being the dominant factor.

To test physiological consequences in our crispants, we examined the effects of loss of *sox11a/b* on gut movement. Interestingly, double-knockout embryos showed a significant increase in the percentage of incoherent gut movement at 10.5 dpf ($N_{sox11a/b} = 7$, $N_{tyr} = 8$) (Fig. 3E and SI Appendix, Fig. S7B). Coherent transmission of gut contraction was observed along the length of the whole gut of *tyr* knock-out embryos, whereas most gut motility was lost in the *sox11a/b* double-knockout embryos in the most extreme cases (Fig. 3F and Movies S1 and S2).

While making stable transgenic lines would be optimal, 95% of *sox11a/b* double-knockout embryos died at ~12 dpf due to the critical nature of the genes in ENS development. However, crispants are frequently used as an alternative to mutant lines^{73–75}, given that they have a permanent loss of the gene of interest and phenocopy the expected mutant phenotype. Nevertheless, the mosaic nature of crispants resulted in a broad variety of phenotypes, which could be resolved with a stable knock-out line.

An *adcyap1b* Enhancer Contains Potential SOX11 Binding Sites.

The phenotype of *sox11a/b* knockout suggests a role for *sox11a/b* in ENS development, specifically in neuronal differentiation. To understand the potential regulatory mechanisms underlying this effect, we next performed bulk ATAC-seq on *Tg(-8.3phox2bb:Kaede)* fish at 3 dpf with the goal of identifying enhancers expressed in the ENS. To this end, separating our samples into *phox2bb*⁺ and *phox2bb*[−] cells and comparing the differences between groups enabled the identification of open chromatin regions that reflect putative enhancers in ENS cells⁷⁶.

To identify possible enhancers that mediated expression of genes characteristic of inhibitory motor neurons, we first looked for open chromatin in the region of the *adcyap1b* gene body. We identified a positive peak around 32 kb downstream of the transcription starting site (Fig. 4A). This putative enhancing region was cloned into a GFP-reporter plasmid to test its ability to mediate GFP expression after injection into one-cell-stage zebrafish embryos⁷⁷ (Fig. 4B). The result revealed that this regulatory region drives GFP expression in the hindbrain starting at 3 dpf (SI Appendix, Fig. S8A

), reminiscent of *adcyap1b* transcript expression⁷⁸, suggesting it is a putative *adcyap1b* enhancer element. In addition to the hindbrain, embryos injected with the putative enhancer exhibited mosaic expression in the ENS that overlapped well with *adcyap1b* at 4.5 dpf (Fig. 4C). Enhancer-driven expression also overlapped with that of *sox11a* and *sox11b* (SI Appendix, Fig. S2).

Next, we applied TOBIAS (transcription factor occupancy prediction by investigation of ATAC-seq signal) to correct Tn5 bias and conduct transcription factor footprinting⁷⁹ (SI Appendix, Fig. S9). We looked for SOX11 binding sites within open chromatin regions in the vicinity of the gene body. In this way, we identified a putative SOX11 binding site in the enhancer of *adcyap1b* (Fig. 4A). To determine if the activity of this enhancer was affected by the putative SOX11 binding site, we mutated the binding site in the GFP-reporter plasmid by replacing the entire site with a random sequence, checking to make sure the random sequence did not generate a new transcription factor binding site (Fig. 4B). After mutating the predicted SOX11 binding motif sequence in the reporter plasmid, we examined the pattern of GFP expression in ENS after its single-cell injection. We found that the GFP signal was significantly reduced in ENS of embryos injected with the plasmid containing the mutated binding site relative to the control intact enhancer ($N_{\text{intact-enhancer}} = 60$, $N_{\text{mutated-enhancer}} = 51$, replicates = 3; $p < 0.001$) (Fig. 4 D and E). Further examining the overlapping pattern of *adcyap1b* and GFP signal via co-staining confirmed this significant reduction ($N_{\text{intact-enhancer}} = 17$, $N_{\text{mutated-enhancer}} = 16$, replicates = 3; $p < 0.05$) (Fig. 4 D and E). This suggests that SOX11 is likely to regulate *adcyap1b* expression in ENS via direct binding to its enhancer.

Next, we established a *Tg(+32adcyap1b:GFP)* line in which we examined enhancer driven expression relative to *adcyap1b* and HuC/D staining. We noted not all GFP⁺ cells are *adcyap1b*⁺, and not all *adcyap1b*⁺ cells are GFP⁺, whereas most GFP⁺/*adcyap1b*⁻ cells are HuC/D⁺ (Fig. 4F), suggesting that the enhancer only regulates a portion of *adcyap1b*⁻ expressing cells, and is also active in neuroblast cells that have not yet differentiated. This is consistent with our observation of incomplete loss of *adcyap1b* signal in *sox11a/b* double-knockout embryos (Fig. 3). However, a few GFP⁺ cells show no coexpression of either *adcyap1b* or HuC/D, which could be due to some small leakiness or background noise of the enhancer reporter line. We also identified three putative enhancers in the vicinity of the gene bodies of *vip* and *nos1* that contain SOX11 binding motifs, two for *nos1* and one for *vip*. To validate their identity, we then constructed GFP-reporter plasmids driven by each potential enhancer respectively, and tested them in vivo. Although GFP signals were observed, no signal in ENS was observed in either (SI Appendix, Fig. S10). Therefore, we successfully identified one putative *adcyap1b* enhancer, and discovered that its activity was affected by the potential SOX11 binding site, suggesting a potential regulatory mechanism of SOX11 in neuronal specification.

***ascl1a* Is Coexpressed with *sox11a* and *sox11b* During Neuronal Differentiation.**

CryoEM structural analysis of nucleosome–SOX11 complex has suggested that it may act as a “pioneer” factor to open chromatin and initiate the recruitment of other factors⁸⁰. To probe potential transcription factors that may bind cooperatively with SOX11, we

returned to our scRNA-seq data to examine other transcription factors expressed in similar patterns in the ENS that also showed predicted binding motifs in the same enhancer as SOX11 from our bulk ATAC data. We then calculated the percentage of enhancers containing predicted motifs for these genes and SOX11 throughout the genome. We found SOX4 sites that have highly similar motifs to SOX11 in more than 70% of enhancers with SOX11 sites. In addition, the proneural gene ASCL1 (MASH1) also shared more than 30% of enhancers with SOX11 (Fig. 5A). With our scRNA-seq data capturing the gene expression profile during the transition from ENS progenitor cells to mature neurons in zebrafish developing ENS, *sox4a*, the zebrafish ortholog of human SOX4, was mainly expressed in the progenitor cells cluster, while *ascl1a*, the zebrafish ortholog of human ASCL1, was expressed together with *sox11a* and *sox11b* in the neuroblast clusters (Fig. 5 B and C). Since both *sox4a* and *ascl1a* are expressed at early stage of ENS development, we used CRISPR-Cas9 to knock out these genes and examined ENS development in F0 crispants at 3.5 dpf, respectively (SI Appendix, Fig. S11 and Dataset S2). No significant difference was observed in ENS neurons in the *sox4a* crispants (SI Appendix, Fig. S11A). In contrast, the *ascl1a* knockout showed a near complete loss of HuC/D signal ($N_{ascl1a} = 6$, $N_{tyr} = 6$, replicates = 2) (Fig. 5D) together with other specific neuronal markers, including *adcyap1b* (Fig. 5E and SI Appendix, Fig. S11C) at 3.5 dpf. In contrast, *Phox2b* was unchanged (SI Appendix, Fig. S11B). This indicates that *ascl1a* regulates neurogenesis in ENS. This finding agrees with a previous study, where *Ascl1*^{-/-} caused a significant delay in neurogenesis in ENS development in

mouse⁸¹, although no significant difference in the PHOX2B expression pattern was observed.

To further probe the relationship between *ascl1a* and *sox11a/b* in ENS development, we performed HCR costaining, which revealed overlapping expression of *ascl1a*, *sox11a*, and *sox11b* at 3 dpf (Fig. 5C). Furthermore, TOBIAS footprinting analysis reveals a putative motif for ASCL1 in the enhancer of *adcyap1b* that lies adjacent to the SOX11 motif identified above (Fig. 5F). As *ascl1a* does not affect *sox11a/b* expression at 3.5 dpf (SI Appendix, Fig. S12), these findings suggest that *ascl1a* likely cooperates with *sox11a/b* in regulating *adcyap1b* expression in the ENS. When we mutated the sequence of the predicted ASCL1 motif within the *adcyap1b* enhancer, and injected the plasmid containing the mutated enhancer driven GFP at single-cell stage, we noted a significant decrease in GFP signal in the ENS compared to embryos injected with an intact *adcyap1b* enhancer ($N_{\text{mutated-enhancer}} = 56$, replicates = 4; $p < 0.001$) (Fig. 5G). We observed a significant difference in the GFP signal in the hindbrain as well ($N_{\text{mutated-enhancer}} = 182$, replicates = 4; $p < 0.05$) (SI Appendix, Fig. S8B). Altogether, our findings suggest that *ascl1a* may function cooperatively with *sox11a/b* via acting on the *adcyap1b* enhancer in the ENS.

Discussion

In zebrafish, *sox11a* and *sox11b* have been shown to be transiently expressed in the CNS and somites⁶⁹. Similarly, in mouse and chicken embryos, *Sox11* is widely expressed in the CNS and peripheral nervous system, including the olfactory epithelium, dorsal root,

and trigeminal ganglia, suggesting a possible role in neural development of amniote embryos^{82–84}. Functional studies of mouse *Sox11* have demonstrated that it affects neurogenesis in the CNS, including oligodendrocyte differentiation, dendritic morphogenesis, and proliferation^{82,84–87}. *Sox11* has also been shown to affect neuronal specification in the sympathetic nervous system⁸⁸. However, the function of *Sox11* was not previously examined in the ENS, though it appears in recent scRNA-seq data of chicken and mouse ENS^{12,33}.

By probing our zebrafish ENS scRNA-seq dataset, we found that *sox11a* and *sox11b* are expressed in the developing ENS, specifically in the progenitors and early differentiating inhibitory neurons that are characterized by *adcyap1b* expression (Fig. 6A). This is consistent with scRNA-seq data from other organisms. For example, *Sox11* expression is found in both progenitor clusters and neuronal clusters in scRNA-seq data of developing chicken ENS and in neuroblasts clusters in scRNA-seq data of developing murine ENS, suggesting that it may have a conserved role in vertebrates^{12,33}. Using multiplex HCR in situ hybridization, we validated the expression of *sox11a/b* in the zebrafish ENS and found that it was transiently expressed with the highest expression at 3 dpf. At 5 dpf, we observed a low level of *sox11a* expression in *vip*-expressing neurons, a subcluster of *adcyap1b*-expressing neurons.

Our loss of function analysis further suggests a critical role for *sox11a* and *sox11b* during ENS development. After CRISPR-Cas9 knockout, we noted an initial delay in the development of ENS, with a significant reduction of *Phox2b* and *HuC/D* expression in

the hindgut of *sox11a/b* crispants at 4.5 dpf. This is consistent with previous findings in the chicken spinal cord, where *Sox11* affects neuron maturation via the establishment of pan-neuronal protein expression⁸⁶. However, by 6.5 dpf, when a mature and functional ENS has developed, we saw no significant difference in Phox2b or HuC/D expression levels in the *sox11a/b* knock-out embryos. Thus, development appeared to have recovered by this time point. Instead, we noted a significant reduction of *adcyap1b* expression throughout the gut of knock-out embryos at 4.5 and 6.5 dpf and region-specific reduction in *nos1* and *vip* expression at 6.5 dpf. This suggests that *sox11a/b* specifically regulates neuronal differentiation of the inhibitory motor neurons subpopulation of the ENS. The region-specific phenotype of *adcyap1b*, *nos1*, and *vip* signal could be related to their different spatial distribution in ENS, and suggests that specific subtypes of neuronal clusters are affected by loss of *sox11a/b*.

Individual knockout of *sox11b* showed a similar but less severe phenotype compared to the double knockout, suggesting that both genes function redundantly, with *sox11b* being the dominant factor. Although our loss of function results utilized crispants rather than mutant lines due to lethality after loss of *sox11* paralogs, our results reveal greater than 90% gene loss after CRISPR mutagenesis, suggesting that these crispants closely phenocopy the putative mutant phenotype. Moreover, individual *sox11a* knockout had no noticeable effect on ENS development, suggesting that stress response or off-target effects are unlikely. CRISPR knockouts also enable efficient screening of potential cofactors that may work together with *sox11* paralogs (e.g., *ascl1a* or *sox4a* experiment). Nevertheless, compared to transgenic knock-out lines, crispants show larger individual

variation and more intragroup inconsistency, as relevant to our studies of quantitation of neuronal quantification and examination of gut motility.

Although *Sox11* is crucial for multiple aspects of neurodevelopment, our findings differ from previous studies in the developing sympathetic nervous system. pH3 and Caspase3 immunostaining results showed no difference in cell apoptosis or proliferation between control and *sox11a/b* knock-out embryos. Previous studies have found that *Sox11* is required for the proliferation of tyrosine hydroxylase-positive cells and that its loss results in a dramatic increase in the rate of cell death in the developing mouse spinal cord⁸⁶. In contrast, our results reveal an essential role for *sox11a/b* in ENS neurogenesis and differentiation that appears neuronal subtype-specific and independent of cell death.

We identified SOX11, SOX4, and ASCL1 motifs within the *adcyap1b* enhancer. Analysis mutating motif sequence suggests that ASCL1 may act cooperatively with SOX11 in the developing ENS (Fig. 6B). CRISPR knockout demonstrated that the loss of *ascl1a* resulted in a delay in neurogenesis, consistent with previous reports⁸¹. The proneural transcription factor, *Ascl1*, has been found to play crucial roles in the specification of neuronal progenitor cells and in regulating specific neuronal differentiation⁸⁹. For example, in mouse ENS development, in addition to its impact on overall neurogenesis, *Ascl1* has been shown to selectively affect neuronal subtypes that express calbindin, Vip, and TH⁸¹. In addition to the previous finding, we noticed a decrease in *adcyap1b* expression with *ascl1a* knockout in zebrafish. Since *vip* and *adcyap1b* are mainly coexpressed in the inhibitory motor neuron clusters (SI Appendix,

Fig. S1), the loss of Vip observed in ENS development in mice could be due to the fate change of the inhibitory motor neuron cluster.

Previous studies have suggested a close connection between *Sox11* and *Ascl1* during neurogenesis. SOX11 and ASCL1 have been suggested to function synergistically in activating neuronal enhancers in cultured human inhibitory interneuron-like progenitors⁹⁰. In chicken spinal cord development, the expression of Sox11 is induced by proneural bHLH proteins, like NGN2 and ASCL1⁹¹. Interestingly, in some cases, a reverse order has been observed; e.g. in both adrenergic neuroblastoma and spinal cord regeneration, *Sox11* appears to act as an upstream regulator of *Ascl1*^{92,93}. Different from both findings, our *ascl1a* knockout resulted in no significant difference in *sox11a/b* expression level. Instead, a predicted ASCL1 motif was found adjacent to the SOX11 motif in the putative *adcyap1b* enhancer. Mutating this predicted motif also resulted in a reduction of GFP reporter expression in ENS. Therefore, we speculate that *ascl1a* may regulate *adcyap1b* expression cooperatively with *sox11* paralogs. However, we cannot rule out the possibility that other transcription factors may also be involved in *adcyap1b* regulation. Taken together, our study demonstrates an essential role for *sox11* paralogs in regulating neuronal differentiation of inhibitory motor neurons in ENS development and suggests its interaction with *ascl1a* may be important for regulating the differentiation of inhibitory neuron subtypes.

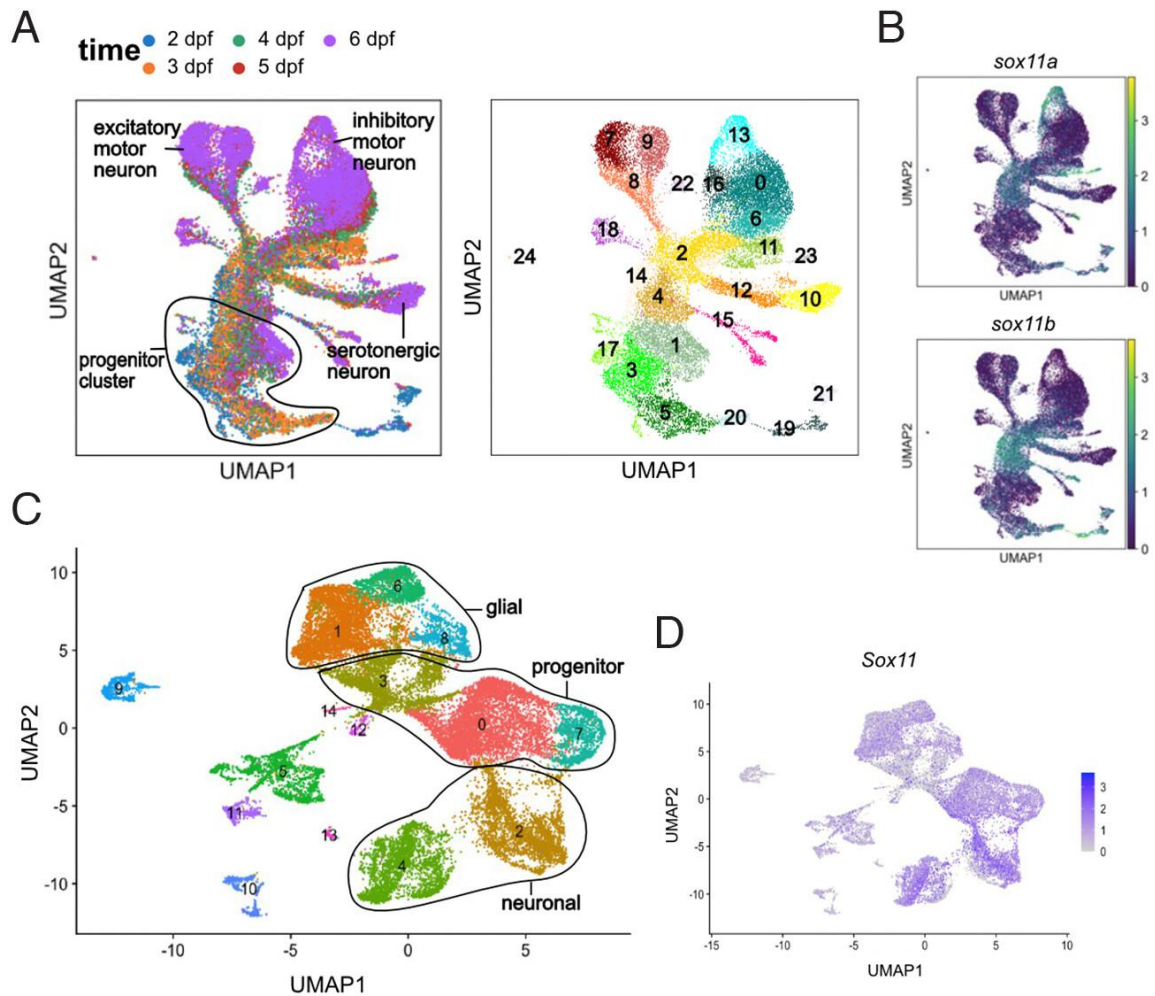


Fig.1 *sox11a* and *sox11b* expression in zebrafish and *Sox11* expression in chicken developing ENS (A) UMAP of single-cell (sc) RNA-seq of zebrafish ENS development. It covers the transition from progenitor cells to mature neurons in ENS development. (B) UMAP showing the expression pattern of *sox11a* and *sox11b* in zebrafish ENS development, suggesting their roles in affecting neuronal differentiation. (C) UMAP of scRNA-seq experiment on chicken ENS development (D) UMAP showing the expression pattern of *Sox11* in chicken ENS development

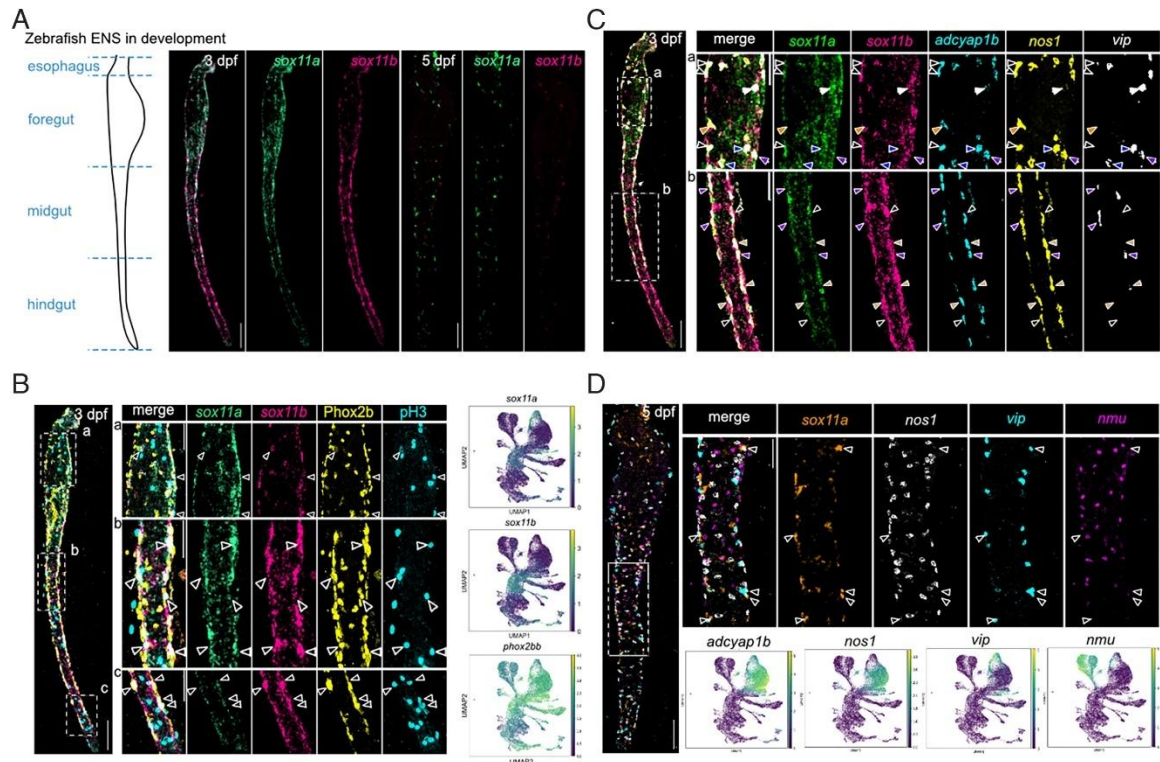


Fig. 2. *sox11a* and *sox11b* expression overlap with inhibitory motor neuron clusters.

All staining images were after “Max Intensity” z-stack projection. (A) Left: Illustration of zebrafish developing ENS separated into foregut, midgut, and hindgut. Right: Transient expression of both *sox11a* and *sox11b* in gut at 3 dpf, and only *sox11a* expression at 5 dpf. (Scale bar, 100 μ m.) (B) Overlapping expression of *sox11a*, *sox11b*, *Phox2b*, and pH3 in the ENS at 3 dpf. The white dash box indicates the zoomed region on the right. Arrows indicate cells coexpressing *sox11a/b*, *Phox2b*, and proliferation marker pH3. (Scale bar, 100 μ m, and 50 μ m for zoomed region.) (C) Coexpression pattern between *sox11a*, *sox11b*, and inhibitory motor neuron markers, *adcyap1b*, *vip*, and *nos1*

at 3 dpf. The white dash box indicates the zoomed region on the right. Arrows point to cells coexpressing *sox11a/b* and neuronal markers:

hollow arrows: *sox11a*⁺/*sox11b*⁺/*adcyap1b*⁺/*nos1*⁺/*vip*⁻ cells,

white arrows: *sox11a*⁺/*sox11b*⁺/*adcyap1b*⁻/*nos1*⁻/*vip*⁺ cells,

orange arrows: *sox11a*⁻/*sox11b*⁺/*adcyap1b*⁺/*nos1*⁺/*vip*⁻ cells,

purple arrows: *sox11a*⁺/*sox11b*⁺/*adcyap1b*⁺/*nos1*⁺/*vip*⁺ cells,

blue arrows: *sox11a*⁺/*sox11b*⁻/*adcyap1b*⁺/*nos1*⁺/*vip*⁺ cells.

(Scale bar, 100 μ m, and 50 μ m for zoomed region.) (D) Coexpression pattern between *sox11a* and only *vip* at 5 dpf. The white dash box indicates the zoomed region on the right. Arrows indicate cells coexpressing *sox11a* and *vip*. (Scale bar, 100 μ m, and 50 μ m for zoomed region.)

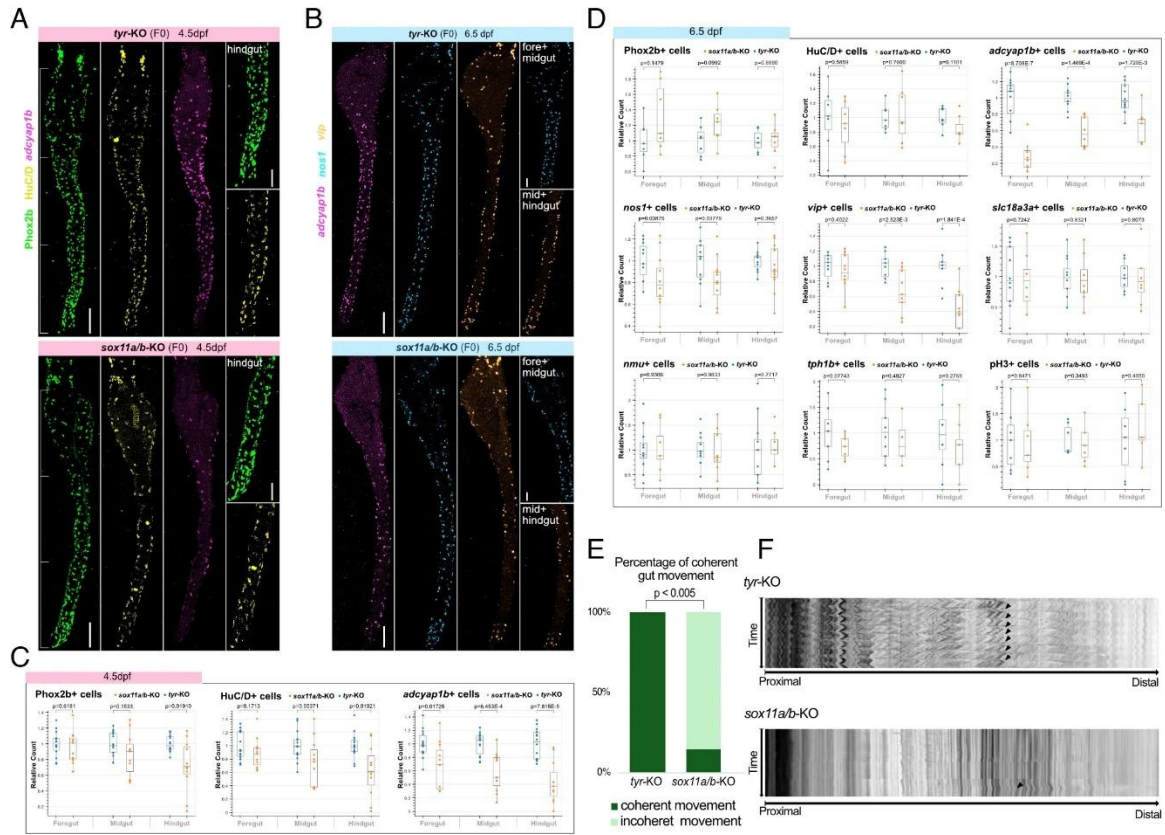


Fig. 3. Quantification analysis reveals differences between *sox11a/b* knock-out and control embryos. All staining images were after “Max Intensity” z-stack projection. (A) Antibody and HCR staining comparing the expression levels of Phox2b, HuC/D, and *adcyp1b* in *sox11a/b* knock-out embryos (Top) with the control embryos (Bottom) at 4.5 dpf. On the right, hindgut regions that showed a significant difference in cells expressing Phox2b and HuC/D were zoomed in. A significant difference in *adcyp1b* expression was observed throughout the whole gut. (Scale bar, 100 μ m, and 50 μ m for zoomed region.) (B) Antibody and HCR staining comparing the expression levels of *adcyp1b*, *nos1*, and *vip* in *sox11a/b* knock-out embryos with the control embryos at 6.5 dpf. On the right, corresponding regions that showed a significant difference in cells

expressing *nos1* and *vip* were zoomed in. A significant difference in *adcyap1b* expression was observed throughout the whole gut. (Scale bar, 100 μ m, and 50 μ m for zoomed region.) (C) At 4.5 dpf, an overall developmental delay was observed in *sox11a/b* knock-out embryos ($N_{sox11a/b} = 11$, $N_{tyr} = 14$, replicates = 3). (D) At 6.5 dpf, a specific reduction of the inhibitory motor neuron cluster was observed in *sox11a/b* knock-out embryos, particularly in *adcyap1b* expression (for Phox2b and HuC/D, $N_{sox11a/b} = 7$, $N_{tyr} = 7$, replicates = 3; For other neuronal markers, $N_{sox11a/b} = 8$, $N_{tyr} = 12$, replicates = 3). (E) A significant increase of incoherent gut movement was found in *sox11a/b* knock-out embryos. ($N_{sox11a/b} = 7$, $N_{tyr} = 8$). (F) Kymographs showing the passage of coherent contraction from proximal to distal in *tyr* knock-out embryos, and little movement at the length of the gut in *sox11a/b* knock-out embryos. Arrows indicate gut contraction.

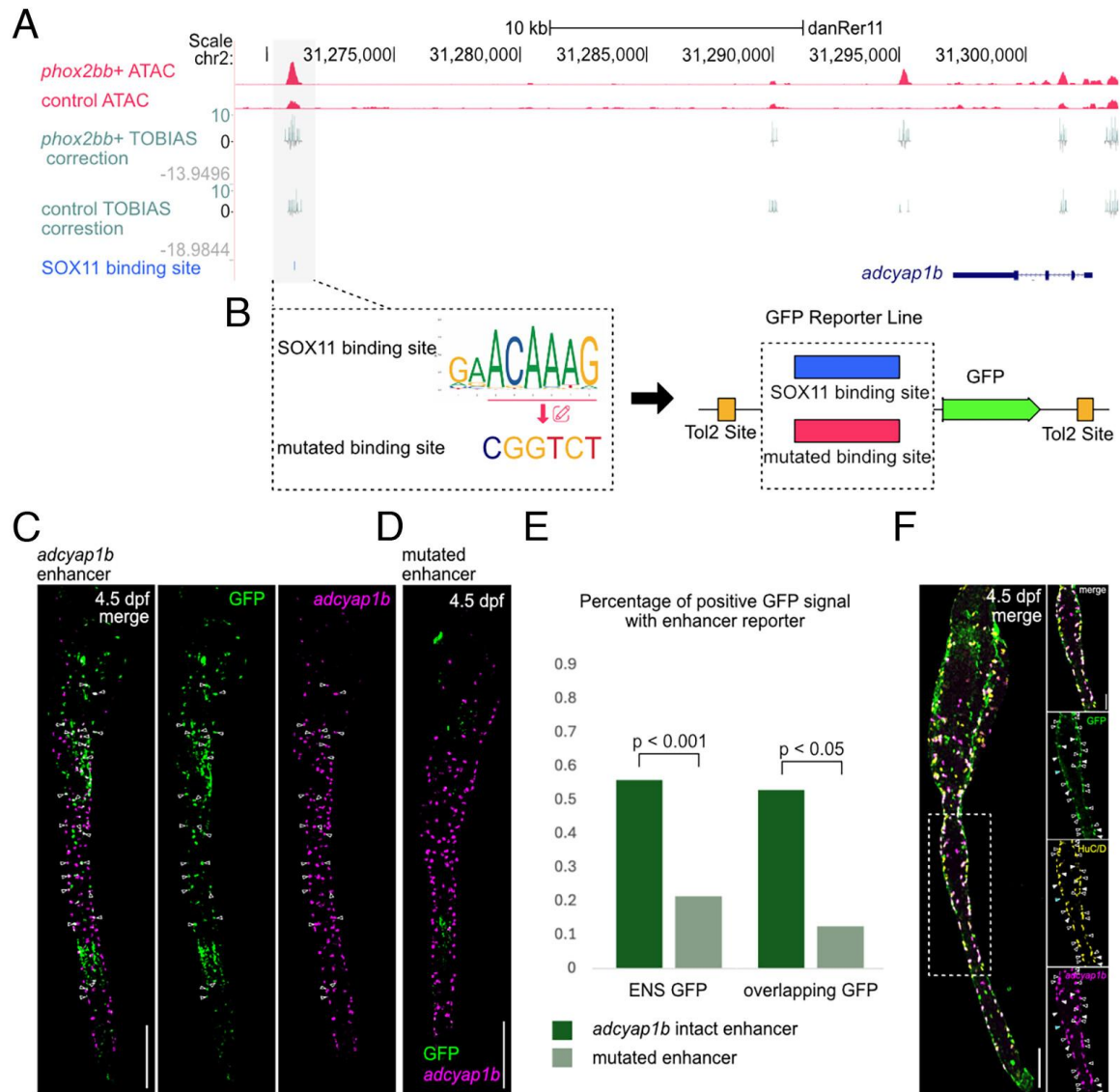


Fig. 4. Putative SOX11 binding site in an *adcyap1b* enhancer. All staining images were after “Max Intensity” z-stack projection. (A) TOBIAS provides Tn5 bias correction of bulk ATAC seq results and SOX11 footprinting. (B) The *adcyap1b* enhancer with either the intact or mutated SOX11 motif was cloned into the GFP reporter plasmid. (C) At 4.5 dpf, embryos showed *adcyap1b* enhancer mediating GFP reporter expression in the gut. Arrows indicate cells coexpressing GFP and *adcyap1b*. (Scale bar, 100 μ m.) (D)

At 4.5 dpf, embryos showed a reduction in *adcyap1b* enhancer-mediated reporter expression after mutation of SOX11 motif. (Scale bar, 100 μ m.) (E) Enhancers with mutated SOX11 motif showed a significant reduction in the percentage of positive GFP signal in ENS (ENS GFP, $N_{\text{intact-enhancer}} = 60$, $N_{\text{mutated-enhancer}} = 51$, replicates = 3; overlapping GFP, $N_{\text{intact-enhancer}} = 17$, $N_{\text{mutated-enhancer}} = 16$, replicates = 3). (F) Antibody and HCR staining comparing coexpression of GFP signals, HuC/D and *adcyap1b* in ENS of *Tg(+32adcyap1b:GFP)* at 4.5 dpf. The white dash box indicates the zoomed region on the right. Hollow arrows point to $\text{GFP}^+/\text{HuC/D}^+/\text{adcyap1b}^+$ cells, white arrows point to $\text{GFP}^+/\text{HuC/D}^+/\text{adcyap1b}^-$ cells, and blue arrows point to $\text{GFP}^+/\text{HuC/D}^-/\text{adcyap1b}^-$ cells. (Scale bar, 100 μ m, and 50 μ m for zoomed region.)

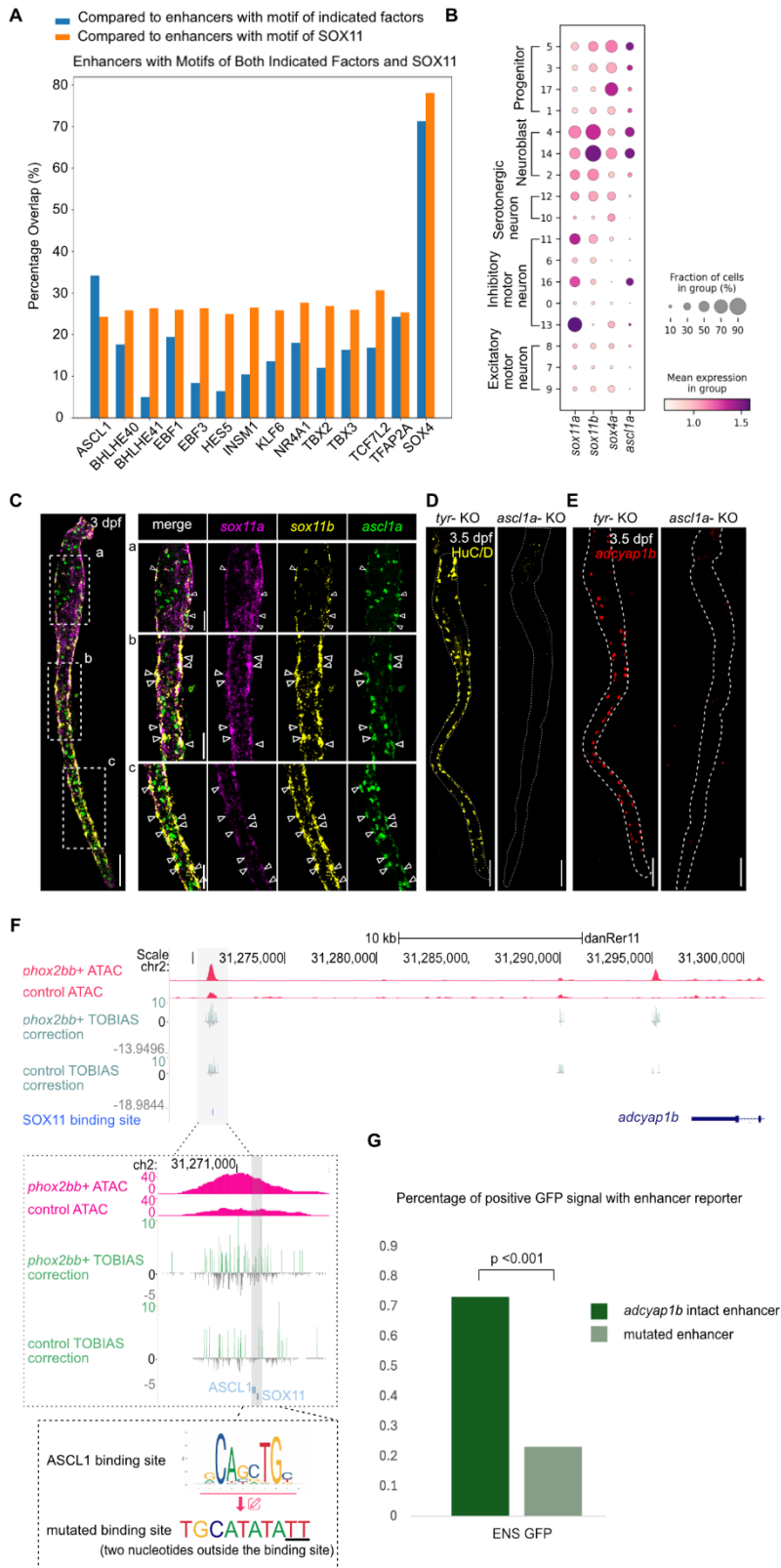


Fig. 5. ASCL1 has a predicted motif adjacent to the putative motif of SOX11. All staining images were after “Max Intensity” z-stack projection. (A) SOX4 and ASCL1 show the top percentage of enhancers that contain motifs for them and SOX11. (B) scRNA-seq analysis suggested that *sox4a* was expressed in progenitor clusters, while *sox11a*, *sox11b*, and *ascl1a* were expressed in ENS neuroblasts. (C) At 3 dpf, *ascl1a* had an overlapping expression with *sox11a/b*, especially with *sox11b*. The white dash box indicates the zoomed region on the right. Arrows indicate overlapping expression between *ascl1a*, *sox11a*, and *sox11b*. (Scale bar, 100 μ m, and 50 μ m for the zoomed region.) (D) Representative image of embryo with near complete loss of HuC/D after *ascl1a* knockout. The dotted line indicates the outline of the gut ($N_{ascl1a} = 6$, $N_{tyr} = 6$, replicates = 2). (Scale bar, 100 μ m.) (E) Representative image of embryo with near complete loss of *adcyap1b* after *ascl1a* knockout. The dotted line indicates the outline of the gut. ($N_{ascl1a} = 6$, $N_{tyr} = 6$, replicates = 2). (Scale bar, 100 μ m.) (F) ASCL1 has a motif in the putative enhancer of *adcyap1b*. A plasmid was generated with a mutated ASCL1 motif. (G) Enhancers with mutated ASCL1 motif showed a significant reduction in the percentage of positive GFP signal in ENS (ENS GFP, $N_{\text{intact-enhancer}} = 60$, $N_{\text{mutated-enhancer}} = 56$, replicates = 4).

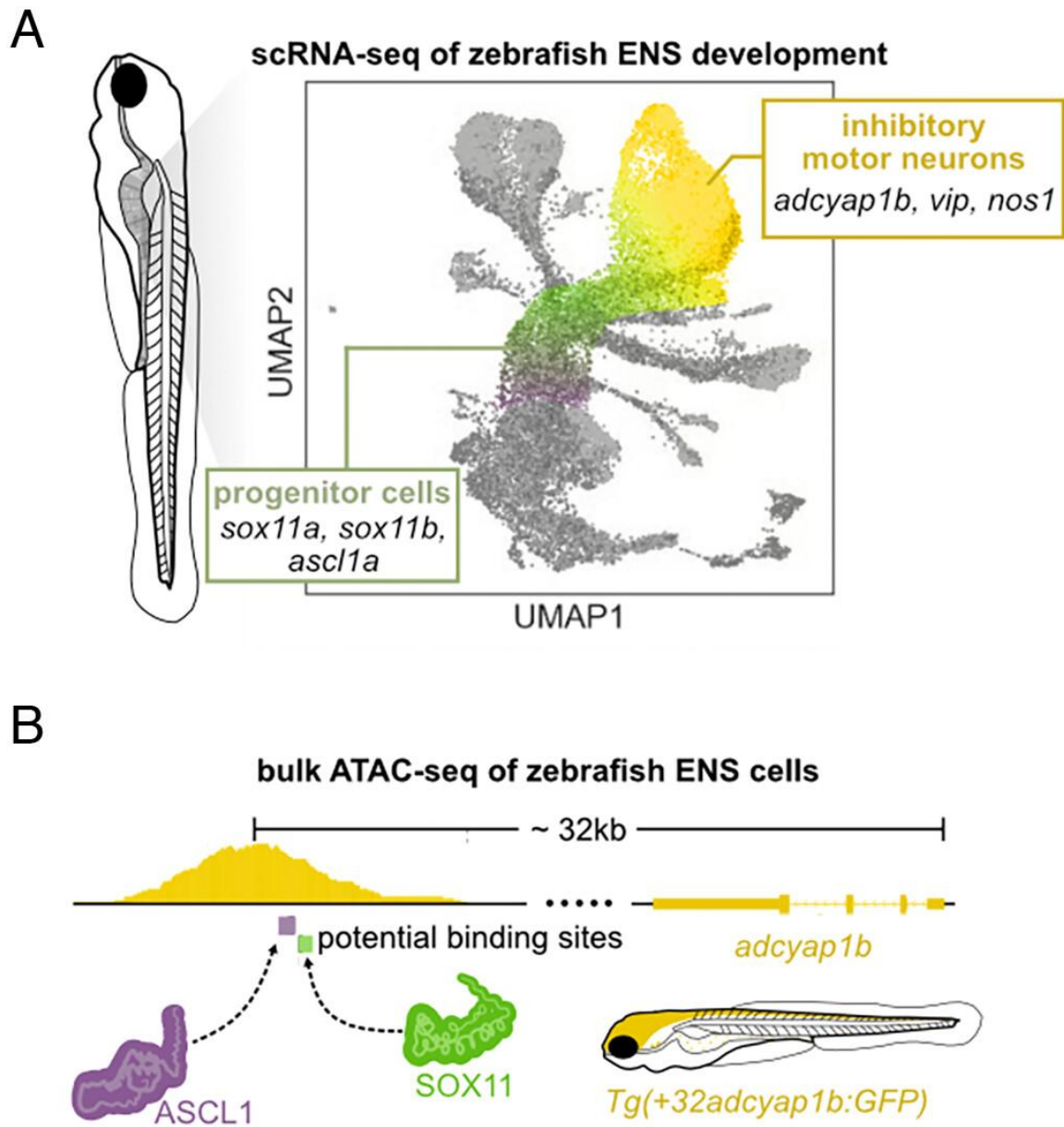


Fig. 6. **Schematic summary of the results.** (A) Identification of *sox11a/b* expression in progenitors and inhibitory neurons via scRNA-seq, confirmed by HCR. (B) ATAC-seq enables identification of a putative *adcyap1b* enhancer that contains critical SOX11 binding sites.

Methods

Zebrafish Lines. Adult zebrafish (*Danio rerio*) and larvae were maintained at 28 °C, with a 13-h light/11-h dark cycle. All experiments complied with the regulations of the California Institute of Technology Institutional Animal Care and Use Committee. Transgenic fishlines used in the study were the ABWT, Tg(Phox2bb:mNeonGreen)³⁵ (Generated in Bronner Lab), Tg(-8.3*phox2bb:Kaede*)⁹⁴ and Tg(+32*adcyap1b:GFP*). Tg(+32*adcyap1b:GFP*) was generated by raising embryos injected with *adcyap1b*-enhancer reporter plasmid to adulthood. F1 embryos were obtained by crossing F0 with ABWT, and used for coexpression staining.

Zebrafish Embryo Fixation for Staining. Zebrafish embryos at selected stages were anesthetized with tricaine (MS222) and placed in Ringer's solution for deyolking. Deyolked embryos were fixed with 4% paraformaldehyde (PFA) overnight at 4 °C. The next day, samples were washed with 1× PBS-0.1% Tween20 solution (3 × 5 min) and then with methanol (1 × 5 min). Samples were then stored in fresh methanol at -20 °C for later usage.

scRNA-Seq Data Processing. The transcriptomic data of the developing zebrafish ENS were obtained from gene expression omnibus (GEO) database, accession number GSE274407³⁵. The reads of five libraries generated from ENS cells collected at 2, 3, 4, 5, and 6 dpf were mapped using Kallisto and bustools programs 81 and 82 to the reference transcriptome based on zebrafish genome assembly GRCz10, and the gene-cell matrices were generated separately. Scanpy package⁸³ was then used for analysis. We

concatenated these five datasets and performed quality control (removing cells with fewer than 400 genes captured, with more than 15,000 counts detected and a ratio of mitochondrial genes to total genes ≥ 0.05 and eliminating genes expressed in fewer than 50 cells). We saved the preprocessed data, and with the remaining 24,677 cells, we normalized the data to 10,000 total-counts per cell and log-transformed the data. Then, we identified 1,768 highly variable genes by setting the threshold with minimum and maximum means equal to 0.0125 and 3, respectively, and with minimum dispersion equal to 0.5. After regressing out the source of variation from the expression of mitochondrial genes, we performed PCA and visualized the data in two dimensions by running the UMAP algorithm with the parameter settings as $n_neighbors = 20$ and $n_pcs = 40$. Cell clusters are further revealed using Leiden algorithm with resolution = 0.8, and 24 clusters in total are discovered. Transcriptomics data of developing chicken ENS were obtained from GEO database, accession number GSE 242228 and were processed as previously described¹². *Sox11* expression in both zebrafish and chicken ENS development was visualized with UMAP.

HCR Multiplex Staining and Immunostaining. Deyolked zebrafish embryos stored in methanol were rehydrated in 50% Methanol/PBS-0.1% Tween20 solution for gut dissection. Dissected guts were then transferred back to fresh methanol solution. Superfrost slides were prepared with a tape boundary and a rectangle window in the middle. Guts were placed inside the boundary and automatically got stuck on the slide after methanol evaporation. The slide was then ready for multiplex staining.

Each round of HCR staining was performed with HCR v3.0 probes following the zebrafish protocol provided by Molecular Technologies. Additional stripping steps were added after each round of HCR staining to strip off the previous probes before starting the next round. For the stripping step, we washed the slide with in situ hybridization buffer without tRNA (50% formamide, 5× SSCT, 50 µg/mL heparin, 0.1% Tween20) at 50 °C (4 × 20 min), followed by washes with 1× PBS-0.1% Tween20 solution (3 × 5 min). Slides were then examined with a confocal microscope to validate that no residue probes were detected before starting the next round of staining. HCR probes for *sox11a* and *sox11b* were produced based on publicized protocol⁹⁵ and all other HCR probes, hairpins, and buffers were designed and ordered through molecular technologies (<https://www.molecularinstruments.com>).

Whole mount HCR staining of ABWT was conducted following the zebrafish protocol provided by Molecular Technologies, with a modified PK treatment time based on the stage of the embryo (ex. 3 min on shaker for 3 dpf embryos).

Immunostaining was performed with either HCR-stained samples after the stripping step. Embryos were transferred to 1× PBS-TritonX-100 (0.5% TritonX-100) solution with washing (3 × 5 min), and blocked with the blocking solution (10% Donkey Serum in 1× PBS- 0.5% TritonX-100) for at least 1 h. Samples were then incubated with the primary antibody in the blocking solution (1:100) overnight at room temperature. The next day, samples were washed with 1× PBS-TritonX-100 (0.5% TritonX-100) solution (8 × 20 min), and incubated in the corresponding secondary antibody (1:1,000) in the blocking

buffer overnight at room temperature. After washing with $1 \times$ PBS-TritonX-100 (0.5% TritonX-100) solution (8×20 min) on the third day, samples are ready to be imaged. Primary antibodies used in the study include mouse anti-Phox2b IgG1 (Santa Cruz Biotechnology, sc-376997), mouse anti-HuC/D IgG2b (Thermo Fisher Scientific, A21271), rabbit anti-phospho-Histone H3 (pH3) IgG (Millipore, 06-570), rabbit anti-active Caspase3 IgG (Biotechne, AF835) antibodies and goat anti-GFP IgG (Rockland, 600-101-215).

Perturbation with CRISPR-Cas9. Guide RNAs of zebrafish *sox11a*, *sox11b*, *sox4a*, *ascl1a*, and *tyr* were designed using CHOP-CHOP (<https://chopchop.cbu.uib.no/>) and ordered from IDT. Assembly of CRISPR-Cas9 complex and F0 injection followed the F0 knockout-single gene V.3 protocol⁷². Embryos showing significant developmental deficiency were filtered out from later analysis. Sequences of guide RNAs used in the experiment are

sox11a-

5'AGACGTGGAAACGGACCTAG3', 5'GGGAGCAAGTCGTCGTCTCA3',
5'CGAGCTCAAAGTTGGGCTGG3';

sox11b-

5'GGAACCTCGAAGTGAGAACCG3', 5'GCGAGAGACCACAGACACCG3',
5'GCATGGCGCGAGCATGTACG3';

sox4a-

5'AGGGCGTCGGAGACGGCGCG3', 5'CTGGCCGGCACC GCGACAGA3',
5'CCTCTTGATGTGTCCCGATG3';

ascl1a-

5'ATTGGGAACGTGTTCGCGGA3', 5'GTAAGCGCGGCATTCCAGTC3',
5'CCGCGTCAAAGCAGGCAAAG3';

tyr-

5'CGTTGGGAAGGTCGGACACC3', 5'TAACTTCACCATCCCGTACT3',
5'GATGCA TTATTACGTGTCCC3'.

DNA Extraction and crRNA Efficiency Analysis. Ten embryos injected with either experimental or control crRNAs were randomly selected and collected in one 1.5 mL eppendorf tube at 3 dpf and freezed at −20 °C for at least 1 d before DNA extraction. For DNA extraction, 50 µL of 50 mM NaOH was added to the sample, and the sample was vortexed and incubated at 95 °C for 15 min. The sample was then cooled to room temperature, and neutralized with 5 µL of 1 M Tris-HCl, pH = 8.0. After centrifugation at 13,000 rpm for 5 min, the supernatant was used for the PCR. For crRNA efficiency detection of *sox11a* and *sox11b*, the predicted cutting region was amplified with forward and reverse primers that bind to ~150 bp upstream and downstream of the gRNA binding site, respectively. Amplicons were then sequenced with Nanopore sequencing by

Plasmidsaurus. Raw fastq data were obtained, and indel mutation efficiency was calculated with CRISPResso2⁹⁶. For crRNA efficiency detection of *ascl1a* and *sox4a*, we conducted qPCR analysis. We added 80 µL of FastStart Universal SYBR Green Master (ROX) (MilliporeSigma, Catalog number 4913850001) into each sample, mixed well, and then distributed it into a 96-well plate with 12.5 µL of the reagent in each well. Primers designed to amplify *actb1* (reference) and three targeted regions of each gene were then added, respectively. Three replicates for each PCR product were conducted. The qPCR program for detecting the efficiency was set as Step 1, 50 °C for 2 min and 95 °C for 10 min; Step 2, 40 cycles at 95 °C for 15 s and 57 °C for 1 min; Step 3, melting curve step for detecting the amplification specificity. We used QuantStudio 3 Real-time PCR System to perform the qPCR experiment.

Gut Motility Analysis. Starting from 6 dpf, both *tyr* knock-out and *sox11a/b* knock-out larvae were fed once per day in a petri dish. At 10.5 dpf, their gut movements were recorded as an 80-frame video with a 4 s interval between each frame, using the Zeiss LSM 980 brightfield channel. The coherent gut contraction was then examined for each embryo using the video, and kymographs were made for better visualization of the process. Kymographs were produced with FIJI-Analysis-Multikymograph.

BULK-ATAC Analysis. Zebrafish embryos of the *Tg(-8.3phox2bb:Kaede)* line were raised to 3 dpf, transferred to the Ringer's solution, and anesthetized with Tricaine (MS222). During tissue dissection, the embryos were deyolked and the head part that had a high level of *phox2bb* expression was removed. The remaining trunk parts were

collected. About 150 embryos were dissected and separately stored in 6 low-binding microcentrifuge tubes on ice. After dissection, the supernatant was removed and 400 μ L Accumax (Invitrogen, Catalog number: 00-4666-56) solution was added into each tube. The tubes were incubated in the Eppendorf ThermoMixer at 30 °C and shaken at 1,000 rpm for about 25 min for cell dissociation. After the dissociation step, the tubes were chilled on ice, and 1 mL of cold HBSS-BSA solution was added to quench the reaction. The solution was passed through a 70 μ m cell strainer into a 50 mL conical tube, and an additional 500 μ L HBSS-BSA solution was used to rinse the tube and subsequently collected into the conical tube. Six tubes of solution were then combined and divided into two conical tubes. The solution was then spun down at 4 °C at 1,200 rpm for 11 min. The supernatant was carefully removed and the pellet in each conical tube was resuspended in 1 mL HBSS-BSA solution. We then performed fluorescence-activated cell sorting and sorted out Kaede-positive and Kaede-negative cells.

The samples were used for the bulk ATAC-seq experiment, following the previously published protocol⁹⁷. The adapter sequence was first removed with Cutdapt⁹⁸. Alignment, postalignment processing and quality control, and peak-calling were performed with Galaxy EU, following its “ATAC-seq data analysis tutorial”⁹⁹. Over 95% overall alignment rate was achieved for both Kaede-positive and Kaede-negative groups after the alignment step.

TOBIAS Analysis. After obtaining the .bw file for both the *phox2bb*⁺ (Kaede positive) and *phox2bb*⁻ (Kaede-negative) groups, TOBIAS (Transcription factor Occupancy

prediction By Investigation of ATAC-seq Signal) (v0.14.0) analysis was conducted according to the protocol described on the GitHub page⁷⁹. ATACCorrect, ScoreBIGwig, and BINDetect analysis were conducted with Vertebrate PFMs (redundant) from JASPAR as the “motifs” parameter. Information on the binding sites was obtained after BINDetect analysis and was used for overlapping binding enhancers search. The putative binding sites of transcription factor and bulk ATAC result were then visualized with UCSC genome browser.

Overlapping Enhancer Analysis. TOBIAS output was scanned to find transcription factors coexpressed with *sox11a/b* in our scRNA-seq data that have binding sites in the putative *adcyap1b* enhancer. Before the comparison, TOBIAS outputs for all motifs of each transcription factor were concatenated. The percentage of overlapping enhancers was calculated by comparing the percentage of shared “peak_id” between the two transcription factors. The analysis was accomplished in Python with the help of ChatGPT 3.0. The code is available upon request.

Reporter Plasmid Construction and Injection. The identified enhancer of *adcyap1b* that is 32 kb downstream of the transcription start site of *adcyap1b* contains the intact motif of SOX11 (ACAAAG) and ASCL1 (GCATCTGCTC), and it was cloned into the E1b-GFP-Tol2 plasmid (Addgene plasmid # 37845)⁷⁷ with BglII digestion and Gibson reaction. Primers for cloning this enhancer are *adcyap1b*-enhancer 5'TGCAGGAGCATCTTTTAACCCTT3', 5'TGGATGTGTGTTGGCTCGAT3'. To mutate the motif of either SOX11 or ASCL1, a random sequence was chosen to replace

them, and JASPAR vertebrate database was used afterward to check that no new binding motif was generated with a threshold value equal to that of SOX11 or ASCL1, respectively. The plasmid was mutated with Gibson reaction: The motif of SOX11 was mutated to CGGTCT and that of ASCL1 was mutated to TGCATATATT. 20 ng/μL of constructed reporter plasmid and 25 ng/μL of Tol2 transposase mRNA were then coinjected into ABWT embryos at the single-cell stage, with 1nl per embryo. Three more putative enhancers adjacent to either the *vip* or *nos1* gene body were also examined, with primers:

vip-enhancer

5'gatgggccctcgagaCTCACTCACTTAAAGTTTGAAAGGTAGC3',

5'tataccctctagagtcgagaGCATTTGAACTGCCGCTCACTG3',

nos1-enhancer1

5'GGCCCTCGAGAGATCGGGGCGGTGTATATGTGTCA3',

5'TAGAGTCGAGAGATCGGCTATCGAGGCACACTCT3',

nos1-enhancer2

5'gatgggccctcgagaCGTCCAATCACAATCAAGCATTCAAG3',

5'tataccctctagagtcgagaGCTGTAAACAACAGCTATCTGCTAAC3'.

Reporter GFP Assay. Quantification of reporter GFP was conducted at 4.5 dpf with injected wild type embryos. The percentage of embryos showing GFP signal in the

hindbrain was first calculated, and these GFP⁺ embryos were then deyolked and fixed with 4% PFA in PB. The embryos injected with the plasmid containing mutated ASCL1 motif were fixed at room temperature for 3 h. After washing with 1× PBS-0.1% Tween20 solution (3 × 5 min), the gut was dissected out and scanned for GFP signal under the confocal microscope. The embryos injected with either the plasmid containing intact *adcyap1b* enhancer, or the plasmid containing mutated SOX11 motif were fixed at 4 °C overnight. The next day, these embryos were washed with 1× PBS-0.1% Tween20 solution (3 × 5 min), followed by 1× MeOH (1 × 5 min), and stored in fresh MeOH at −20 °C. GFP signal in the ENS was examined after staining with GFP antibody, and overlapping expression of GFP and *adcyap1b* was examined after costaining with GFP antibody and *adcyap1b* HCR probe.

Imaging and Image Processing. All images of the whole gut of zebrafish embryos were taken with confocal microscopes. Staining images in Fig. 2 were taken with Zeiss LSM 980. Staining images of loss of function studies were taken with Zeiss LSM 900, and staining images in Figs. 4 and 5 were taken with Leica STELLARIS 8. Widefield images of whole *tyr* knockout, *sox11a/b* knockout, and GFP enhancer zebrafish embryos were taken with Leica M165 FC, and wholemount staining of *sox11a* and *sox11b* was taken with Leica STELLARIS 8. All images were processed with FIJI. Maximum Intensity Projection was conducted, and the brightness and contrast of the image were set to the same for comparison between experimental and control groups. Image alignment from multiplex staining was performed with FIJI plugin “Hwada-CoordinateShift.” Quantitative cell counting was conducted with FIJI plugin “Trainable Weka

Segmentation,” followed by “Analyze particles.” Quantitative signal area analysis was conducted with FIJI, “Analyze”-“Measure.”

Statistical Analysis. Quantification of signal expression pattern in loss of function studies was analyzed by the two-tailed unpaired t test or the Mann–Whitney test, respectively, depending on whether or not the data followed a normal distribution. Quantification of the percentage of GFP-positive embryos in the enhancer assay was analyzed with the χ^2 test.

Data, Materials, and Software Availability. The bulk ATAC data have been deposited in GEO database (GSE303101)⁷⁶. All other data are included in the manuscript and/or supporting information.

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Supplementary

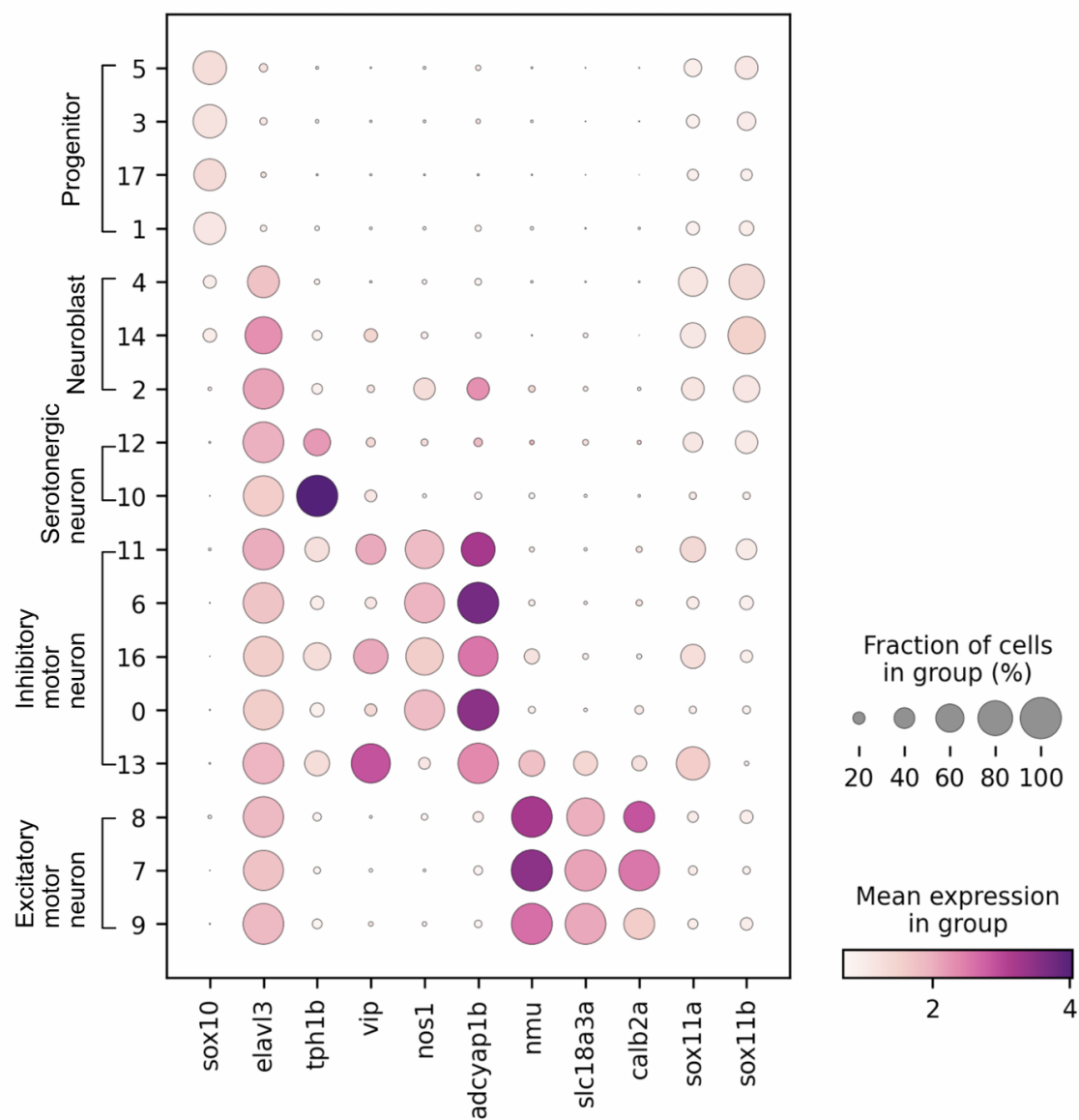


Fig.S1. **Three main mature neuronal clusters were identified based on gene expression profile.**

3dpf

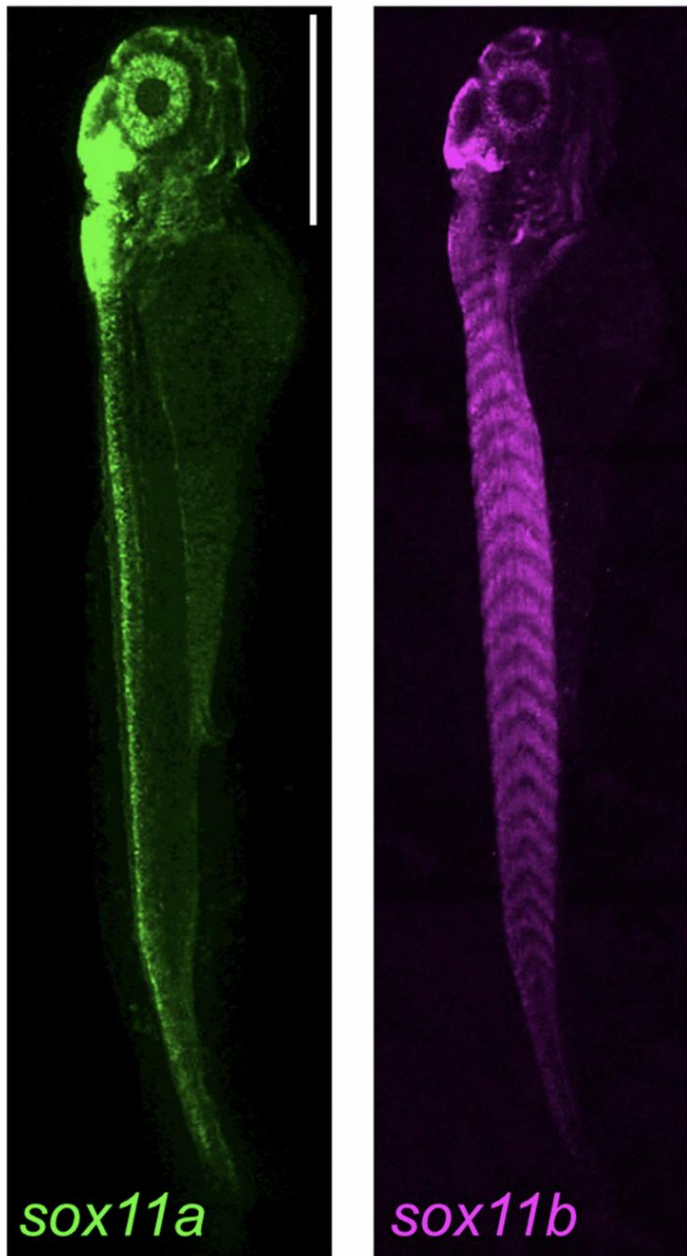


Fig.S2. HCR staining showed *sox11a* and *sox11b* expression in the brain and throughout the body. All staining images were after 'Max Intensity' z-stack projection. Scale bar = 100um.

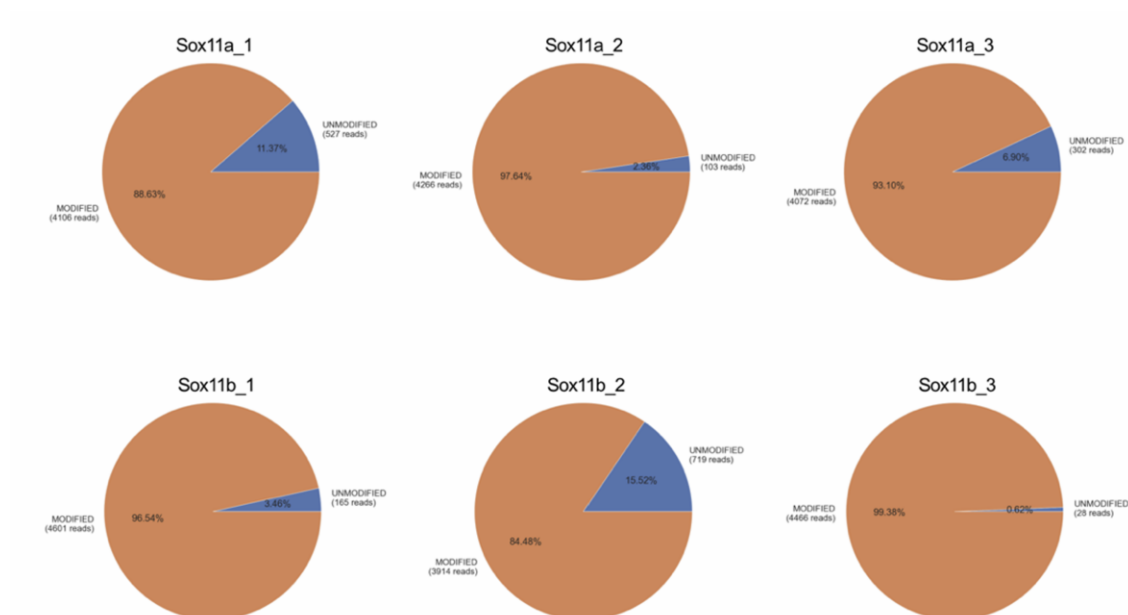


Fig.S3. CRISPR_gRNA injection efficiency was examined with nanopore sequencing, suggesting a high modification rate. N = 10, replicates =2.

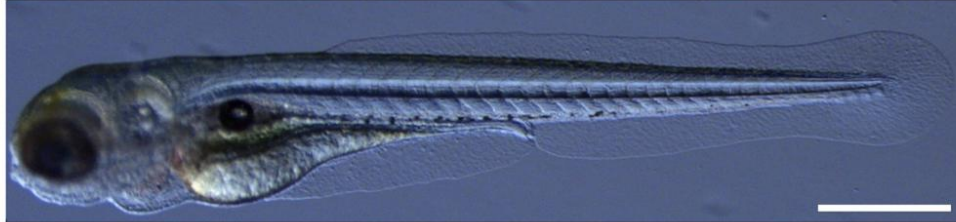
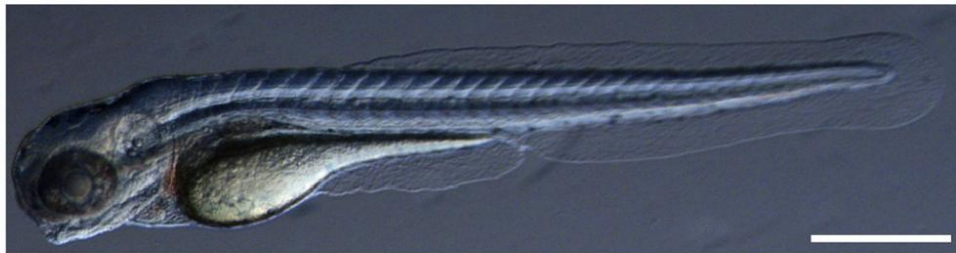
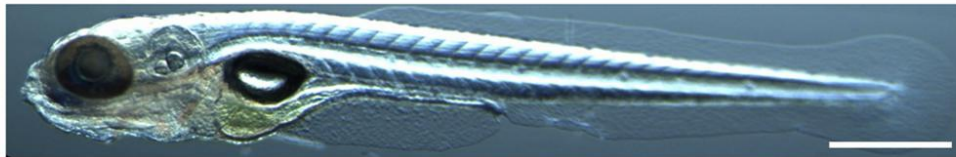
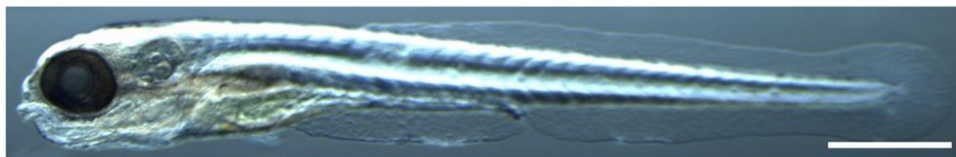
A*Tyr*-KO 4.5dpf*Sox11a/b*-KO**B***Tyr*-KO 6.5dpf*Sox11a/b*-KO

Fig.S4. No obvious phenotypical difference between *tyr*-KO and *sox11a/b*-KO embryos except a delay in development. (scale bar = 0.5mm) (A) Brightfield images of *tyr*-KO and *sox11a/b*-KO at 4.5dpf (B) Brightfield images of *tyr*-KO and *sox11a/b*-KO at 6.5dpf.

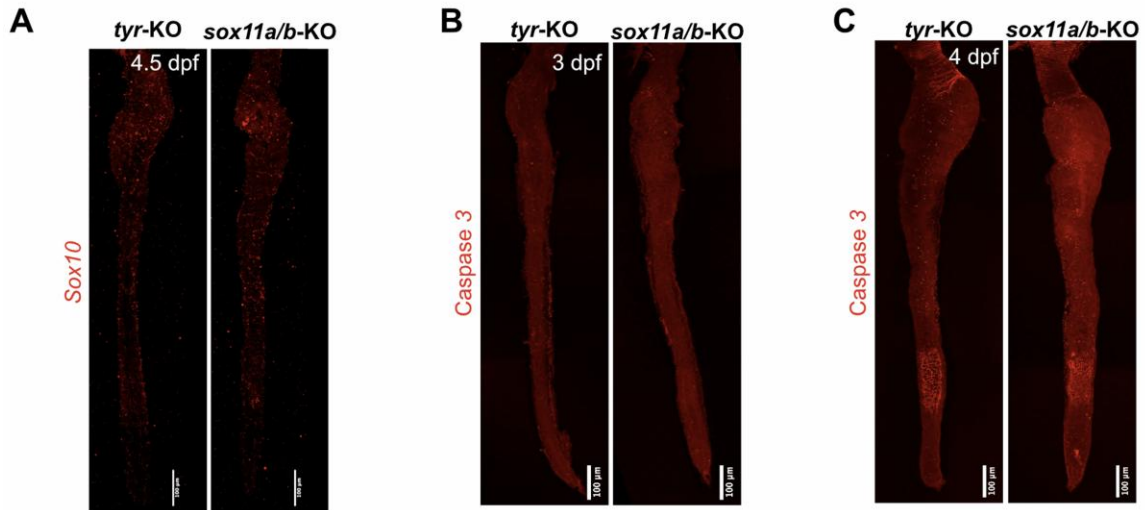


Fig.S5. No significant difference was observed between *sox11* knockout embryos and control embryos in *sox10* expression and cell apoptosis. All staining images were after ‘Max Intensity’ z-stack projection. Scale bar = 100μm. (A) Representative images of HCR staining comparing the expression levels of *sox10* in *sox11* knock-out embryos with the control embryos at 4.5dpf. ($N_{sox11a/b} = 11$, $N_{tyr} = 13$, replicates = 3) (B) Representative images of antibody staining comparing the expression levels of Caspase 3 in *sox11* knock-out embryos with the control embryos at 3dpf. ($N_{sox11a/b} = 4$, $N_{tyr} = 7$, replicates = 2). (C) Representative images of antibody staining comparing the expression levels of Caspase 3 in *sox11* knock-out embryos with the control embryos at 4dpf. ($N_{sox11a/b} = 6$, $N_{tyr} = 9$, replicates = 2)

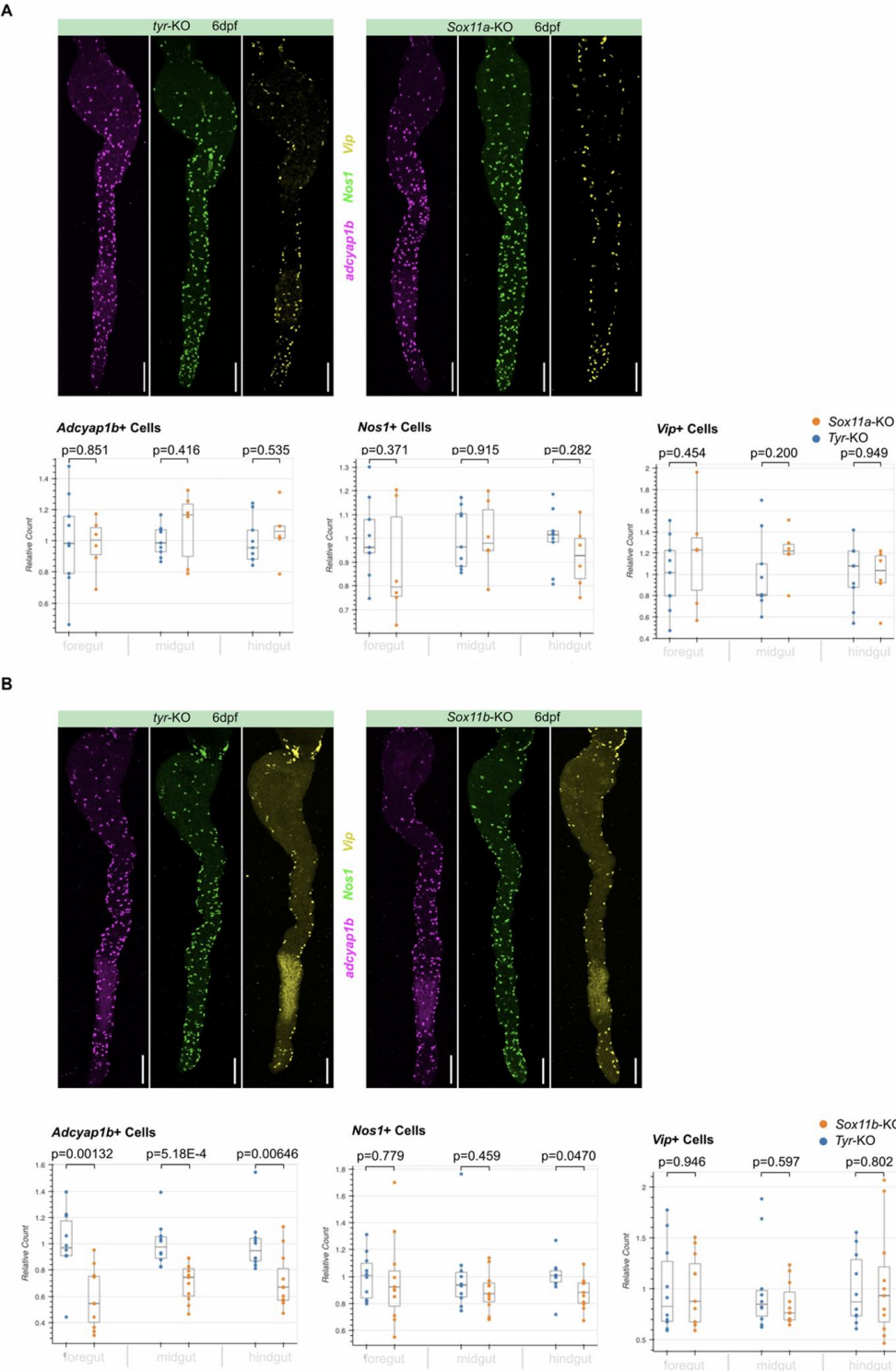


Fig.S6. *sox11a* and *sox11b* single knockout showed a less severe phenotype than the double knockout at 6dpf. All staining images were after 'Max Intensity' z-stack projection. Scale bar = 100um. (A) *sox11a* single knockout resulted in no significant difference in the expression level of a variety of neuronal markers. ($N_{sox11a} = 6$, $N_{tyr} = 9$, replicates = 2). (B) *sox11b* single knockout resulted in a significant reduction of *adcyap1b* expression throughout the gut, and of *nos1* expression in the hindgut region. ($N_{sox11b} = 11$, $N_{tyr} = 10$, replicates = 2)

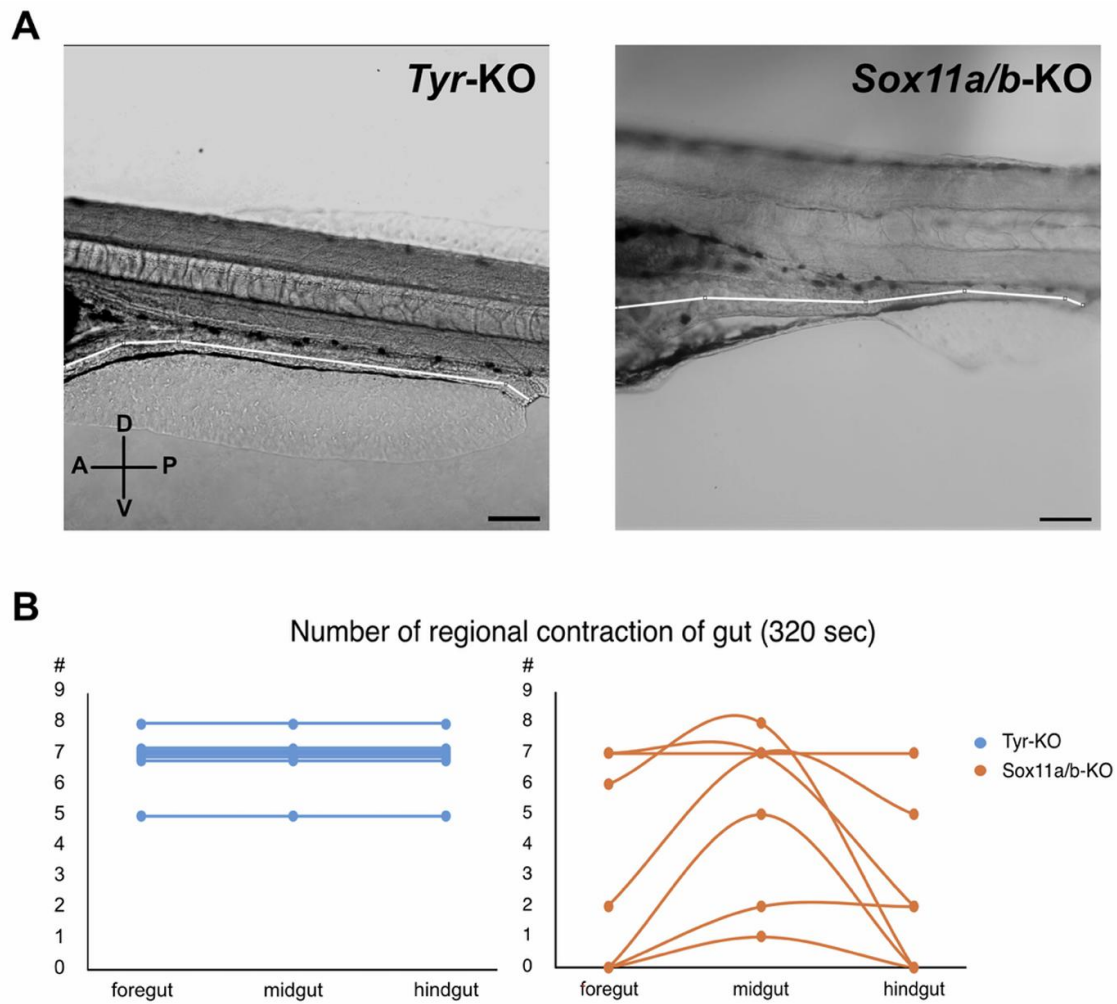


Fig.S7. *sox11a/b*-KO showed a significant increase in incoherent gut motility at 10.5dpf.

(A) Reference line for kymograph. (Scale bar = 100um) (B) The number of regional contractions along the gut was consistent in *tyr*-KO, whereas that of *sox11a/b*-KO shows a large variation. ($N_{sox11b} = 7$, $N_{tyr} = 8$)

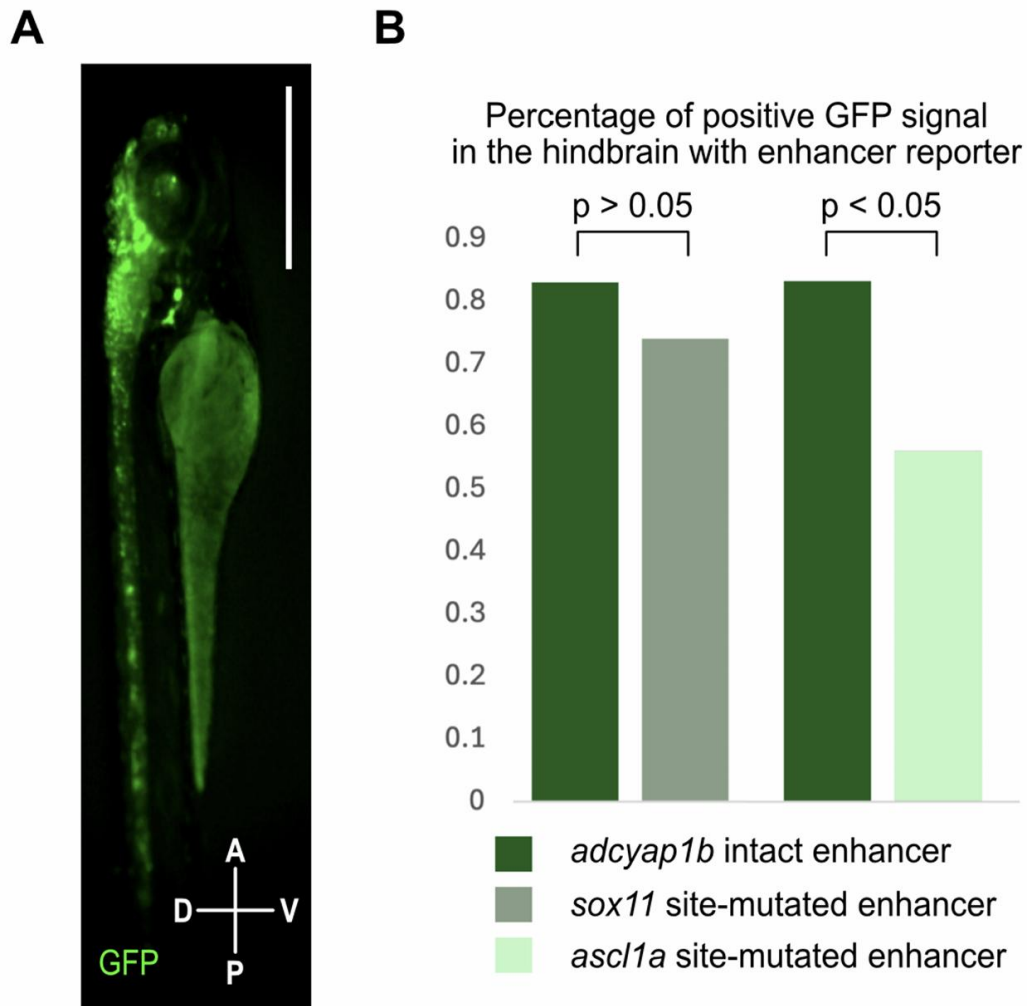


Fig.S8. The *adcyap1b* enhancer mediating GFP expression in the hindbrain was differentially regulated by *sox11* and *ascl1a* motif-mutation. (A) At 3 dpf, embryos showed *adcyap1b* enhancer mediating GFP reporter expression in the hindbrain. Scale bar = 0.5mm. (B) Enhancers with the mutated *ascl1a* motif showed a significant reduction in the percentage of positive GFP signal in hindbrain, whereas the mutated *sox11* motif didn't. ($N_{\text{intact-enhancer}} = 59$, $N_{\text{mutated-sox11-motif}} = 42$, replicates = 3; $N_{\text{mutated-ascl1a-motif}} = 182$, replicates = 4)

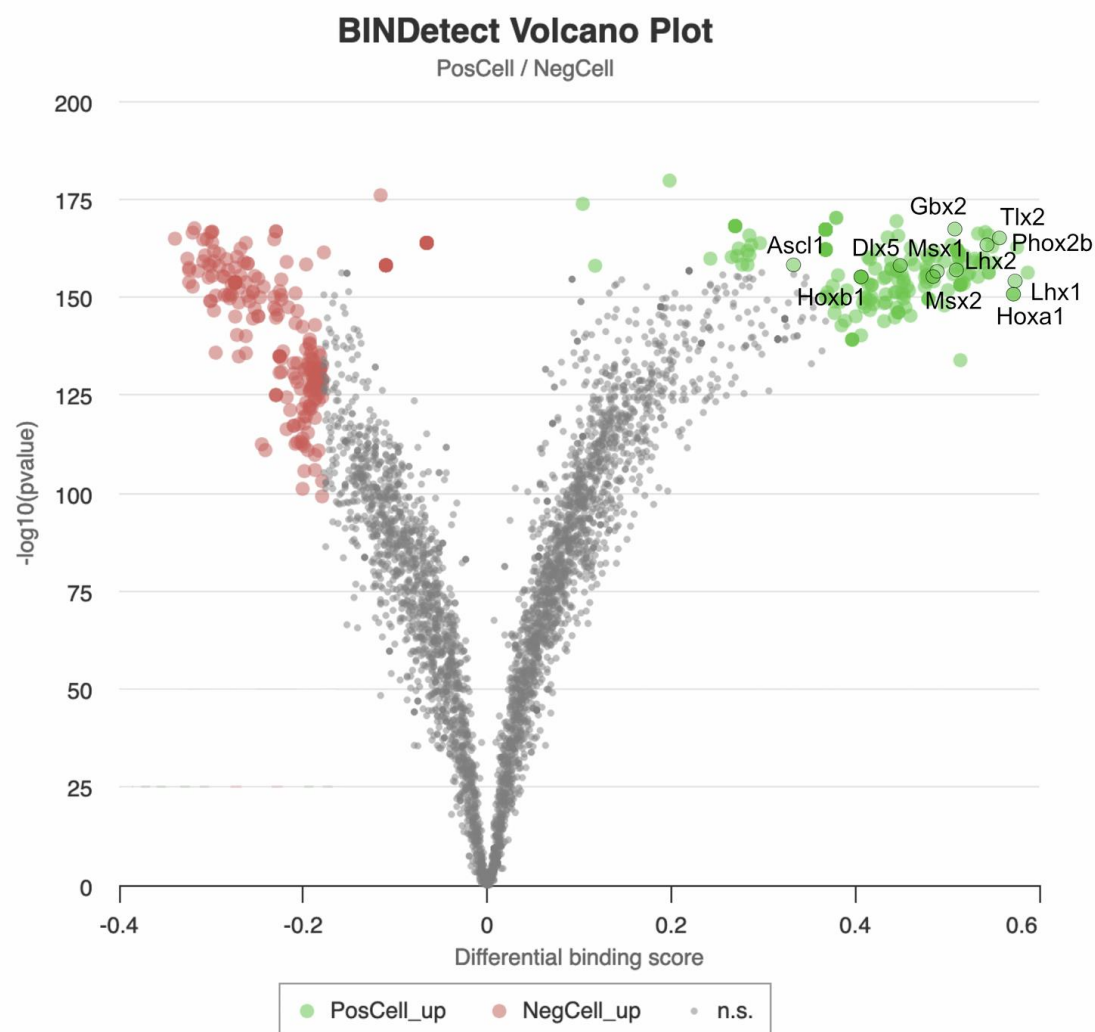


Fig.S9. TOBIAS footprinting analysis showed an increase in multiple transcription factors that participated in neural crest differentiation.

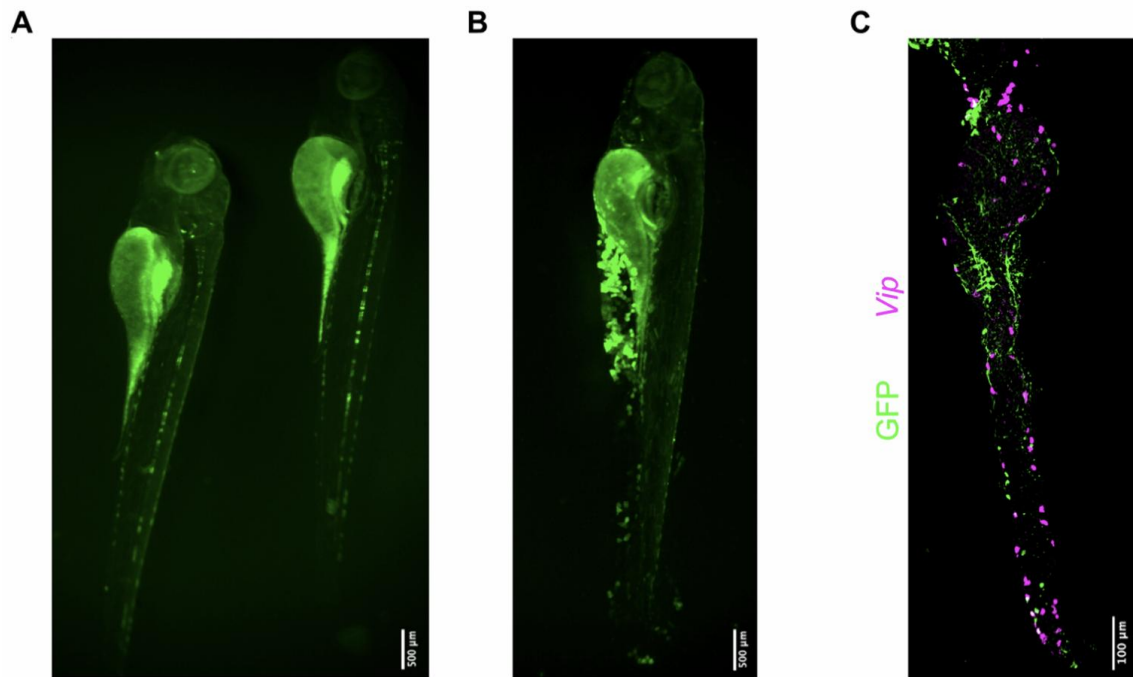


Fig.S10. No GFP signal that overlaps well with the corresponding gene was found in the ENS with reporter assays of three other putative enhancers of either *nos1* or *vip*. (Yolk is autofluorescent in (A),(B)) (A) GFP expression mediated by the *nos1* enhancer 1 sequence showed consistent expression throughout the spinal cord. Scale bar = 0.5mm. (B) GFP expression mediated by the *nos1* enhancer 2 sequence showed consistent expression in epidermal cells. Scale bar = 0.5mm. (C) GFP expression mediated by the *vip* enhancer sequence showed sporadic expression and little reporter expression in the ENS. Scale bar = 100um.

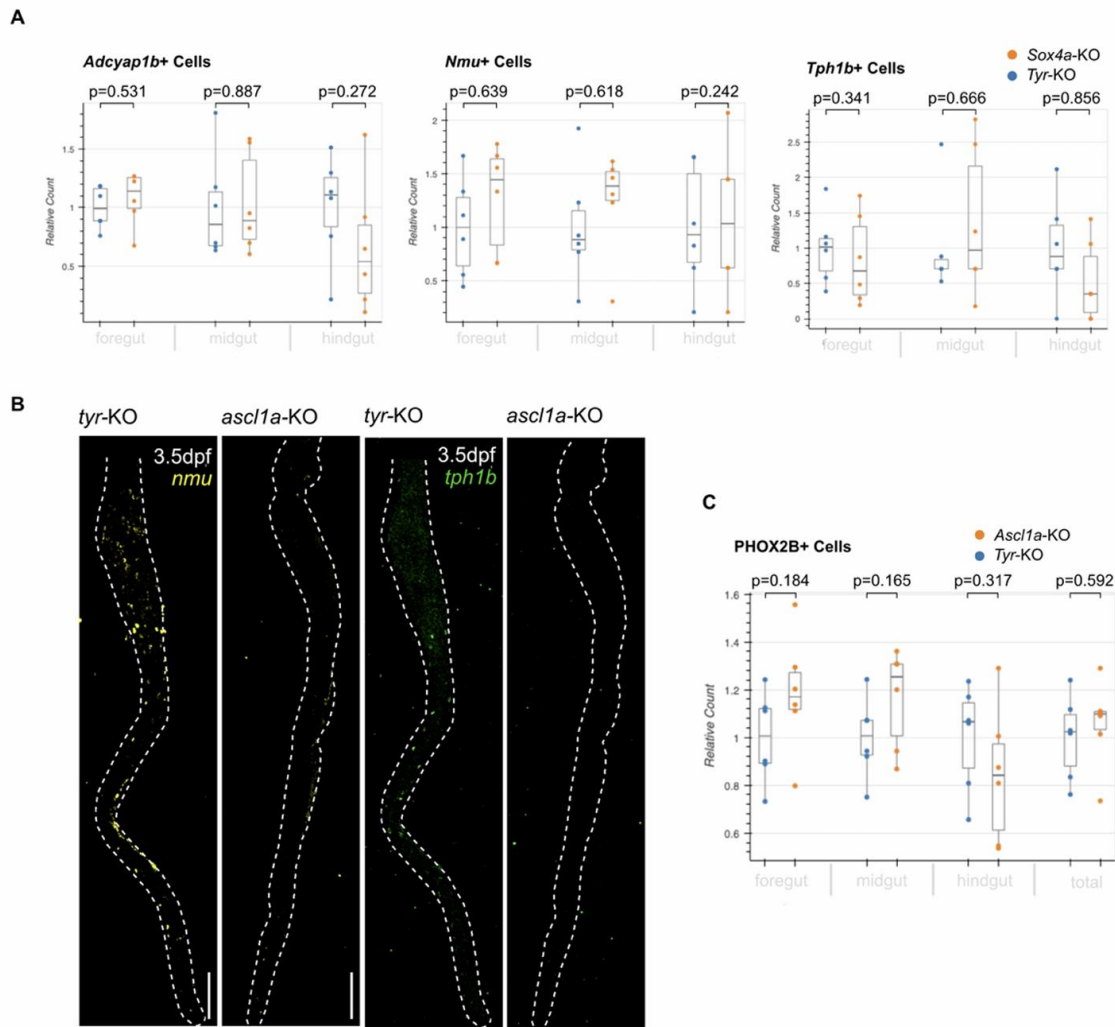


Fig.S11. While *sox4a* didn't affect ENS development, *ascl1a* knockout significantly impacted neurogenesis in ENS development at 3.5dpf. All staining images were after 'Max Intensity' z stack projection. (A) *sox4a* knockout showed no significant difference in the expression level of a variety of neuronal markers. ($N_{sox4a} = 6$, $N_{tyr} = 6$, replicates = 2) (B) Representative images of a near complete loss of both *nmu* and *tph1b* signal was observed in *ascl1a* knockout. ($N_{ascl1a} = 6$, $N_{tyr} = 6$, replicates = 2) Representative images were selected. Scale bar = 100um. (C) No significant difference was found in Phox2b expression in *ascl1a*

knockout, suggesting *ascl1a* affected neurogenesis instead of the migration of ENS progenitor cells. ($N_{ascl1a} = 6$, $N_{tyr} = 6$, replicates = 2)

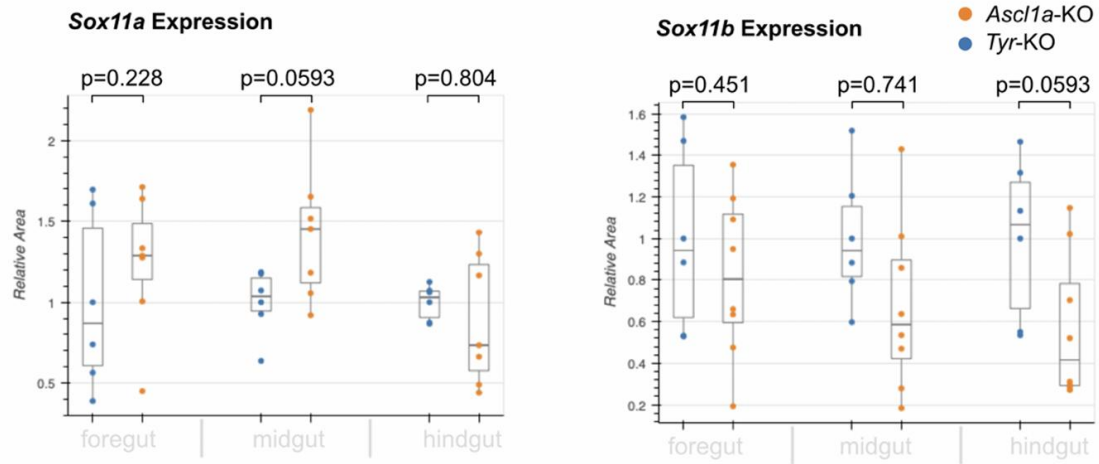


Fig.S12. *ascl1a* knockout didn't cause a significant difference in either *sox11a* or *sox11b* expression level at 3.5dpf in ENS. ($N_{ascl1a} = 8$, $N_{tyr} = 6$, replicates = 2)

Other Supplementary Materials

Document S1 (separate file). Detailed CRISPR sequencing result of *sox11a* and *sox11b* crRNA

Document S2 (separate file). qpcr result of *ascl1a* and *sox4a* crRNA

Movie S1 (separate file).

Gut motility assay for *tyr*-KO embryos at 10.5dpf

Movie S2 (separate file).

Gut motility assay for *sox11a/b*-KO embryos at 10.5dpf

NEURONAL ATLAS OF ZEBRAFISH ENS AT MULTIPLE TIME POINTS REVEAL THE TRANSITION OF ENS COMPOSITION DURING DEVELOPMENT

Background

To better understand the distribution of neuronal subtypes in the enteric nervous system, we applied multiplex Hybridization Chain Reaction (HCR) in situ hybridization to identify transcripts characteristic of a variety of neurotransmitters within the zebrafish ENS. By studying both embryonic and adult stages, we sought to capture the dynamics of the neuronal cluster formation during the course of zebrafish ENS development. A total of 16 markers of neuropeptides and neurotransmitters were selected according to both the scRNA-seq data of zebrafish embryo ENS from our lab³⁵ and the bulk RNA-seq of adult zebrafish ENS⁴⁶ (Fig.7). Probes to the following markers were used: *vip*, *vipb*, *nos1*, *adcyap1b*, *tph1b*, *nmu*, *slc18a3a*, *calb2a*, *galn*, *tac1*, *pnoca*, *cart2*, *tac3a*, *vgf*, *chata*, *nppc*, and a pan-neuronal marker for cell segmentation HuC/D. Most selected markers are highly expressed in both embryonic and adult ENS, while some show differential expression patterns. For example, *tac1*, Tachykinin Precursor 1, a gene that encodes mainly Substance P and Neurokinin A, is not expressed in the embryonic ENS, but is upregulated in adult ENS according to the bulk RNA-seq. Interestingly, *tac3a*, the zebrafish ortholog of another tachykinin gene that is mainly responsible for another neuropeptide, Neurokinin B, is downregulated in adult zebrafish

ENS. This suggests that the adult zebrafish ENS may employ a different signaling pathways from embryonic versus adult neuronal genes in the ENS. Furthermore, *galn* (*gal*), encoding galanin, was observed in the adult glial cell population but was seen in the embryo in a neuronal cluster expressing both nitrergic marker, *nos1*, and cholinergic marker, *slc18a3a*.

While published research in our lab examined the spatial localization of neuronal subtypes of embryonic zebrafish ENS³⁵, we expanded the original 9 neuronal markers to 15 for finer neuronal subclusters identification, and applied a modified version of the technique to the adult zebrafish ENS to capture the dynamic change of ENS at different developmental stages. Due to its relative transparency and small size that enable imaging of the entire length of the zebrafish gut, zebrafish serves as a good model for ENS visualization. Moreover, it allows examination of region-specific difference within the ENS, which is a significant advantage over other vertebrate systems.

Result

Multiplex HCR enables visualization and co-expression analysis of up to 15 neurotransmitters

We adopted a method of ENS dissection¹⁰⁰ that was coupled with an HCR technique¹⁰¹, together with our multiplex staining protocol to achieve multiplex HCR staining for adult zebrafish ENS. Briefly, the myenteric plexus along with the longitudinal muscle was separated from the rest of the gut and fixed onto a slide. After immobilizing the myenteric

plexus layer onto the slide, we conducted 6 sequential rounds of HCR plus one round of immunostaining in the end, enabling us to examine expressions of all 16 markers of neurotransmitters and neuropeptides in the adult zebrafish ENS (Fig.S13-S28). In our pilot study, we examined expressions of 5 neuronal markers, *nos1*, *vipb*, *vip*, *tph1b*, and *cart2*, within the most rostral part of adult gut. We were able to identify 6,985 neurons in the examined region and divided them into 13 main neuronal clusters based on their expression profile (Fig. 8). The contribution of each neuronal subclusters were then calculated. In comparing the corresponding neuronal distribution of embryonic zebrafish ENS at 6 dpf, *nos1*⁺/*vipb*⁺ neurons were found to be the major contributors to both adult and embryonic zebrafish ENS. In contrast, *cart2* was restricted to the hindgut in of zebrafish embryos, but observed throughout the rostral region of adult ENS³⁵. Together, the pilot study not only validated the feasibility of our imaging method but also suggested both similarity and difference of the ENS composition between embryonic and adult stages.

Spatial examination of *calb2a*, *galn* and *nos1*

We further optimized our pipeline by adding steps for sample bleaching, image deconvolution, manual-supervised tile stitching and cell segmentation to improve imaging quality and analysis stringency. We applied this to the whole gut of adult zebrafish (Fig. 9). As proof of principle, we used the optimized pipeline to analyze expression of 3 neuronal markers, *calb2a*, *galn* and *nos1*, in both the embryonic and adult gut. We were able to identify 266 neurons in embryonic zebrafish ENS at 6.5dpf, and 16k neurons in the adult zebrafish

ENS, and assign them to neural clusters based on their expression profile of neurotransmitter and neuropeptides.

At embryonic stages, *nos1*, a major marker for nitrergic neurons, is expressed widely along the entire length of the gut. On the contrary, only few cells in the esophagus express either *galn* or *calb2a* that encodes an intracellular calcium binding protein (Fig. 10A). In the adult ENS, while *galn* is still only expressed in the most rostral region of the gut (Fig. 10B), a total of 4344 neurons expressing *calb2a* were found distributed along the entire length of the adult gut. Thus, our pipeline successfully captured neurons expressing three markers (Fig.10C), including one rare clusters that is *calb2a*⁺/*nos1*⁺.

We divided the length of adult zebrafish gut into four subregions, rostral intestinal bulb (RIB), mid-intestine (MI), caudal intestine (CI), and the most caudal part of CI (CCI) that lacks any folding, based on the gut morphology⁴³. Interestingly, we observed spatial differences between neuronal clusters, with *calb2a* more highly expressed rostrally in the RIB and *nos1* expressed throughout all regions (Fig. 11). A transition of neuronal cluster contribution was also identified when comparing the result between adult and embryonic ENS. By including all 15 markers, we would create the first neuronal atlas for the adult zebrafish ENS. The richness of spatial-temporal information enables us to better understand ENS development (Fig.11).

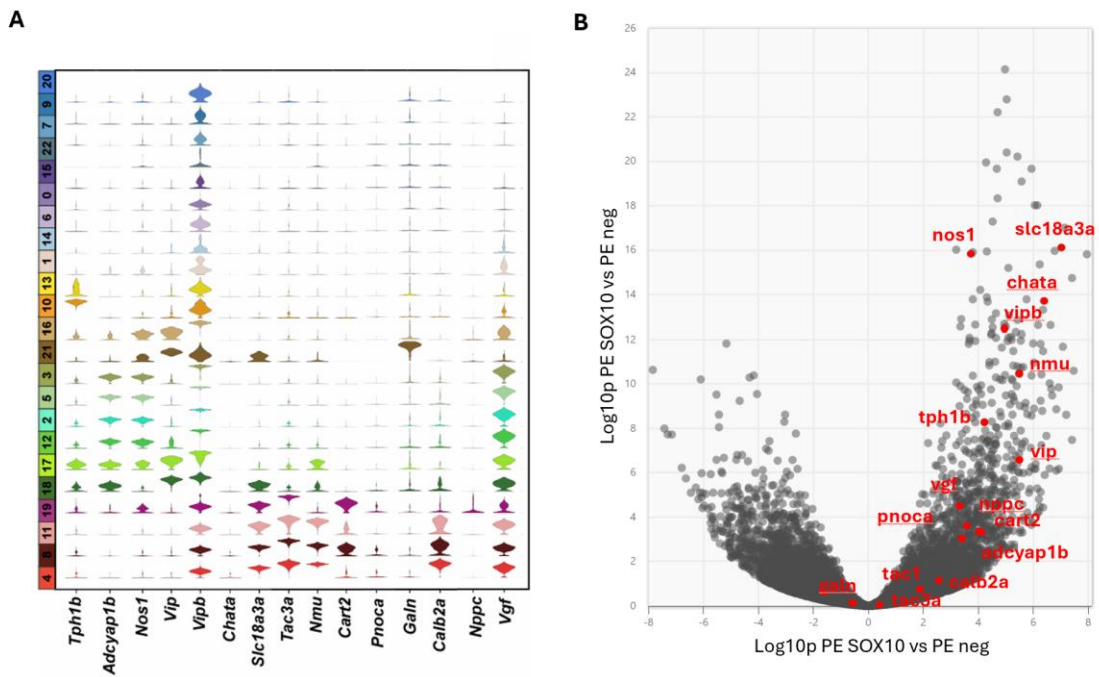


Fig.7 Markers of neurotransmitters/ neuronal peptides were selected based on two zebrafish datasets. (A) scRNA-seq dataset on zebrafish embryonic ENS during 2-6 dpf. (B) bulk RNA-seq dataset on adult zebrafish ENS.

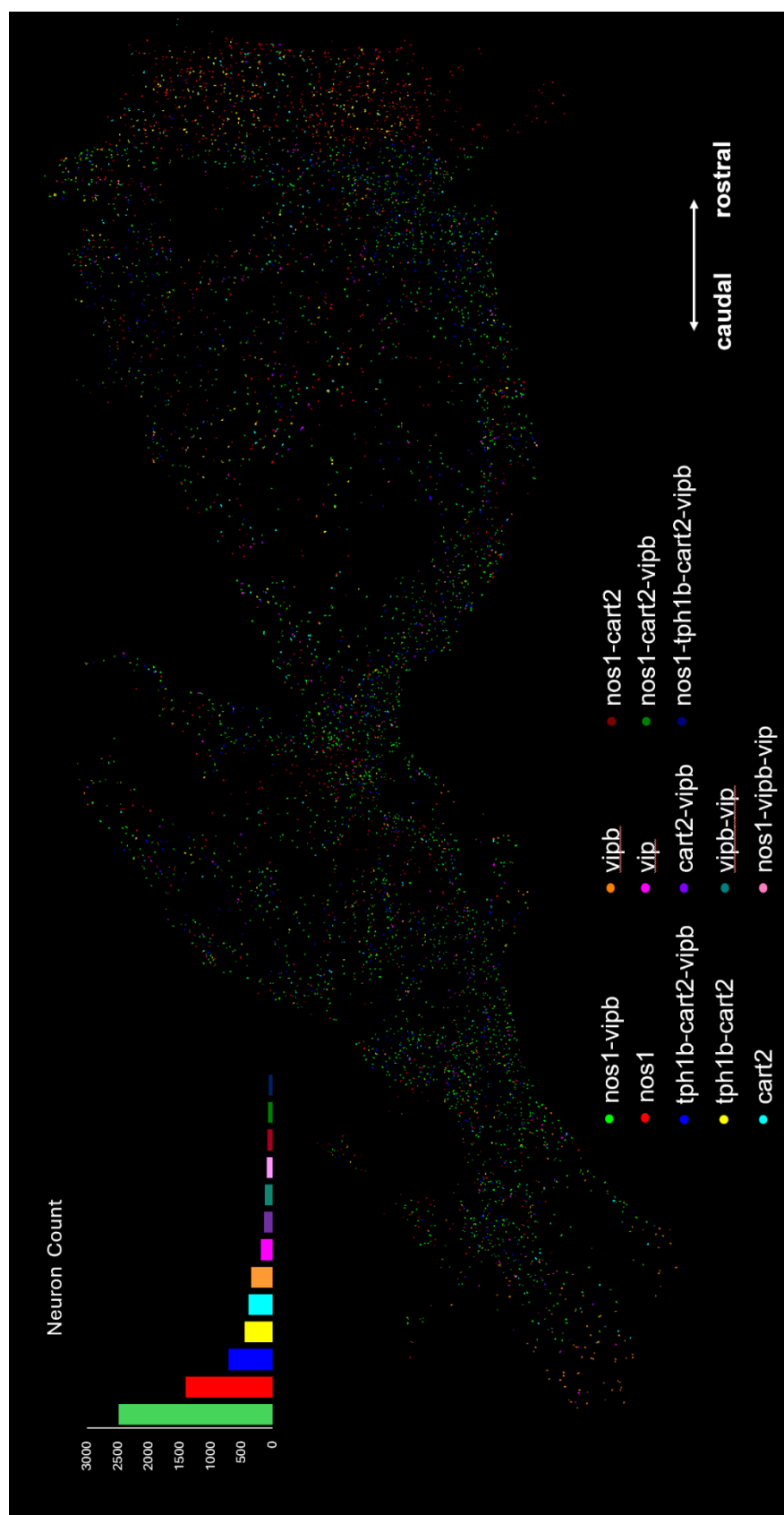


Fig.8 Neuronal atlas of the rostral region of adult zebrafish ENS with 5 neuronal markers. Thirteen neuronal clusters were identified, and their neuron number and spatial distribution were analyzed.

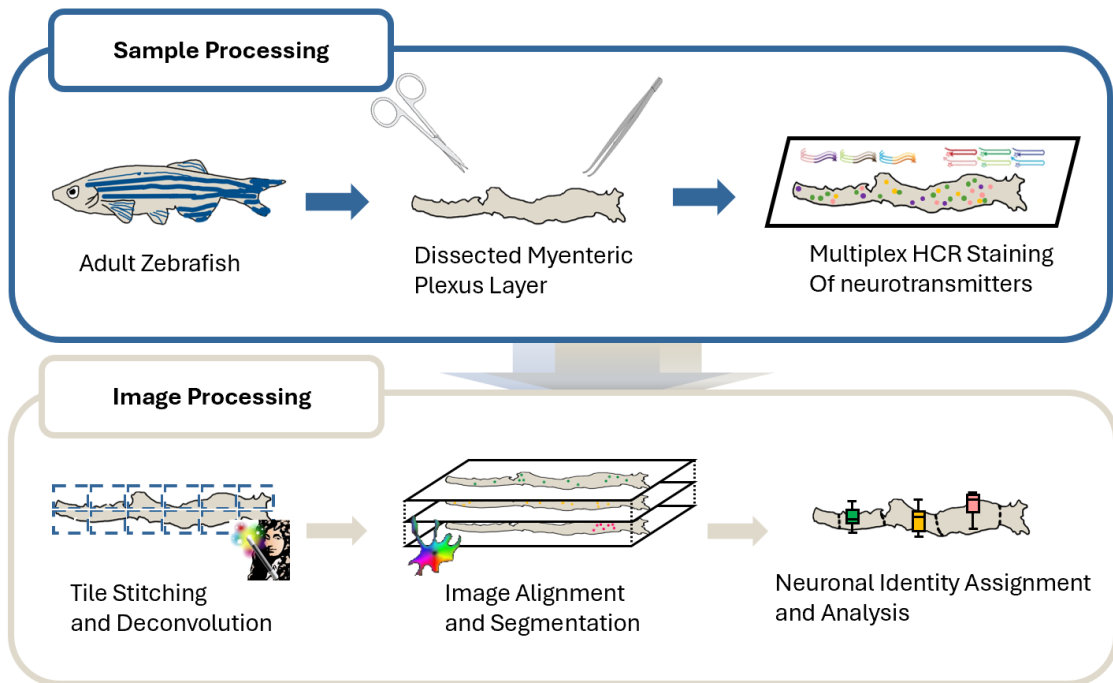


Fig.9 The optimized pipeline includes sample processing and image processing.

Sample Processing: Adult zebrafish was first euthanized, and its myenteric plexus layer was isolated and immobilized on the slide for multiplex HCR staining. Image Processing: Confocal images underwent tile stitching, deconvolution, image alignment and segmentation, and eventually neuronal identification.

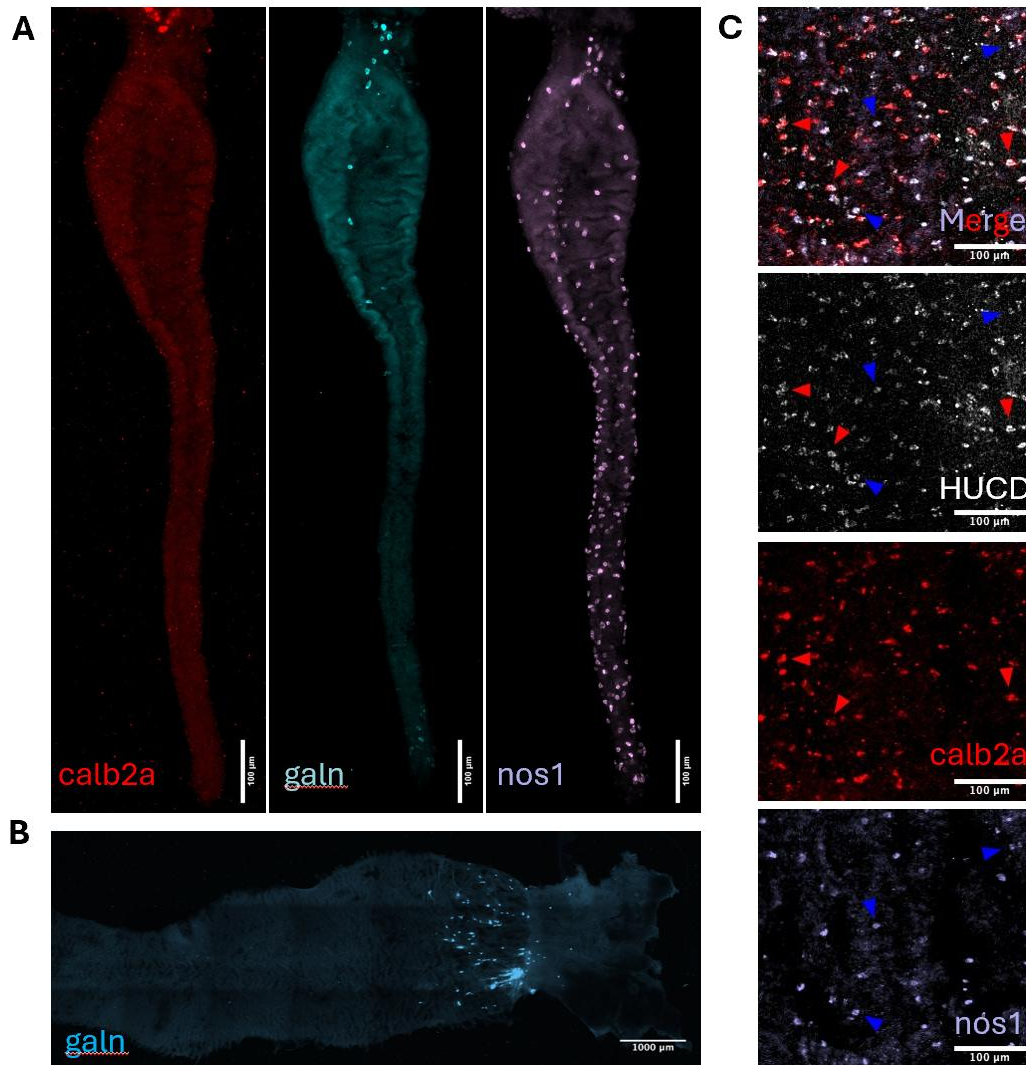


Fig.10 Proof of principle study reveals both temporal and spatial differences between neuronal markers. (A) The expression of *calb2a*, *galn*, and *nos1* in 6.5dpf zebrafish embryo. (B) In adult zebrafish, *galn* is only observed around esophagus. (C) Processed images were aligned successfully. (Blue arrows: neurons expressing *nos1*. Red arrows: neurons expressing *calb2a*)

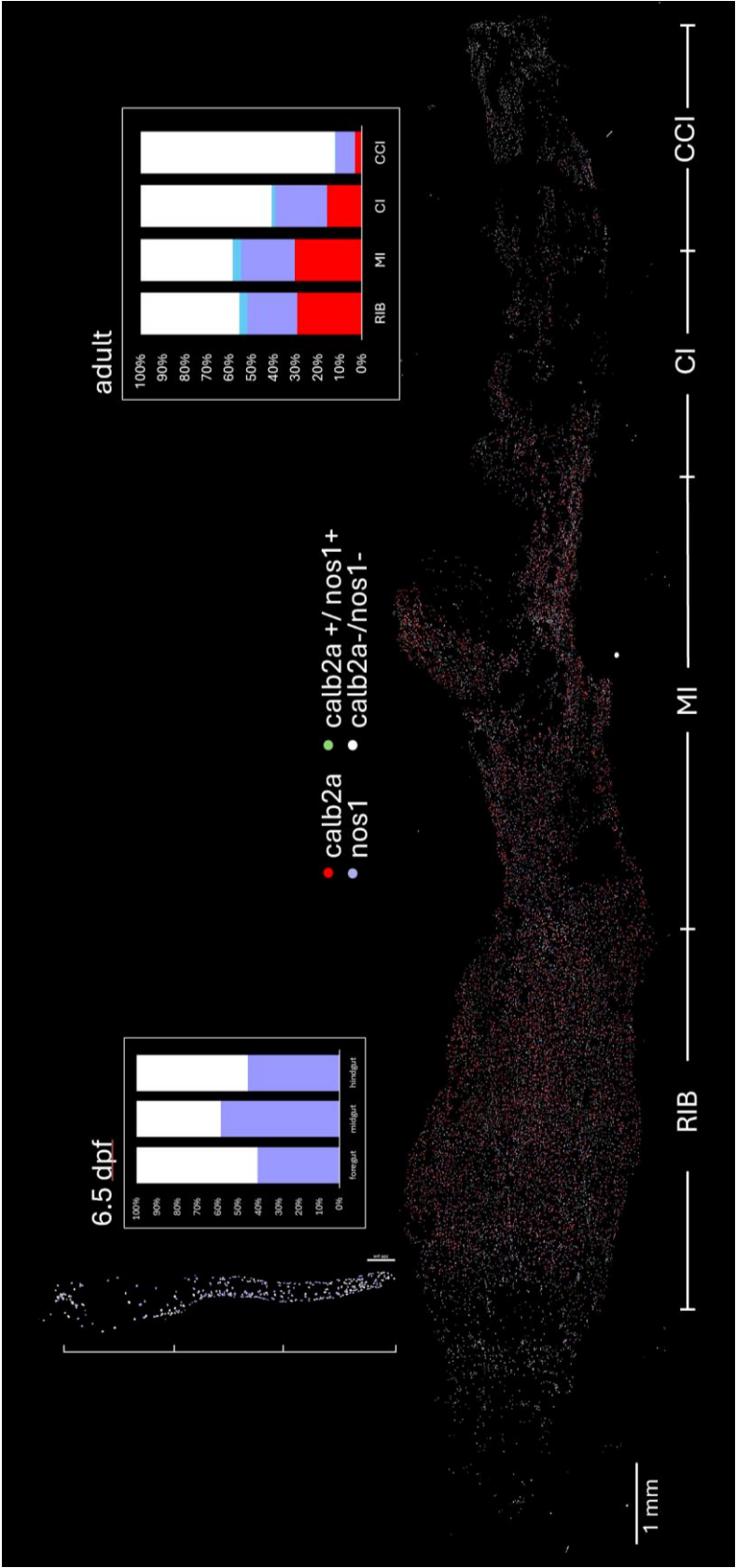


Fig.11 Neuronal atlas of the entire length of adult and embryonic zebrafish gut show expression pattern of *nos1* and *calb2a*. Relative proportion of each neuronal clusters show regional differences.

Methods

Sample Preparation The myenteric plexus, along with longitudinal muscle was dissected out according to the published protocol¹⁰⁰. It was then placed in the middle of the taped well onto a slide, and was fixed with 4% paraformaldehyde (PFA) at room temperature for 15 minutes. The PFA was then dried out to immobilize the sample on the slide, which later got overnight fixation with 4% PFA at 4°C. The next day, the sample was washed with 1× PBS solution for 3×10 min, and went through dehydration with 100% MeOH for 2×10 min. It was then stored in fresh MeOH at -20°C for later usage.

Multiplex HCR and Immunostaining The protocol of HCR staining and immunostaining is the same as the previous project. Except, based on the cycle HCR protocol¹⁰¹, we modified our stripping step between each round of staining to 2×17 min wash with the stripping buffer (80% formamide in 4× SSC, with 0.1% Triton) at 32°C.

Image Acquisition Each round of HCR staining includes three channels for imaging: Alexa 488, Alexa 546 and Alexa 647. For adult zebrafish gut, a background image of all three channels, and all following stained images were acquired with LSM 980 that was equipped with a 10× Air Lens. For 6.5dpf zebrafish gut, the LSM 980 with a 20× Air Lens was used for imaging.

Image Processing The confocal image was composed of multiple tiles, and they were stitched with FIJI stitching plugins. The stitched image was then deconvolved with Huygen Deconvolution software for speckle removal and resolution improvements.

Processed signal images were then aligned to the HUC/D image with FIJI-BigWarp plugin, and cell segmentation was then conducted on aligned image with Cellpose. By exporting cell segmentation profile as ROIs, we calculated overlapping between ROIs with FIJI Macros, and assigned neuronal identity for further analysis.

Supplementary

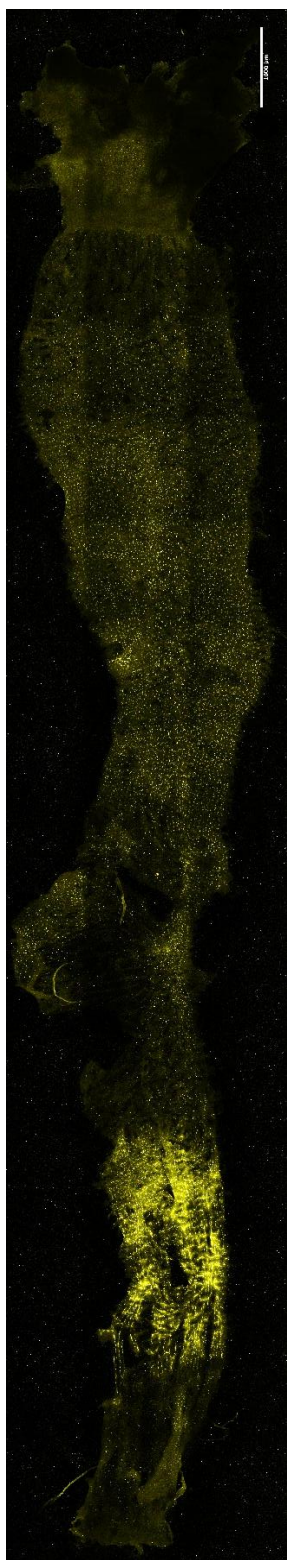


Fig. S13 HCR staining of *calb2a* with adult zebrafish gut



Fig. S14 HCR staining of *cart2* with adult zebrafish gut



Fig. S15 HCR staining of *tac1* with adult zebrafish gut

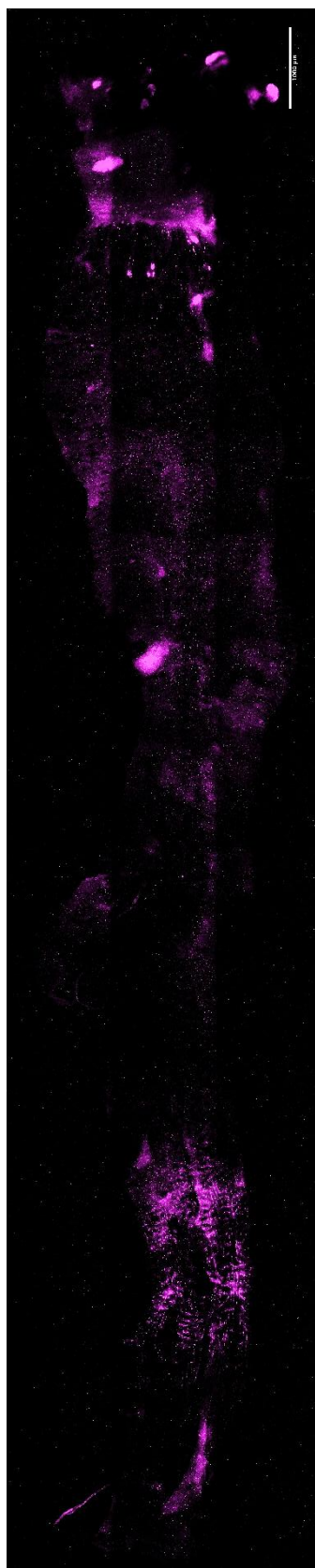


Fig. S16 HCR staining of *chata* with adult zebrafish gut

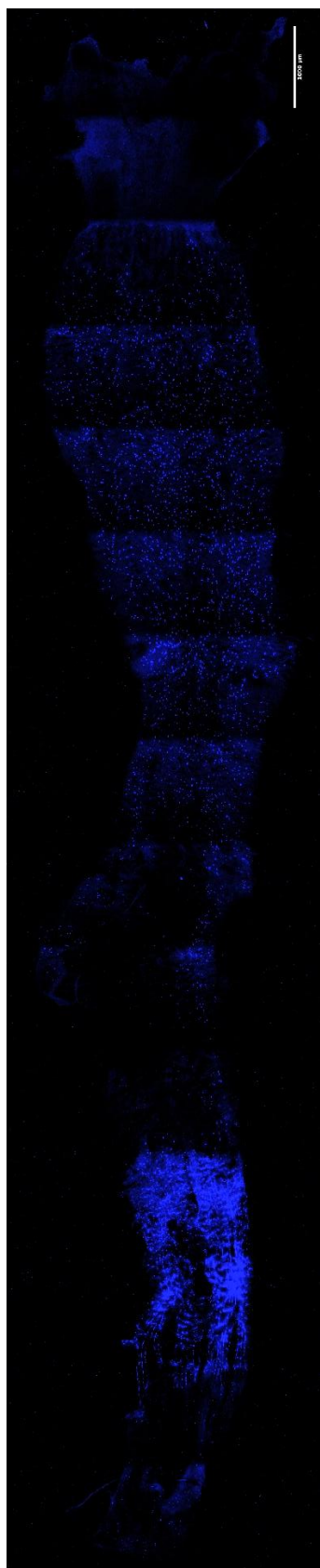


Fig. S17 HCR staining of *tph1b* with adult zebrafish gut



Fig. S18 HCR staining of *slc18a3a* with adult zebrafish gut

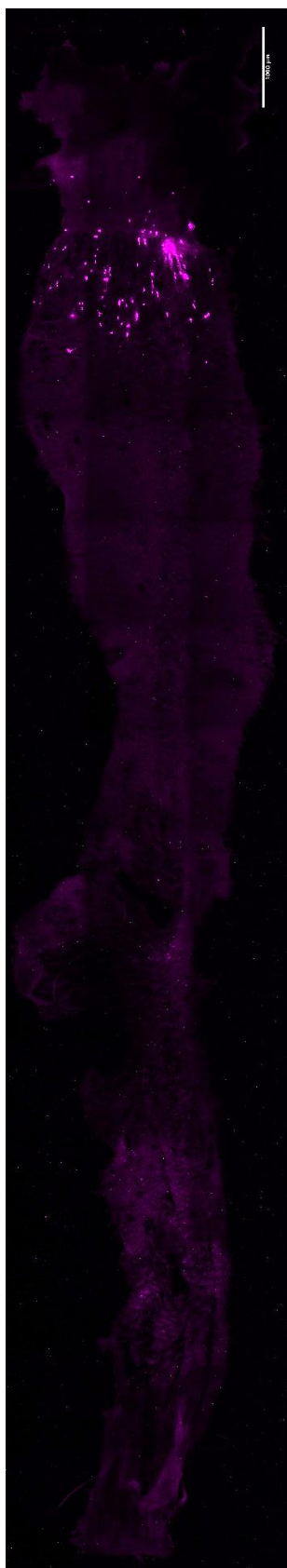


Fig. S19 HCR staining of *galn* with adult zebrafish gut

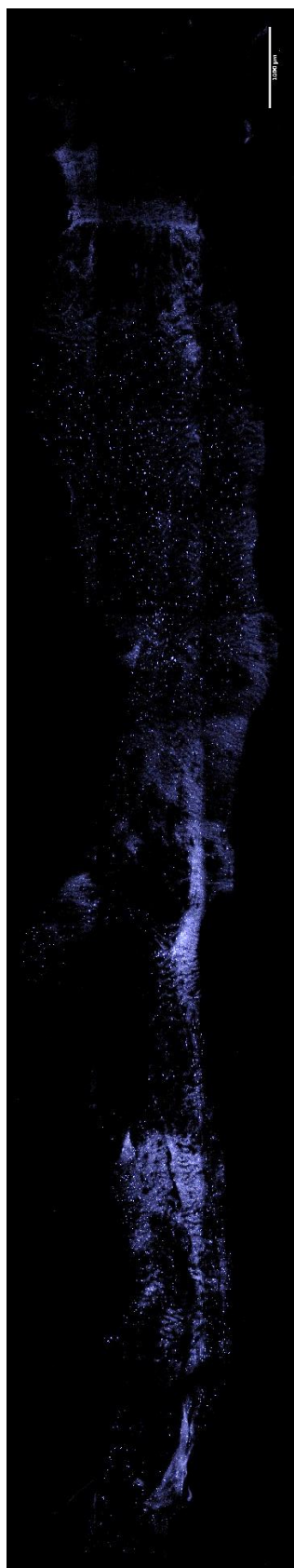


Fig. S20 HCR staining of *pnoca* with adult zebrafish gut

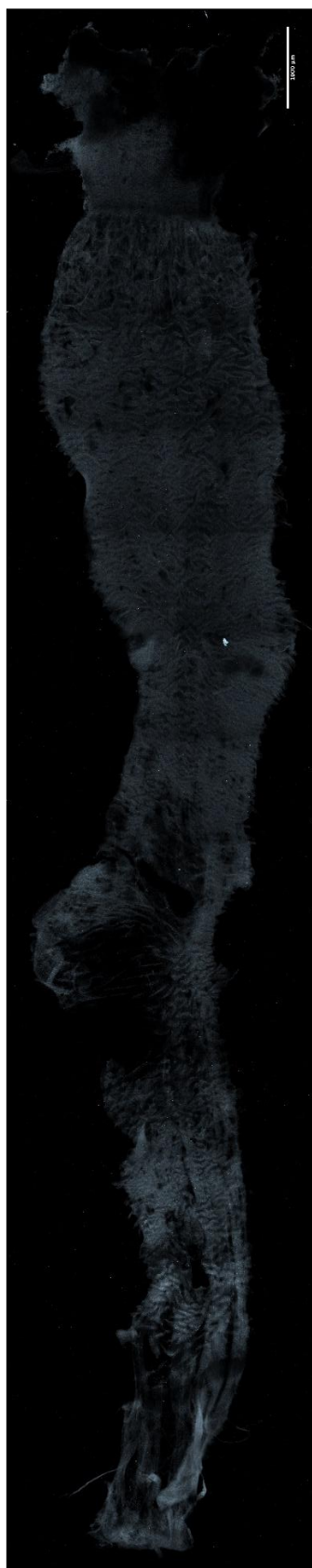


Fig. S21 HCR staining of *nppc* with adult zebrafish gut

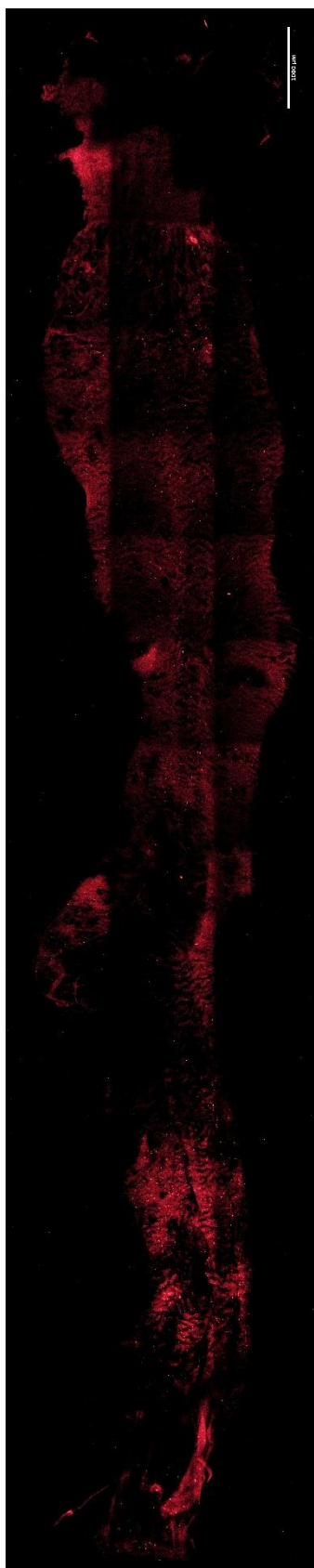


Fig. S22 HCR staining of *tac3a* with adult zebrafish gut

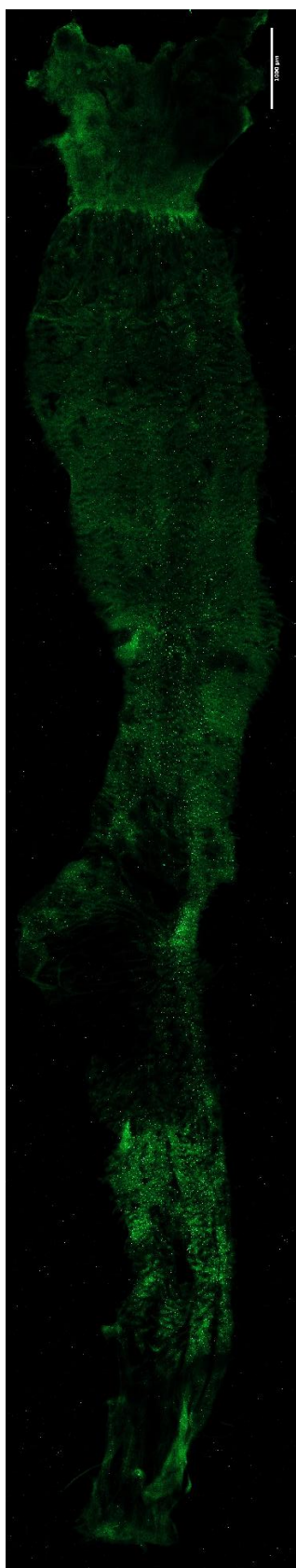


Fig. S23 HCR staining of *adcyap1b* with adult zebrafish gut

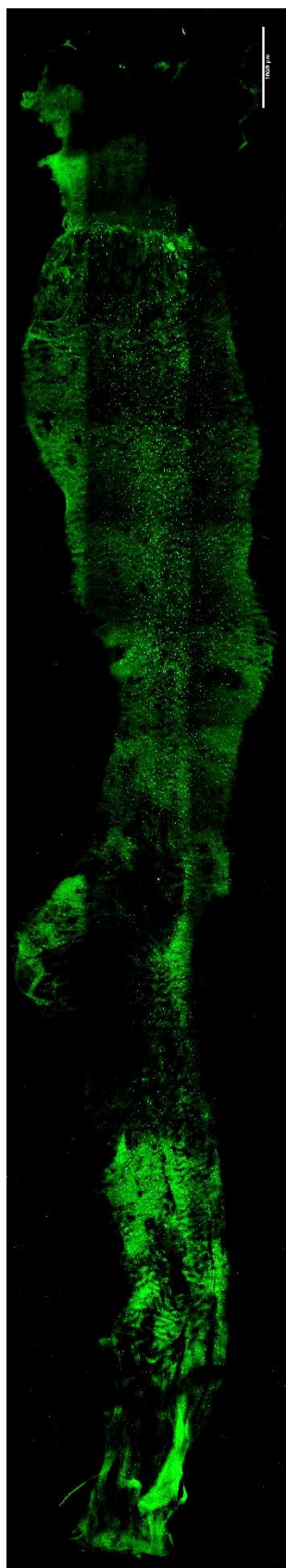


Fig. S24 HCR staining of *nos1* with adult zebrafish gut



Fig. S25 HCR staining of *nmu* with adult zebrafish gut

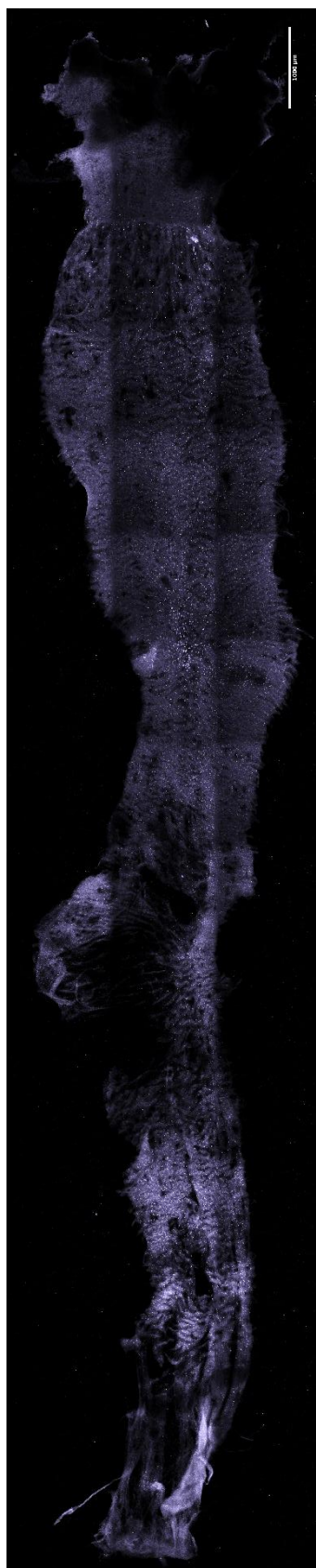


Fig. S26 HCR staining of *vgf* with adult zebrafish gut

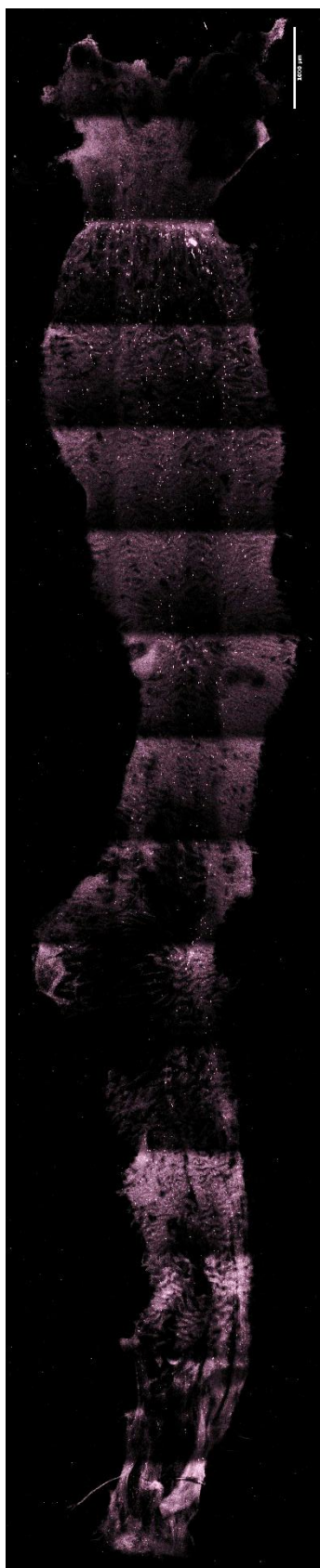


Fig. S27 HCR staining of *vip* with adult zebrafish gut



Fig. S28 HCR staining of *vipb* with adult zebrafish gut

DISCUSSION

During the course of my PhD thesis, I successfully identified *sox11a/b* as a novel transcription factor that affects neuronal specification during zebrafish ENS development, and also developed an imaging method to construct neuronal diversity atlas of zebrafish ENS at multiple developmental stages. Cumulatively, this work has provided insight into spatiotemporal neuronal distribution and function of ENS development.

My first project aimed at identifying key transcription factors that regulate the neuronal fate choice at branch points of the developmental trajectory of zebrafish ENS, identified from our lab's previous scRNA-seq of enteric neurons and neuronal precursors³⁵. We established a pipeline that included expression and functional validation of candidate genes in zebrafish embryonic ENS, and the revelation of their underlying molecular mechanism at the enhancer level. This helps elucidate understanding of the GRN underlying ENS development. The high fecundity of zebrafish and the high efficiency of F0 crispants⁷² enable large-scale screenings of potential transcription factors. However, our research was limited because the loss of function phenotypes are mosaic due to performing CRISPR knockouts at the single-cell stage. To obtain a more comprehensive analysis of the function of transcription factors of interest in ENS development, we have generated a transgenic fishline that expressed Cas9 driven by the *Phox2bb* promoter. In future, with a heat shock-inducible Cre/Lox system¹⁰², this Cas9 fishline can be crossed with another fishline that contains gRNAs for targeted genes¹⁰³. This will enable an ENS-specific and temporally-controllable knockout of the target genes in their progeny ENS. This approach not only provides a more consistent and precise description of the effect of genes of interest on ENS development, but also will allow the examination of their function at multiple developmental stages.

In the first project, we used TOBIAS for footprinting analysis of transcription factors to reveal their potential occupancy on crucial enhancers that are activated during ENS development. However, the current database of TF motifs was a mixed vertebrate database mainly composed of human and mouse data. The caveat is that predicted TF activity does not necessarily infer real activity during zebrafish development. Although CHIP-seq has the potential to map high-resolution protein-DNA interactions in vivo, it is limited by the low availability of antibody that recognize zebrafish proteins compared to mammals⁶⁸. Optimally, an improved database of zebrafish-specific TF motifs could be curated computationally based on matching the DNA-binding domains (DBD) of protein sequence between zebrafish TF to that of mouse and human¹⁰⁴. Researchers have successfully obtained cell-type specific enhancers in human, mouse and chicken telencephalon through applying trained sequence-based deep learning models on single-cell multiome¹⁰⁵. By expanding such methods to zebrafish ENS datasets, a more powerful TF footprinting could be achieved, and further used for a more comprehensive analysis of enhancer codes in zebrafish ENS development.

My second project was designed to further capture the complexity of zebrafish ENS formation by including the spatial information, which was crucial due to the highly dynamic nature of ENS development. The purpose of the project was not limited to the construction of neuronal atlas at multiple developmental stages, but also offered a pipeline for broader usage, such as examining transient spatial-expression of TFs, evaluation of temporal-specific interaction between ENS progenitor cells with surrounding environment, and characterization of disease models.

In the future, a method that could associate the identity of neurons to their axon projection would greatly improve understanding the wiring of ENS. Currently, neurotransmitter-specific label of neuron bodies together with their axons could be achieved by injecting AAV with corresponding Cre-system³⁶. Perhaps AAVs with enhancers targeting specific neuronal subtypes could help visualize all labeled neurons together rather than one cell-type at a time and build an ENS wiring map. Building upon my project, which provides multiple

‘snapshots’ of zebrafish ENS development, introducing a lineage tracing approach would further our understand of the developmental program at an individual-neuron level.

Lastly, my projects demonstrated the advantage of using zebrafish in understanding ENS development. However, one main difference of composition of the ENS between zebrafish and amniotes is the contribution of sacral neural crest cells. Since sacral neural crest cells have only been identified in amniotes¹⁰⁶, and they only contribute to ENS formation at the caudal end and at a later developmental stage than vagal neural crest cells in amniotes, we could reasonably assume that our understanding of ENS development through zebrafish is mostly applicable to the rostral portion of the ENS of amniotes. How and why the ENS has gained additional complexity by addition of the sacral neural crest cells is an interesting open question, highlighting the need to examine emergence of evolutionary complexity to the ENS that can only be gleaned by looking at multiple species on the vertebrate tree of life.

Taken together, this research has provided useful information in characterizing and deciphering the gene regulatory program of ENS development in zebrafish.

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