1 An atlas of neural crest lineages along the posterior developing zebrafish at 2 single-cell resolution

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ABSTRACT

Neural crest cells (NCCs) are vertebrate stem cells that give rise to various cell types throughout the developing body in early life. Here, we utilized single-cell transcriptomic analyses to delineate NCC-derivatives along the posterior developing vertebrate, zebrafish, during the late embryonic to early larval stage, a period when NCCs are actively differentiating into distinct cellular lineages. We identified several major NCC/NCC-derived cell-types including mesenchyme, neural crest, neural, neuronal, glial, and pigment, from which we resolved over three dozen cellular subtypes. We dissected gene expression signatures of pigment progenitors delineating into chromatophore lineages, mesenchyme subtypes, and enteric NCCs transforming into enteric neurons. Global analysis of NCC derivatives revealed they were demarcated by combinatorial *hox* gene codes, with distinct profiles within neuronal cells. From these analyses, we present a comprehensive cell-type atlas that can be utilized as a valuable resource for further mechanistic and evolutionary investigations of NCC differentiation.

Key words: neural crest, sox10, single-cell, zebrafish, enteric neuron, hox

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65 INTRODUCTION

66 Unique to vertebrates, neural crest cells (NCC) are an embryonic stem cell population
67 characterized as transient, highly migratory, and multipotent. Following their birth from the
68 dorsal neural tube, NCCs migrate extensively, dorsolaterally or ventrally along the main axial
69 levels of the embryo; the cranial, vagal, trunk, and sacral regions (Graham et al., 2004; Le
70 Douarin and Teillet et al., 1974). Depending on the axial level of their origination, NCCs give
71 rise to different cell types within many critical tissues; such as the cornea, craniofacial cartilage
72 and bone, mesenchyme, pigment cells in the skin, as well as neurons and glia that comprise
73 peripheral ganglia (Hutchins et al., 2018; Epstein et al., 1994; Kuo and Erickson, 2011; Hall and
74 Hörstadius, 1988; Le Douarin and Kalcheim, 1999; Theveneau and Mayor, 2012; Williams and
75 Bohnsack, 2015; Yntema and Hammond, 1954).

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77 During their development, NCCs undergo dramatic transcriptional changes which lead to
78 diverse cellular lineages, making their transcriptomic profiles highly dynamic (Simões-Costa et
79 al., 2014; Martik et al., 2017; Soldatov et al., 2019; Williams et al., 2019). In support of the
80 model that complex transcriptional programs govern NCC ontogenesis, gene regulatory
81 networks involved in early development of NCCs into broad cell types has been studied at a
82 high level using a combination of transcriptomics, chromatin profiling and enhancer studies,
83 especially during pre-migratory and early migratory NCC specification along cranial axial
84 regions, across amniotes (Martik et al., 2017; Simoes-Costa and Bronner, 2016; Green et al.
85 2016; Lumb et al., 2017; Williams et al., 2019; Hockman et al., 2019). For example, during pre-
86 migratory stages the transcription factors FoxD3, Tfap2a and Sox9 are important for NCC fate
87 specification and in turn regulate the expression of Sox10, a conserved transcription factor that
88 is expressed along all axial levels by early migrating NCCs and within many differentiating
89 lineages (Sauka-Spengler and Bronner-Fraser, 2008; Martik et al., 2017). Gene regulatory
90 networks that are important for select NCC cell fates, like melanocytes and chondrocytes, have
91 been well characterized (reviewed in Martik and Bronner, 2017). Recently, the regulatory
92 circuitry behind glial, neuronal, and mesenchymal fates of vagal NCC was described (Ling and
93 Spengler, 2019) where *Prrx1* and *Twist1* have been described as key differentiation genes for
94 mesenchymal fate. Despite this progress, however, comprehensive knowledge of the genes that

are expressed and participate in NCC lineage differentiation programs during later phases of embryogenesis remains to be fully characterized, particularly for posterior tissues (reviewed in Hutchins et al., 2018). Indeed, altered gene expression during NCC differentiation can cause several neurocristopathies, such as DiGeorge syndrome, neuroblastoma, Hirschsprung disease, Auriculo-condylar syndrome, and Klein-Waardenburg syndrome (Barlow, 1984; Bolande, 1997; Brosens et al., 2016; Escot et al., 2016; Vega-Lopez et al., 2017; Wang et al., 2014), further highlighting the need to understand NCC spatiotemporal gene expression patterns during their differentiation into diverse cellular types.

Previous single-cell transcriptomic studies in zebrafish have laid a strong foundation to globally map early lineages of a majority of cell types through early to middle embryonic development (Wagner et al. 2018; Tambalo et al. 2020), and recently this has been extended into the larval stage (Farnsworth et al., 2020). With respect to the posterior NCC fates, however, many of these cells undergo differentiation programs during the embryonic to larval transition, a developmental stage that emerges between ~48 hpf to 72 hours post fertilization (hpf). Transcriptomic analysis during this transitional phase therefore would enhance our understanding of the dynamic shifts in cell states that may regulate cellular differentiation programs.

In this study, we leverage the power of single-cell transcriptomics and curate the cellular identities of *sox10*-expressing and *sox10*-derived populations along the posterior zebrafish during development. We have utilized the Tg(-4.9*sox10*:EGFP) (hereafter referred to as *sox10*:GFP) transgenic fish line to identify NCCs and their recent derivatives (Carney et al., 2006). Using *sox10*:GFP⁺ 48-50 hpf embryos and 68-70 hpf larvae, we identified eight major classes of cells: mesenchyme, NCC, neural, neuronal, glial, pigment, muscle, and otic. Among the major cell types, we annotated over 40 cellular subtypes. By leveraging in depth analysis of each time point separately, we remarkably captured the dynamic transition of several NCC fates, most notably we discovered over a dozen mesenchymal subtypes and captured the progressive differentiation of enteric neural progenitors into maturing enteric neurons. Using Hybridization Chain Reaction (HCR) and *in situ* hybridization, we validated the spatiotemporal expression patterns of various subtypes. By computationally merging our 48-50 hpf and 68-70 hpf datasets, we generated a comprehensive atlas of *sox10*⁺ cell types spanning the embryonic to larval transition, which can also be used as a tool to identify novel genes and mechanistically

test their roles in the developmental progression of posterior NCCs. Using the atlas, we characterized a *hox* signature for each cell type, detecting novel combinatorial expression of *hox* genes within specific cell types. Our intention is that this careful analysis of posterior NCC fates and resulting atlas will aid the cell and developmental biology communities by advancing our fundamental understanding of the diverging transcriptional landscape during the NCC's extensive cell fate acquisition.

RESULTS

*Single-cell profiling of *sox10*:GFP⁺ cells along the posterior zebrafish during the embryonic and larval stage transition*

To identify *sox10*-expressing and *sox10*-derived cells along the posterior zebrafish during the embryonic to larval transition, we utilized the transgenic line *sox10*:GFP (**Figure 1A**) (Carney et al., 2006; Kwak et al., 2013). Tissue posterior to the otic vesicle, encompassing the vagal and trunk axial region (**Figure 1B**), was dissected from 100 embryonic zebrafish at 48-50 hpf and 100 larval zebrafishes at 68-70 hpf. Dissected tissues were dissociated and immediately subjected to fluorescence-activated cell sorting (FACS) to isolate *sox10*:GFP⁺ cells (**Figure 1B**; **Figure 1-figure supplement 1A,B**). Isolated cells were then input into 10X Genomics Chromium scRNA-seq assays and captured at a depth of 2300 cells from the 48-50 hpf time point and 2580 cells from the 68-70 hpf time point (**Figure 1C**; **Figure 1-figure supplement 1C**). We performed cell filtering and clustering (**Figure 1-figure supplement 1D-I**) of the scRNA-seq datasets using Seurat (Butler et al., 2018; Stuart et al., 2019) to computationally identify cell populations based on shared transcriptomes, yielding 1608 cells from the 48-50 hpf time point and 2410 cells from the 68-70 hpf time point, totaling 4018 cells for final analysis (**Figure 1-figure supplement 1C**). We detected cell population clusters with transcriptionally unique signatures, as shown in heatmap summaries that revealed the top enriched gene signatures per cluster, with 19 clusters (0-18) from the 48-50 hpf time point (**Figure 1-figure supplement 2A**) and 23 clusters (0-22) from the 68-70 hpf time point (**Figure 1-figure supplement 2B**), totaling 42 clusters across both time points. Datasets were visualized with the t-Distributed Stochastic Neighbor Embedding (tSNE) method, which spatially grouped cells in each cluster, for both time points examined (**Figure 1D,E**). The top significantly enriched

markers for each cluster at 48-50 and 68-70 hpf are provided in a table in **Figure 1-source data 1**.

Major classification of *sox10:GFP*⁺ cell states

To assess the proliferative state of *sox10:GFP*⁺ cells, we determined their G1, S or G2/M phase occupancy, based on expression of proliferative cell cycle marker genes (**Figure 1-figure supplement 3I**). At 48-50 hpf, 52% of *sox10:GFP*⁺ cells were in G1 phase, 31% were in the S phase and 17% in G2/M phase (**Figure 1-figure supplement 3G**), collectively indicating that 48% of the cells in the 48-50 hpf time point were proliferative. At 68-70 hpf, 64% of cells were in G1 phase, 24% of cells were in the S phase and 12% in G2/M phase (**Figure 1-figure supplement 3G**), indicating that 36% of the cells were proliferative. The cell cycle occupancy distributions were visualized in tSNE plots, revealing congregations of proliferative and non-proliferative *sox10:GFP*⁺ cells (**Figure 1-figure supplement 3A,B**); *aurkb* and *mcm3* confirmed general occupancy in the G2/M and S phase (**Figure 1-figure supplement 3C-F**). Together, these data of cell cycle state reflect a general decrease in proliferative cells among *sox10:GFP*⁺ populations between 48 and 70 hpf, in agreement with prior observations (Rajan et al., 2018).

Using a combination of gene expression searches of the literature and bioinformatics sources, examination of the scRNA-seq transcriptomes indicated that *sox10:GFP*⁺ cells exist in several major cell type categories. Major cell type categories were based on the presence of signature marker genes (**Figure 1F,G**; **Figure 1-figure supplement 2E,F**). These major cell type categories included: neural, neuronal, glial, mesenchyme, pigment cell, NCC, otic, and muscle; their respective fraction of the datasets were also calculated (**Figure 1D-G**; **Figure 1-figure supplement 2C,D**; **Figure 1-figure supplement 3H**). Neuronal refers to cells predominantly expressing neuron markers, such as *elavl3/4*, while neural cells are defined by a multipotent state with potential towards fates of either glial or neuron identity, and marked by expression of factors such as *sox10*, *dla* and/or *ncam1a*.

Notably, mesenchyme tissue identity among clusters represented the largest proportion of the datasets at 61% and 53% of the cells at 48-50 and 68-70 hpf, respectively (**Figure 1-figure supplement 3H**). Mesenchyme clusters were identified by a combination of mesenchymal gene markers, including: *snaila/b*, *twist1a/b*, *prrx1a/b*, *meox1*, *foxc1a/b*, *cdh11*, *sparc*, *colec12*,

and/or *pdgfra*, as recently described in amniotes (Soldatov et al., 2019). Pigment cell development markers *tyrp1a/b*, *dct*, and *mitfa* were expressed in 5% of the 48-50 hpf dataset and increased to 14% at 68-70 hpf (**Figure 1-figure supplement 3H**), during which time the pigment cells diverged and expanded into distinct chromatophore lineages, as discussed further in **Figure 2**. A NCC cluster, Cluster 5 at 48-50 hpf, which we also discuss further in **Figure 4**, was identified by the combined expression of *sox10*, *crestin*, *foxd3*, *ngfrb*, and *tfap2a*. Cells with an otic vesicle and muscle signature were also detected (**Figure 1D-G**; **Figure 1-figure supplement 3H**; **Figure 1-figure supplement 5**), as has previously been described in the *sox10*:GFP line (Carney et al., 2006; Rajan et al., 2018; Rodrigues et al., 2012; Kwak et al., 2013). Overall, major cell type cluster identities and top signature marker genes are summarized in a table in **Figure 1-figure supplement 4**.

Annotation of posterior *sox10*:GFP⁺ cell subtypes

Analysis of the 42 cluster gene signatures among the two time points revealed distinct subpopulations of posterior *sox10*:GFP⁺ cells and their transcriptomes in the developing zebrafish. Indeed, we identified previously described *sox10*-derived cell subtypes. The *sox10*:GFP line has been shown to transiently label sensory dorsal root ganglion (DRG) progenitors between the 1st and 2nd day of development (McGraw et al., 2008; Rajan et al., 2018). We observed sensory neuronal/DRG gene expression in Cluster 17 at 48-50 hpf (**Figure 1-figure supplement 4**; **Figure1-figure supplement 6**) by the markers *neurod1*, *neurod4*, *neurog1*, *six1a/b*, *elavl4* (Carney et al 2006; Delfino-Machín et al., 2017). In addition, the scRNA-seq transcriptome datasets at both time points exhibited gene expression indicative of previously described NCC-derived lineages (summarized in Hutchins et al., 2018) including mesenchymal cells (Le Lievre and Le Douarin, 1975; Kague et al., 2012; Soldatov et al., 2019; Ling and Sauka-Spengler, 2019), pigment cells (Reedy et al., 1998; Higdon et al., 2013), and enteric neurons (Kelsh and Eisen, 2000; Kuo and Erickson, 2011; Lasrado et al., 2017), which we describe in further detail for both time points in Figures 2-5.

Identification of pigment cell chromatophore lineages

With robust genetic lineage details published on pigment cell differentiation in zebrafish (Kelsh, 2004; Lister, 2002; Quigley and Parichy, 2002), we sought to validate our scRNA-seq analysis by assessing if we could resolve pigment cell gene expression states. Pigment cell development has been broadly studied in the developing zebrafish, where NCCs give rise to

three distinct chromatophore populations: melanophores, xanthophores, and iridophores (**Figure 2A**). Melanophores, the best characterized population, express a combination of markers throughout their development including *mitfa*, *dct*, *tyrp1a/b*, and *pmela* (Du et al., 2003; Lister et al., 1999; Ludwig et al., 2004; Quigley and Parichy, 2002). Similarly, the genes *pnp4a*, *tfec*, *gpnmb*, and *atic* are all enriched in iridophores and are critical for their maturation (Higdon et al., 2013; Lister et al., 2011; Petratos et al., 2018, 2019). Finally, differentiating xanthophores express *gch2*, *pax7a/b*, *xdh*, *mitfa*, and *aox5* (Nord et al., 2016; Parichy et al., 2000; Saunders et al., 2019; Minchin and Hughes, 2008; Lister et al., 1999).

Our cluster analysis of *sox10*:GFP⁺ single cell datasets revealed the robust presence of pigment cell lineages (**Figure 2B-J; Figure 1-figure supplement 2**). At 48-50 hpf, melanophores were detected in Cluster 8 based on expression of *mitfa*, *dct*, *tyrp1b*, and *pmela* (**Figure 1F; Figure 2A**), reflected by the dot and tSNE plots (**Figure 2B,C**). At 68-70 hpf, we resolved discrete pigment cell populations that included xanthophore, iridophore, and two distinct melanophore clusters (**Figure 2D-G, Figure 1-figure supplement 4**). The xanthophores mapped to Cluster 15 and were enriched with *xdh*, *aox5*, *pax7b*, *mitfa*, and *gch2* (**Figure 2A,D,F**). Cluster 16 was identified as iridophores, which presented the well characterized markers: *tfec*, *pnp4a*, *gpnmb*, and *atic* (**Figure 2A,D,G; Figure 1-figure supplement 4**).

The use of cell cycle markers revealed that two different melanophore clusters at 68-70 hpf (Clusters 4 and 18) were present in different proliferative states. While the majority of cells in Cluster 4 were in G1, Cluster 18 contained the presence of S and G2/M markers, such as *pcna* and *aurkb*, suggesting that this population is proliferating melanophores (**Figure 1-figure supplement 3B,E,F; Figure 2-source data 1**). These data corroborate and extend previous observations where melanophores in distinct proliferative and differentiating states have been described during the larval stage at 5 days post fertilization (dpf) (Saunders et al., 2019). Overall, genes shared between Cluster 4 and Cluster 18, as well as their unique genes, are summarized in **Figure 2-source data 1**.

At 68-70 hpf, we were able to identify a pigment progenitor population, where iridophore and melanophore markers were co-expressed in Cluster 13: an irido-melano progenitor (**Figure 2A,D**). These undifferentiated pigment progenitor cells express *tfec* in combination with *mitfa* and have been described recently at 24, 30, and 48 hpf (Petratos et al. 2018). Additionally,

Cluster 13 expressed *tfap2e*, *gpx3*, and *trpm1b* (**Figure 1-figure supplement 4**) whose expression patterns have been previously reported in pigment progenitors (Saunders et al., 2019). Finally, a population of pigmented muscle (Cluster 9) was also found with a weak melanophore signature coupled with expression of the muscle markers *ckmb*, *tpma*, *tnnc2* and *tnnt3b* (**Figure 1-figure supplement 4; Figure 1-figure supplement 5**).

We next performed whole mount HCR to assess the spatial expression of *mitfa*, *tfec* and *xdh*, and to validate the *sox10*:GFP datasets. When examining *mitfa* and *tfec* at 48-50 hpf (**Figure 2H**), we detected GFP⁺ cells that expressed *mitfa*, identifying the melanophores (**Figure 2H**; white arrowhead), and cells that expressed both *mitfa* and *tfec*, defining the pigment progenitors (**Figure 2H**; red arrowhead). At 68-70 hpf, we confirmed the four distinct pigment populations we identified through Seurat (**Figure 2B-G**): GFP⁺ melanophores expressing *mitfa* only (**Figure 2I**; white arrowhead), iridophores only expressing *tfec* (**Figure 2I**; blue arrowhead), and pigment progenitors expressing both *mitfa* and *tfec* (**Figure 2I**; red arrowhead) were detected. When examining *xdh* and *mitfa* expression patterns, GFP⁺ xanthophores were found to be expressing both markers (**Figure 2J**; orange arrowhead).

Taken together, these results show that the scRNA-seq datasets effectively identify discrete subpopulations, and coupled with our HCR analysis, effectively shows we are able to validate these cell populations *in vivo*.

Mesenchyme in the posterior embryo and larvae exists in various subpopulations

Heatmap analysis of gene expression groups depicted that mesenchyme cells clustered together globally within the datasets (**Figure 1-figure supplement 2C,D; Figure 3A,B**), with *twist1a* expression broadly labeling all mesenchyme cells (**Figure 1-figure supplement 2E,F**). In addition to *twist1a*, mesenchyme cells also expressed *prrx1a/b*, *twist1b*, *foxc1a/b*, *snaila/b*, *cdh11*, *sparc*, *colec12*, *meox1*, *pdgfra* (**Figure 3A,B**), and other known mesenchymal markers such as *mmp2* (**Figure 1-figure supplement 4**) (Janssens et al., 2013; Theodore et al., 2017). In whole mount embryos at 48 hpf, we observed broad expression of *foxc1a* and *mmp2* along the posterior pharyngeal arches and ventral regions of the embryo via *in situ* hybridization (**Figure 3-figure supplement 1C,D**; arrowheads), confirming their expression territories within posterior-ventral mesenchymal tissues.

Analysis of the mesenchyme clusters revealed various subtypes were present in the scRNA-seq datasets. Among these, we detected 9 chondrogenic cell subtypes—identified by expression of mesenchymal signature genes, as well as the chondrogenic markers *barx1* and/or *dlx2a* (Sperber et al., 2008; Sperber and Dawid, 2008) (**Figure 3C,D**). All chondrogenic clusters expressed *barx1*, regardless of the time point, which is expressed in developing mesenchymal tissues and required for development of osteochondral progenitor cells and their tissue condensation therein (Sperber et al., 2008; Sperber and Dawid, 2008; Ding et al., 2013; Barske et al., 2016). Within the 9 chondrogenic subtypes, we discovered genes indicative of heterogeneous cell states; ranging from proliferative, progenitor/stem-like, migratory to differentiating signatures (**Figure 1-figure supplement 4**).

All other mesenchyme subtypes were classified into various progenitor and differentiation categories. Among these categories, 7 clusters expressed either proliferative progenitor markers, differentiation signatures, or general migratory mesenchymal markers (**Figure 1-figure supplement 4**). Additionally, Cluster 14 at 48 hpf and Clusters 1 and 7 at 68-70 hpf exhibited a general mesenchymal signature, but also expressed fin bud marker genes (*hand2*, *tbx5a*, *hoxd13a*, *prrx1a*) (**Figure 1-figure supplement 6**) (Yelon et al., 2000; Lu et al., 2019, Nakamura et al., 2016; Feregrino et al., 2019). The fin bud cells formed distinct groupings, as depicted in tSNE analysis (**Figure 1-figure supplement 6**).

Visualization of clusters with general mesenchyme and chondrogenic identities using a cluster tree further highlighted potential similar subtypes between the time points (**Figure 3C**). For example, the cluster tree showed proximal location of Cluster 8 at 68-70 hpf and Cluster 2 at 48-50 hpf, which we noted contained clear proliferative chondrogenic gene signatures (**Figure 3D; Figure 1-figure supplement 4**). Further, the tree depicted the closeness of Cluster 1 and 6 at 48-50 hpf with Cluster 2 at 68-70 hpf. These clusters present with varying proliferative/migratory/differentiation states, suggesting that transcriptionally-related mesenchymal cells captured in our datasets were in various stages of dynamic differentiation, proliferation, and migration.

Feature plot exports confirmed that a sub-population of chondrogenic cells (*barx1*⁺) were present in relation to all other mesenchyme (*prrx1b*⁺, *twist1a*⁺) cells in the datasets (**Figure 3E,L**). To confirm the spatial co-expression of *prrx1b*, *twist1a*, and *barx1* at 48-50 hpf and 68-

70 hpf, we utilized HCR analysis (**Figure 3F-K,M-R**). The expression of *prrx1b*, *twist1a*, and *barx1* was seen within *sox10*:GFP⁺ cells along the posterior pharyngeal arches (white arrowheads) and fin bud mesenchyme (yellow arrowheads) at both time points (**Figure 3F-K,M-R**). While *prrx1b* and *twist1a* labeled the arches and fin buds (**Figure 3F,H,M,O**), *barx1* was observed in the arches, but not the fin buds (**Figure 3I,G,P,N**).

Overall, these analyses reveal great diversity among the mesenchyme and suggest that mesenchymal cells in the posterior zebrafish exist in various subpopulations and exhibit dynamic transcriptional states during their development. Furthermore, these results show that our dataset analysis can pinpoint previously unknown discrete *sox10* subpopulations.

sox10-derived cells during the embryonic to early larval transition reveal enteric progenitor to enteric neuron progression

At 48-50 hpf, cells with NCC identity gene signatures were notably detected in Cluster 5, defined by expression of the core markers *sox10*, *foxd3*, *crestin*, and *tfap2a* (**Figure 1F; Figure 4A, Figure 1-figure supplement 4**) (Dutton et al., 2001; Luo et al., 2001; Knight et al., 2003; Stewart et al., 2006). In addition to the core genes, Cluster 5 cells also contained genes previously shown to be expressed in zebrafish NCCs; including *vim*, *snailb*, *sox9b*, *zeb2a*, *mych*, and *mmp17b* (**Figure 4D**) (Cerdà et al., 1998; Heffer et al., 2017; Hong et al., 2008; Leigh et al., 2013; van Otterloo et al., 2012; Wang et al., 2011; Rocha et al., 2020). We reasoned that many of the NCCs had started their respective differentiation programs and were beginning to assume specified genetic profiles. Therefore, we sought to determine if the NCC cluster also contained gene expression profiles of known differentiating NCC types along the posterior body, such as enteric progenitors, also known as enteric neural crest cells (ENCCs).

During NCC diversification, ENCCs fated to give rise to the enteric nervous system (ENS) express a combination of NCC and enteric progenitor marker genes over developmental time (reviewed in Nagy and Goldstein, 2017; Rao and Gershon, 2018), which occurs between 32 to 72 hpf in zebrafish (reviewed in Ganz, 2018). Enteric markers in zebrafish include *sox10*, *phox2bb*, *ret*, *gfra1a*, *meis3*, and *zeb2a* (Dutton et al., 2001; Shepherd et al., 2004; Elworthy et al., 2005; Delalande et al., 2008; Heanue and Pachnis, 2008; Uribe and Bronner, 2015). Given the developmental timing of early ENS formation in zebrafish as occurring between 32 to 72 hpf, we expected to capture a population of ENCCs within our 48-50 hpf dataset. Indeed, within

Cluster 5 we observed expression of the enteric markers *phox2bb*, *ret*, *gfra1a*, *meis3*, *sox10*, and *zeb2a* (**Figure 4B-D**). Using whole mount *in situ* hybridization, we confirmed the expression of *sox10* and *phox2bb* within ENCCs along the foregut at 48 hpf (**Figure 3-figure supplement 1A,A',B,B'**; arrowheads). Furthermore, gene orthologs known to be expressed in ENCC in amniotes were detected within Cluster 5, such as *ngfrb* (orthologue to p75) (Anderson et al., 2006; Wilson et al., 2004) and *hoxb5b* (orthologous to *Hoxb5*) (Kam and Lui, 2015) (**Figure 4B-D**).

HCR analysis of 48 hpf embryos validated the co-expression profiles of several ENCC markers along the foregut (**Figure 4E-F**; foregut in grey box). Co-expression analysis demonstrated that a chain of *crestin*⁺ cells localized in the foregut contained a subpopulation of cells expressing *ngfrb*, *phox2bb*, and *gfra1a* (**Figure 4E**; white arrowheads), or expressing *foxd3*, *ngfrb*, and *gfra1a* (**Figure 4F**; white arrowheads). Together, these HCR data reveal a subpopulation of ENCCs within the zebrafish gut, confirming ENCC markers are co-expressed.

We next asked if we could resolve discrete differentiating enteric neurons over time. In zebrafish, by 72 hpf ENCCs have migrated throughout the length of the gut and begun early neuron differentiation and neural patterning (Elworthy et al., 2005; Olden et al., 2008; Harrison et al., 2014; Uribe and Bronner, 2015; Taylor et al., 2016). During early neuronal differentiation, ENCCs display differential enteric progenitor gene expression patterns (Taylor et al., 2016) and neurochemical signatures representative of varying stages of neuronal differentiation and subtype diversification (Poon et al., 2003; Holmqvist et al., 2004; Uyttbroek et al., 2010). Zebrafish early differentiating enteric neurons have been characterized by the RNA expression of *sox10*, *phox2bb*, *gfra1a*, *fgf13b*, and *ret*, as well as the immunoreactivity of Elavl3/4 (Shepherd et al., 2004; Heanue and Pachnis, 2008; Uyttbroek et al., 2010; Taylor et al., 2016). In addition, at this time, enteric neurons express multiple neurochemical markers, with *Nos1* being most prominent (Olden et al., 2008; Uyttbroek et al., 2010), a finding consistent with studies performed within the amniote ENS (Hao and Young, 2009; Matini et al., 1995; Qu et al., 2008; Heanue et al., 2016). In light of these previous observations, our 68-70 hpf dataset was expected to contain the transcriptomes of ENCCs captured at various stages of their progressive differentiation into the diverse subtypes of the ENS.

389 tSNE analyses identified differentiating enteric neurons within the 68-70 hpf dataset based off
 390 of the combinatorial expression of *elavl3*, *phox2bb*, *ret*, and *gfra1a* (**Figure 4G**), which mapped
 391 to the neural/neuronal major cell type regions of the dataset (**Figure 1E**), comprising Clusters 5
 392 and 12 (**Figure 1E**). Transcripts that encode for the neurochemical marker *nos1*, and the
 393 neuropeptides *vip* and *vipb*, a paralogue to *vip* (Gaudet et al., 2011), were found in a
 394 subpopulation of enteric neurons localized to a distal group of the neuronal cluster, likely
 395 indicative of a differentiating enteric neuron subtype (**Figure 4G**; red arrows). We then queried
 396 for the presence of a combination of pan-neuronal and enteric neuron markers (**Figure 4H**). The
 397 pan-neuronal markers *tuba2*, *elavl3*, *stx1b*, and *gng2/3*, as well as the autonomic neuron
 398 markers, *phox2a* and *phox2bb* (Gou et al., 2018; Hans et al., 2013), were present in both
 399 Clusters 5 and 12 (**Figure 4H**). However, the enteric neuron markers, *gfra1a*, *ret*, *hoxb5b*,
 400 *ngfrb*, *fgf13b*, *nos1*, *vipb*, and *vip* were mostly confined to Cluster 12, suggesting that this
 401 cluster contained differentiating enteric neurons (**Figure 4H**). Indeed, whole mount HCR
 402 analysis validated the spatiotemporal expression of *phox2bb*, *nos1*, *vipb*, and *elavl3* transcripts
 403 throughout the foregut of the zebrafish embryo by 69 hpf (**Figure 4I**; yellow arrowheads).
 404 These results suggest that *elavl3*⁺/*phox2bb*⁺ early differentiating enteric neurons in the foregut
 405 display an inhibitory neurochemical signature, consistent with prior observations in zebrafish
 406 and mammalian ENS (Olden et al., 2008; Hao and Young, 2009).

407

408 In an effort to examine the enteric neuron populations with finer resolution, Clusters 5 and 12
 409 were subset from the main dataset in Seurat, re-clustered and visualized using a tSNE plot,
 410 producing 5 new clusters (**Figure 5A**). The gene markers from each new Sub-Cluster are
 411 provided in **Figure 5-source data 1**. Following this, the previously mentioned enteric neuron
 412 markers, with the addition of *etv1*, a recently identified marker of enteric intrinsic primary
 413 afferent neurons (IPANs) in mouse (Morarach et al., 2020), were queried and visualized using
 414 dot and feature plots allowing the identification of Sub-Cluster 3 as a differentiated enteric
 415 neuron cluster (**Figure 5A-C**). *nos1*, *vipb* and *vipb* were enriched in Sub-Cluster 3 (**Figure**
 416 **5B,C; Figure 5-figure supplement 1**). Interestingly, while expressed at lower average levels
 417 than in Sub-Cluster 3, the enteric combination markers were also present in Sub-Cluster 1
 418 (**Figure 5B-C**). Sub-Cluster 1 formed a central point from which Sub-Cluster 3 could be seen
 419 emanating as a distal population (**Figure 5A**). Given the developmental timing, this is likely

420 depicting enteric neurons captured at different stages along their progressive differentiation,
 421 which would suggest that the distal most population represents the more mature enteric neurons.
 422

423 Given our hypothesis that the enteric neurons further along a differentiation program were
 424 localized to the distal tip of Sub-Cluster 3, we asked whether this population of cells contained
 425 additional neurochemical or neuron subtype specific differentiation genes. As expected, a small
 426 pocket of these cells was found to contain the two acetylcholine associated genes, *acetylcholine*
 427 *esterase* (*ache*) (Bertrand et al., 2001; Huang et al., 2019) and *vesicular acetylcholine*
 428 *transferase* (*slc18a3a*) (Hong et al., 2013; Zoli, 2000) (**Figure 5E**; red arrowheads). Within
 429 Sub-Cluster 3, we detected the expression of *calb2a* and *pbx3b* (**Figure 5D-E**; **Figure 5-figure**
 430 **supplement 1**), two genes that have previously been shown to denote myenteric IPANs in
 431 mammals (Furness et al., 2004; Memic et al., 2018). Corroborating our single-cell findings,
 432 HCR analysis revealed the co-expression of *pbx3b*, *calb2a*, *vipb*, *nos1*, and *slc18a3a* in discrete
 433 differentiating enteric neurons within the foregut region of the zebrafish gut at 68 hpf (**Figure**
 434 **5G**). In particular, *calb2a*, *vipb*, *nos1*, and *slc18a3a* were all found to be co-expressed (**Figure**
 435 **5G**; yellow arrowheads). While *pbx3b* expression was found in combination with *calb2a*, *vipb*,
 436 and *nos1* (**Figure 5G**; white arrowheads), we were unable to observe detectable levels of
 437 *slc18a3a* within the *pbx3b* expressing cells. Taken together, these results regarding enteric
 438 neuron subpopulation gene expression patterns suggest a model (**Figure 5F**), whereby *nos1*,
 439 *vipb*, *calb2a*, and *pbx3b* are co-expressed within early enteric neurons, and that through lineage-
 440 restricted gene expression, *pbx3b* expression may promote the assumption of an IPAN signature
 441 characterized by the presence of *calb2a*, *ache*, and *slc18a3a* and the loss of inhibitor markers
 442 *nos1* and *vipb*. Therefore, our single-cell analysis in zebrafish suggests that the transcriptional
 443 emergence of specific enteric neuron subtypes may be conserved between vertebrate species.
 444

445 In order to identify the presence of novel gene pathways within the developing enteric neuron
 446 population, the significantly enriched gene list from Sub-Cluster 3 was processed using gene
 447 ontology (GO) pathway enrichment analysis. We found that three opioid signaling pathways
 448 were among the top 10 highest fold enriched pathways (**Figure 5-source data 2**) (Mi et al.,
 449 2019). Further investigation into these pathways led to the identification of G-protein-coupled
 450 receptors, *opr1l* and *opr1b*, respectively representing nociception/orphan FQ (N/OFQ) peptide
 451 (NOP)-receptor and delta-opioid receptor (DOR) subtype members within the opioid receptor
 452 superfamily (**Figure 5H**) (Donica et al., 2013; Sobczak et al., 2014). Specifically, feature plots

showed that the expression of the opioid receptors was tightly confined within the pocket of enteric neuron progenitors we identified as undergoing sensory lineage-specification, suggesting the expression of opioid receptor genes within both excitatory and inhibitory neurons at the early stages of enteric neuron differentiation within the zebrafish ENS (**Figure 5E and H**). Confirming this suggestion using HCR analysis, we observed the combinatorial expression of *oprl1*, *vipb* and *phox2bb* within migrating enteric neuron progenitors at 68 hpf along the foregut (**Figure 5I-N**). The presence of opioid receptors within immature enteric neurons undergoing lineage-specification helps us to better understand the complexity of early ENS signaling and highlights an area that requires further investigation.

Based on our observation that the enteric neuron population that comprised Sub-Cluster 3 only made up one of five *phox2bb*⁺ Sub-Clusters (**Figure 5A-C**), we suspected that the remaining Sub-Clusters were made up of closely related autonomic neurons. In order to better visualize specific differences between the Sub-Clusters, we viewed them using UMAP (Becht et al., 2019; Mcinnes et al., 2018) (**Figure 5-figure supplement 2**). While the identity of the previous tSNE Sub-Clusters were maintained, UMAP analysis allowed us to better visualize the separation between the Sub-Clusters, which we were able to broadly classify as autonomic neurons based on their shared expression of *ascl1a*, *hand2*, *phox2a* and *phox2bb* (**Figure 5-figure supplement 2A,B,D**). Within the population of autonomic neurons, we were able to distinguish a population of sympathetic neurons within Sub-Cluster 2 based on their combinatorial expression of *th*, *dbh*, *lmo1* and *insmla* (**Figure 5-figure supplement 2B,E**), which could be clearly distinguished from the enteric neuron Sub-Cluster 3 (**Figure 5-figure supplement 2B,F**). Using a cluster tree, we were able to confirm the distinction between enteric neuron Sub-Cluster 3 and sympathetic neuron Sub-Cluster 2 (**Figure 5-figure supplement 2C**). Overall, these results suggest that Sub-Cluster 0 cells may represent a pool of immature sympatho-enteric neurons, and that Sub-Clusters 1 and 4 both represent better resolved, yet still immature, pools of enteric and sympathetic neurons.

Atlas of sox10:GFP⁺ cell types encompassing the embryonic-to-larval transition

To describe the dynamic relationship between *sox10:GFP*⁺ cells across both time points, we merged the 48-50 hpf and 68-70 hpf datasets using Seurat's dataset and Integration and Label Transfer utility (Stuart et al., 2019). The merged datasets were visualized via UMAP, which allowed us to describe the transition between cell types by making both inter- and intra-cluster

486 comparisons. From the merged datasets, we detected 27 clusters (**Figure 6-figure supplement**
487 **1A**). We observed that every cluster identified in the 48-50 hpf dataset mapped proximally to
488 clusters at 68-70 hpf (**Figure 6-figure supplement 2A**). We labeled each cell using the major
489 cell type categories: neural, neuronal, glial, NCC, pigment, mesenchyme, otic, and muscle,
490 forming a major cell type atlas (**Figure 6-figure supplement 1B**). Further refinement of the cell
491 identities based on our previous curation (**Figure 1-figure supplement 2**) allowed us to form a
492 higher resolution atlas for each cell type (**Figure 6A**). The genes for each major cell type
493 category in the atlas is provided in **Figure 6-source data 1**.

494
495 The overall architecture of the UMAP revealed that the cells of each major cell type
496 congregated together, strongly supporting that our formerly described characterizations of each
497 time point are accurate and predictive. For example, cells with mesenchyme identity
498 constellated together, forming three large mesenchyme regions in the atlas: general,
499 chondrogenic, and fin bud (**Figure 6A,B**). NCCs mapped precisely to the top end of a
500 neural/neuronal region of the atlas, while differentiating neurons positioned at the base (**Figure**
501 **6A,D**). Additionally, pigment cell types aligned adjacent to one another in the atlas (**Figure**
502 **6A,C**).

503
504 Comparison of individual subsets of clusters provided deeper analysis of each cell type, with
505 respect to changing cell states across time. We examined three of the largest regions of the atlas
506 in detail: mesenchyme, pigment, and neural/neuronal cell types. We observed consistency
507 across the larger mesenchyme population, which excluded the fin bud mesenchyme, in both the
508 cluster identity shown in the UMAP and gene expression profiles. For example, 48h-Cluster 2
509 and 68h-Cluster 8 both showed a very high degree of similarity, as well as consistent *barx1*,
510 *dlx2a*, and *twist1a* expression (**Figure 6B**), consistent with our prior analysis (**Figure**
511 **3C,D**). The central node of the pigment region within the atlas was marked by the 48h-Cluster
512 8, which resolved into respective pigment chromatophore clusters at 68-70 hpf (**Figure 6C**).
513 We were able to globally discern that pigment cell types displayed expression of *sox10*,
514 regardless of time point. As expected from our previous analysis, we observed the early
515 specified melanophore population at 48h-Cluster 8 branched into later stage melanophore
516 populations (68h-Clusters 4 and 18), both of which expressed *mitfa* and *dct*. Further, we
517 observed that the common bi-potent pigment progenitor population (68h-Cluster 13) bridged
518 both melanophore clusters and the iridophore 68h-Cluster 16. This nested positioning of 68h-

Cluster 13 supports its dual progenitor identity. Xanthophores, marked by *xdh*, segregated tightly away from the remaining pigment populations, reflective of their earlier and distinct lineage (**Figure 2A**).

Cells within the neural/neuronal clusters of the atlas assembled such that progenitor cells bridged into differentiating neurons spatially from the top to the bottom of the neural/neuronal region of the atlas (**Figure 6D**). 6 clusters (Clusters 0, 5, 7, 13, 15, 17) were represented from 48-50 hpf, and 5 clusters (Clusters 3, 5, 10, 12, 14) from 68-70 hpf. The 68-70 hpf neural progenitor populations (Clusters 3 and 10) shared common gene expression with the 48-50 hpf NCC population (48h-Cluster 5), reflected largely by their co-expression of *sox10*, *notch1a*, *dla*, and *foxd3* (**Figure 6D**; **Figure 6-figure supplement 1D**). We confirmed the spatiotemporal expression domains of *notch1a* and *dla* along the hindbrain, spinal cord, and in NCC populations along the post-otic vagal domain at 48 hpf (**Figure 3-figure supplement 1E,F**; arrowheads), in particular with *dla* in the ENCCs along the foregut (**Figure 3-figure supplement 1F**; arrow), a pattern similar to the ENCC makers *sox10* and *phox2bb* (**Figure 3-figure supplement 1A,B**). Delineated from the neural progenitor cells, we observed a bifurcation in cell states; with one moving towards a Schwann/glial cell fate, while the other branched towards neuronal. The glial arm followed a temporal progression of earlier cell fates at 48-50 hpf (48h-Cluster 15) toward the more mature fates at 68-70 hpf (68h-Cluster 14), both denoted by expression of *olig2* and *pou3f1*, respectively (**Figure 6D**). From 48h-Cluster 13, we observed the beginning of the neuronal populations, namely 48h-Clusters 0, 7, 13, and 17 and 68h-Clusters 5 and 12. Cells in these clusters patterned in the atlas UMAP such that the progenitor clusters (48h-Clusters 0, 13, and 17; 68h-Cluster 5) formed a spectrum of cell states leading toward the neuronal populations (48h-Cluster 7; 68h-Cluster 12). Among the neuronal populations, we observed a clear autonomic signature, indicated by *phox2a* and *phox2bb* (**Figure 6D**; **Figure 6-figure supplement 1D**). We also detected a large fraction of enteric progenitors, indicated by *ret*, *ngfrb*, and *hoxb5b* expression, (**Figure 6D**; **Figure 6-figure supplement 1D**) supporting our previous observations (**Figure 4,5**). The enteric progenitors culminated into a pool of enteric neurons, with the specific neural signature: *vipb*, *nos1*, *gfra1a*, *fgf13b*, and *etv1* (**Figure 6D**; **Figure 6-figure supplement 1D**).

Closer inspection of the pigment, mesenchymal, and neural/neuronal clusters separated by time highlighted both predicted and novel changes in gene expression patterns (**Figure 6 -**

figure supplement 2). Analysis of each of these sub-setted cell type lineages revealed temporal differences between the two stages, as clearly exemplified in the pigment subcluster (**Figure 6 - figure supplement 2B**). For example, the xanthophore differentiation marker *xdh* demonstrated restricted expression to 68-70hpf. Further, we identified genes with no known roles in pigment cell development differentially expressed between the two stages, such as *rgs16* and *SMIM18*. Within the mesenchyme lineages, both *barx1* and *snailb* followed expected temporal expression trends, while *abracl* and *id1* both demonstrated novel differential gene expression profiles within the mesenchyme (**Figure 6 - figure supplement 2C**). Lastly, the neural/neuronal lineage showed expected differential gene expression of genes such as *etv1* and *vipb*, while revealing novel expression of *nova2* and *zgc:162730*, which have previously uncharacterized roles in *sox10*-derived cells (**Figure 6 - figure supplement 2D**). Together, these findings demonstrate the power of the *sox10* atlas not only at cataloguing lineages, but also at characterizing novel gene expression changes across developmental stages.

A *hox* gene signature within *sox10*-derived cells in the posterior zebrafish

A common theme examined by many recent and insightful single cell profile studies of the NCC (Dash and Trainor, 2020; Soldatov et al., 2019) is that patterns of Homeobox transcription factors, known as *hox* genes, display discrete expression between various cell lineages, such as in the cranial NCC. We wondered whether specific *hox* signatures were expressed within posterior NCC and their recent derivatives. To analyze if we could detect *hox* gene patterns within the atlas, we queried all the known canonical *hox* genes within zebrafish as listed on zfin.org (Ruzicka et al., 2019). We detected broad expression of 45 of the 49 zebrafish *hox* genes across the atlas, with 85% of the cells in the atlas expressing at least one *hox* gene (**Figure 7-figure supplement 1A,J**). The four undetected *hox* genes (*hoxc1a*, *hoxc12b*, *hoxa11a*, and *hoxa3a*) were not examined further.

A dot plot revealed that specific *hox* gene expression patterns demarcated distinct tissues, with specific robustness in the neural fated cells (**Figure 7A**). Common to the neural lineages, we observed a core *hox* profile which included *hoxb1b*, *hoxc1a*, *hoxb2a*, *hoxb3a*, *hoxc3a*, *hoxd3a*, *hoxa4a*, *hoxd4a*, *hoxb5a*, *hoxb5b*, *hoxc5a*, *hoxb6a*, *hoxb6b*, and *hoxb8a* (**Figure 7A**). One of the top expressed constituents of the core signature, *hoxa4a*, was also widely expressed in several other lineages (**Figure 7A; Figure 7 - figure supplement 1O**). The *hox* signature applied to the NCC, neural progenitor, enteric progenitor, enteric neuron, glial progenitor,

autonomic neuronal progenitor, and CNS lineages described in the atlas. Clustering of all the atlas lineages relying only on *hox* gene expression highlighted the robustness of the core *hox* signature to distinguish the neural lineage fates, grouping the differentiating (autonomic neuronal progenitors, enteric neurons, and enteric progenitors, and CNS neurons) and progenitor (neural progenitors and glial progenitors) lineages into neighboring clades (**Figure 7B**). Building on the core neural signature unifying the neural fates, slight variations in *hox* expression between autonomic and enteric lineages distinguished them from one another, which are summarized in **Figure 7E**. Most notably, considering the lineages in increasing specificity of cell fate, there was a detectable shift in *hox* expression among the autonomic neural progenitors to the enteric neuronal lineage, demarcated by the increase in *hoxb2a*, *hoxd4a*, *hoxa5a*, *hoxb5a*, and *hoxb5b*, accompanied by diminished expression of *hoxc3a*, *hoxc5a*, *hoxb6a* and *hoxb8a*, which formed a distinctive enteric *hox* signature (**Figure 7 - figure supplement 1 A, K-N**).

In order to better understand the complexities of *hox* codes within specific lineages, we performed a pairwise comparison of each *hox* gene for autonomic and enteric lineages, counting the number of cells which coexpressed each *hox* pair. Examining the autonomic neuronal progenitors (**Figure 7C**) and the enteric neuron populations (**Figure 7D**), both lineages demonstrated pervasive fractions of co-positive cells for combinations of the core *hox* signature. For example, autonomic neuronal cells were enriched with a high fraction of pairwise combinations for *hoxc1a*, *hoxa4a*, *hoxb3a*, and/or *hoxb5b* (**Figure 7C**). The enteric signature was highly enriched in the unique expression of *hoxa5a*, with co-expression for *hoxb5a* (36%) and *hoxb5b* (36%), as well as strong co-expression of *hoxd4a* or *hoxb5a* with *hoxb5b* (**Figure 7D**).

To confirm that *hox* core genes were co-expressed within enteric neurons, we sought to validate their co-expression patterns using HCR probes. As previously described, *hoxd4a*, *hoxa5a*, *hoxb5a*, and *hoxb5b* all exhibited strong hindbrain expression (**Figure 7 - figure supplement 1 B-I**) (Barsh et al., 2017), confirming the specificity of our probes. At 70-72 hpf, as predicted by our analysis, enteric neurons along the level of the midgut, marked by *phox2bb* expression (**Figure 7 F,J**), were *hoxb5a*⁺/*hoxb5b*⁺ (**Figure 7 G-I**) and *hoxd4a*⁺/*hoxa5a*⁺ (**Figure 7 K-M**). These data confirm that enteric neurons co-express enteric *hox* code genes during their early development.

618

619 With respect to the remaining cluster identities (**Figure 7A**), many of the populations showed
 620 varied *hox* expression profiles. Both the chondrogenic and general mesenchyme clusters
 621 demonstrated *hoxa2b* expression, as well as weak expression for *hoxb2a*, *hoxb3a*, and *hoxd4a*.
 622 Our detection of these *hox* expression profiles was consistent with prior reports that they are
 623 expressed within NCC targets toward the posterior pharyngeal arches, as well as migrating
 624 NCC (Minoux and Rijli, 2010; Parker et al., 2018, 2019). We detected the distinct identity of
 625 the fin bud mesenchyme (Ahn and Ho, 2008; Nakamura et al., 2016) through the expression of
 626 *hoxa9b*, *hoxa10b*, *hoxa11b*, *hoxa13b*, *hoxd9a*, and *hoxd12a* (**Figure 7A; Figure 7 - figure**
 627 **supplement 1 P-Q**). The pigment populations, including the pigment progenitors,
 628 melanophores, iridophores, and xanthophores, contained generally low levels of *hox* gene
 629 expression. Despite this, we still observed a slight variation of *hox* expression among the
 630 pigment populations. For example, low levels of *hoxa4a*, *hoxb7a*, *hoxb8a*, *hoxc3a*, and *hoxd4a*
 631 were detected among the iridophore population, while only *hoxb7a* was detected within a high
 632 fraction of xanthophores (**Figure 7 - figure supplement 1A**). Interestingly, these expression
 633 profiles are not shared by the melanophore population, which displayed uniformly very low
 634 levels of detectable *hox* expression. Lastly, the muscle, otic, and unidentified cells showed
 635 almost no *hox* expression profile, which serves a foil for the specificity of the signatures
 636 outlined. We noted that the “pigmented muscle” cluster weakly mirrored the general neural *hox*
 637 signature, likely a shared signature more reflective of the axial position of the muscle cells
 638 rather than a shared genetic profile, as corroborated by their distinct separation of the clusters
 639 on the atlas UMAP (**Figure 6A**).

640

641 Overall, these above described *hox* signatures detected within our scRNA-seq atlas indicates
 642 that distinct cell types express unique *hox* combinations during their delineation. Description of
 643 the *hox* signatures within the *sox10* atlas provides further tools to identify these discrete cell
 644 populations, as well as exciting new avenues for further mechanistic investigation.

645

646 DISCUSSION

647 We present a single cell transcriptomic atlas resource capturing diversity of posterior-residing
 648 *sox10*-derived cells during the embryonic (48-50 hpf) to early larval transition (68-70 hpf) in
 649 zebrafish. From our analysis, we identified a large number of cell types; including pigment
 650 progenitor cells delineating into distinct chromatophores, as well as NCC, glial, neural,

651 neuronal, and mesenchymal cells, extending prior whole embryo-based zebrafish single cell
 652 studies (Farnsworth et al., 2020) and expanding the resolution at which these cells have been
 653 described to date. We discovered that distinct *hox* transcriptional codes demarcate
 654 differentiating neural and neuronal populations, highlighting their potential roles during cell
 655 subtype specification. We also uncovered evolutionarily-conserved and novel transcriptional
 656 signatures of differentiating enteric neuron cell types, thereby expanding our knowledge of ENS
 657 development. Corroborating our transcriptomic characterizations, we validated the
 658 spatiotemporal expression of several key cell type markers using HCR. Furthermore, our
 659 datasets captured otic vesicle and muscle cells, populations which the *sox10*:GFP line has been
 660 characterized as marking, and may be useful for investigating these cell types in the future.
 661 Collectively, this comprehensive cell type atlas can be used by the wider scientific community
 662 as a valuable resource for further mechanistic and evolutionary investigation of posterior *sox10*-
 663 expressing and NCC-derived cells during development and the ontogenesis of
 664 neurocristopathies. The atlas is available via an interactive cell browser ([https://zebrafish-](https://zebrafish-neural-crest-atlas.cells.ucsc.edu/)
 665 [neural-crest-atlas.cells.ucsc.edu/](https://zebrafish-neural-crest-atlas.cells.ucsc.edu/)).

666

667 The study of NCC-derived posterior cell types has recently gained increased attention due to
 668 their complex and essential roles in vertebrate development (Gandhi et al., 2020; Hutchins et
 669 al., 2018; Soldatov et al., 2019; Ling and Sauka-Spengler, 2019). Characterizing the
 670 differentiation of NCC-derived cells is important to understand as it will enhance our concept of
 671 human health, especially to fields such as stem cell therapeutics and regenerative medicine.
 672 Prior studies provide incredible insight into their own respective research systems. Our paper is
 673 the first single-cell transcriptomic analysis covering detailed description of the early
 674 development of ENS in zebrafish, in addition to analysis of the *sox10*⁺ mesenchyme and
 675 pigment cells present during the late embryonic to larval phase. The developmental window we
 676 examined, the embryonic to larval transition, is regarded as an ephemeral phase (Singleman and
 677 Holtzman, 2014) and as such is expected to contain the dynamic cell differentiation states that
 678 we observed within our atlas.

679

680 Analysis of pigment populations demonstrated the accuracy and specificity of our transcriptome
 681 datasets and identified distinct NCC-derived differentiating chromatophore lineages during the
 682 embryonic to larval transition, extending on previous descriptions of pigment cell lineage
 683 development performed in older larval and juvenile zebrafish at single cell resolution (Saunders

et al., 2019). Specifically, melanophores were identified in the 48-50 hpf dataset (**Figure 2B,C**), while at 68-70 hpf we identified iridophore, xanthophore, pigment progenitor, and two distinct melanophore populations (**Figure 2D-G**). We employed the robust characterization of these pigment populations to validate technical aspects of integrating both time points into a single, cohesive atlas (**Figure 6A**). The atlas shows a common progenitor population branching into both the iridophores and the melanophores, which are composed of the melanophore progenitor cluster from 48-50 hpf and the two melanophore clusters at 68-70 hpf (**Figure 6C**). We validated the results regarding pigment population gene signatures using whole mount HCR on 48-50 hpf and 68-70 hpf embryos (**Figure 2H-J**). Thus, our validation of pigment populations highlights that the *sox10* atlas can be used to identify cell lineages and discover new information regarding their development in zebrafish.

Our datasets captured the transition from enteric neural progenitor to differentiating enteric neuron subtype (**Figure 4,5,6**). We found that in both 48-50 and 68-70 hpf datasets, the expression of *elavl3*, *phox2bb*, *ret*, and *gfra1a* transcripts were present (**Figure 4,5**); however, enteric progenitor populations at 48-50 hpf still retained a NCC signature, marked by *crestin* and *foxd3*, among others (**Figure 4**). The combined atlas revealed broad transcriptional states captured within enteric neural progenitors and enteric neurons (**Figure 6D**), whereby *elavl3*, *phox2bb*, *ret*, *ngfrb*, and *gfra1a* could be seen extending throughout the neural/neuronal regions of the atlas. A similar enteric progenitor population consisting of *Sox10*, *Ret*, *Phox2b*, and *Elavl4* was identified by scRNA-seq in the mouse (Lasrado et al., 2017), indicating zebrafish express conserved enteric programs. Notably, genes that encode for neurochemicals within enteric neurons were detected in the enteric clusters, with *nos1* and *vipb* being most prominent (**Figure 6D**) and co-expressed in a subset of cells among the enteric neuron population along the foregut (**Fig 5A-C**). Collectively, these results regarding enteric populations suggest that the atlas likely reflects cells captured across a spectrum of differentiation states, with immature neurons reflecting the onset of *elavl3* expression, while others, such as the *nos1*⁺/*vipb*⁺ subpopulation, representing cells further along a differentiation trajectory.

A recent scRNA-seq study performed using E15.5 mice, a time point further along in ENS development when compared to our zebrafish study described here, suggests that *Nos1*⁺/*Vip*⁺ cells represent a post-mitotic immature neuron population capable of branching into excitatory and inhibitory neurons via subsequent differentiation mediated by lineage-restricted gene

717 expression (Morarach et al., 2020). Their model posits that *Nos1*⁺/*Vip*⁺/*Gal*⁺ enteric neurons are
 718 capable of assuming an intrinsic primary afferents neuron (IPAN) signature, characterized by
 719 the loss of *Vip* and *Nos1*, and the gain of *Calb*, *Slc18a2/3*, and *Ntng1*; a process regulated by
 720 transcription factors, *Pbx3* and *Etv1*. This model of IPAN formation appears congruent with a
 721 previous birth dating study performed in mice, where researchers demonstrated the transient
 722 expression of *Nos1* in enteric neurons (Bergner et al., 2014). We wondered if the IPAN gene
 723 expression signature was evolutionarily conserved in zebrafish. Testing this spatiotemporal
 724 gene signature model in our own datasets, we asked if the *nos1*⁺/*vipb*⁺ population represented a
 725 snapshot of immature enteric neurons. We found that *pbx3b*, *etv1*, *calb2a*, *slc18a3a*, *ache*, *vipb*,
 726 and *nos1* were all expressed in differentiating enteric neuron clusters (**Figure 5D-E; Figure 5-**
 727 **figure supplement 1A**), likely reflecting their transition to an IPAN fate in our 68-70 hpf
 728 dataset. Intriguingly, we discovered that the markers tightly mapped to a subpopulation of cells
 729 in an enteric neuron sub-cluster (**Figure 7E**, red arrows) and that *nos1* was either absent or
 730 expressed at lower levels than other enteric subpopulations (**Figure 5C; Figure 5-figure**
 731 **supplement 1**), a finding that corroborates the proposed mammalian model. Our observations
 732 in zebrafish suggest that we captured a transitional time point where subsequent differentiation
 733 is just being initiated and suggests an evolutionarily-conserved mechanism of ENS formation
 734 across vertebrate species.

735

736 Within the enteric neuron subpopulation in our dataset, we discovered the enrichment of opioid
 737 pathway members. Opioids have been known effectors of gastrointestinal function for centuries
 738 based on their use as an anti-diarrheal medicine (De Luca and Coupart, 1996). Modern research
 739 has allowed us to understand the mechanism by which opioids exert their effect on gut function,
 740 namely, through the signaling mediated by the superfamily of G-protein coupled opioid
 741 receptors that serve to regulate neurotransmission within the adult ENS (Holzer, 2004; De Luca
 742 and Coupart, 1996; Wood and Galligan, 2004). This superfamily of opioid receptors is
 743 comprised of mu- (MOR), delta- (DOR), kappa- (KOR), and nociception/orphan FQ (N/OFQ)
 744 peptide (NOP)-receptors (Donica et al., 2013; Holzer, 2004). The expression of opioid
 745 receptors has previously been shown in inhibitory interneurons found within the adult ENS and
 746 are believed to inhibit gut peristalsis and secretion by inducing hyperpolarization of inhibitory
 747 neurons, resulting in the deregulation of the excitatory circuits that drive smooth muscle
 748 contraction within the gut (DiCello et al., 2020; Lay et al., 2016; Wood and Galligan, 2004).

749 According to the CDC, licit and illicit opioid abuse has risen 400% in pregnant women between
750 1999-2014, which has in turn led to a 400% increase in the number of infants born with
751 neonatal abstinence syndrome (NAS), a multisystemic clinical condition characterized by a
752 wide array of symptoms including autonomic nervous system and gastrointestinal dysfunction
753 (Raffaelli et al., 2017).

754

755 While the presence and inhibitory effect of opioid receptors is well characterized within the
756 adult ENS, the role of opioid signaling during the earliest stages of enteric neuron maturation
757 and ENS formation has yet to be investigated. Indeed, a recent study performed in zebrafish
758 found that the NOP-receptor, *oprll* was expressed within 7 dpf enteric neurons following bulk
759 RNA sequencing (Roy-Carson et al., 2017). However, this data represents a developmental
760 stage where zebrafish are characterized as free swimming larva that display feeding behavior
761 and digestive capability representative of a functioning and more mature ENS (Cassar et al.,
762 2018). As such, our 68-70 hpf dataset, in which we detected the expression of the two receptors,
763 *oprll* and *oprdbl*, respectively representing (NOP) and (DOR) class opioid receptors (**Figure**
764 **5H**), represents the earliest stage in which these opioid receptors have been shown to be
765 expressed within the early developing ENS. The presence of opioid receptors within the ENS at
766 68-70 hpf, a time when immature enteric neurons are continuing to migrate and pattern within
767 the developing embryo, highlights an important area of research focusing on the interplay
768 between opioid use and fetal ENS development, a reality that has been on the rise in recent
769 decades as opioid abuse continues to increase.

770

771 Finally, we have elucidated a comprehensive, combinatorial code of Hox transcription factor
772 expression which define specific cell lineages within the context of the *sox10* atlas. The fin bud
773 mesenchyme, pigment populations, and neural fates presented the most specific *hox* codes
774 (**Figure 7**). The previously well characterized signature identified in the fin bud served as
775 validation of our dataset's curation for further analysis of the *hox* signatures. Among the neural
776 lineages, we identified a previously undescribed *hox* signature demarcating the developing
777 enteric neuron population in zebrafish: high relative expression of *hoxb2a*, *hoxa5a*, *hoxd4a*,
778 *hoxb5a* and *hoxb5b*, while also exhibiting low expression of *hoxc3a*, *hoxc5a*, *hoxb6a* and
779 *hoxb8a* (**Figure 7**). While previous bulk microarray studies of differentiating enteric neurons
780 from humans and mice have been shown to express orthologs to the former signature (Heanue
781 and Pachnis, 2006; Memic et al., 2018), our analysis extends knowledge to comprehensively

show for the first time specific combinatorial *hox* expression at a single cell resolution, thereby providing a heretofore unknown readout of *hox* heterogeneity among nascent enteric cells. The conservation of an enteric *hox* signature between zebrafish and other systems point toward larger conservation of function, which may facilitate translation of future findings between models. These findings imply interesting potential models wherein combinations of *hox* codes may represent a molecular address designating the axial site of origination for migrating NCC or indicate dynamic expression profiles which are modified during the NCC to enteric neuron developmental course. Further work is required to test these possible models as well as the functional requirement of the constituent members of the *hox* code the developing zebrafish ENS. Considered collectively, these data lend support to a model in which overlapping expression domains of *hox* genes may facilitate enteric neural subtype differentiation, similar to their function in hindbrain and spinal neurons (Philippidou and Dasen, 2013).

In summary, our study greatly increases our foundational understanding of NCC-derived cell fates, as well as other *sox10*⁺ posterior cell types in zebrafish, thereby complementing ongoing studies in mammalian models and expanding fundamental knowledge of how cells diversify in developing organisms. The spatiotemporal information contained within our zebrafish atlas will serve as a resource for the developmental biology, stem cell, evolutionary biology and organogenesis communities.

METHODS & MATERIALS

KEY RESOURCE TABLE

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
recombinant DNA reagent	<i>phox2bb</i>	Uribe and Bronner, 2015		
recombinant DNA	<i>sox10</i>	Dutton et al. 2001		

reagent				
recombinant DNA reagent	<i>mmp2</i>	Strausberg et al., 2002		
sequence-based reagent	<i>notch1a</i>	Uribe Lab		Forward 5'- CAGTGGACTCAGCAGCAT C-3' Reverse 5'- CCTTCCGACCAATCAGAC AAG-3'
sequence-based reagent	<i>dla</i>	Uribe Lab		Forward 5'- CAGCCAAGTTGCTCAGAG- 3' Reverse 5'- GTACAGAGAACCAGCTCA TC-3'
sequence-based reagent	<i>foxc1a</i>	Uribe Lab		Forward 5'- ATACGGTGGACTCTGTGG- 3' Reverse 5'- CAGCGTCTGTTCAGTATCG- 3'
Genetic reagent (Danio rerio)	AB	Zirc		Wild-type zebrafish
Genetic reagent (Danio rerio)	<i>Tg(-4.9sox10:eGFP)ba2Tg</i>	Carney et al., 2006		GFP Labeled Neural Crest Cells
peptide, recombinant protein	<i>mitfa</i>	Molecular Instruments	NM_130923.2	
commercial assay, kit	<i>tfec</i>	Molecular Instruments	NM_001030105.2	
commercial assay, kit	<i>xdh</i>	Molecular Instruments	XM_683891.7	
commercial assay, kit	<i>phox2bb</i>	Molecular Instruments	NM_001014818.1	

commercial assay, kit	<i>ngfrb</i>	Molecular Instruments	NM_001198660.1	
commercial assay, kit	<i>gfra1a</i>	Molecular Instruments	NM_131730.1	
commercial assay, kit	<i>crestin</i>	Molecular Instruments	AF195881.1	
commercial assay, kit	<i>foxd3</i>	Molecular Instruments	NM_131290.2	
commercial assay, kit	<i>vipb</i>	Molecular Instruments	NM_001114555.1	
commercial assay, kit	<i>elavl3</i>	Molecular Instruments	NM_131449	
commercial assay, kit	<i>opr11</i>	Molecular Instruments	NM_205589.2	
commercial assay, kit	<i>barx1</i>	Molecular Instruments	NM_001024949.1	
commercial assay, kit	<i>pbx3b</i>	Molecular Instruments	BC131865.1	
commercial assay, kit	<i>prrx1b</i>	Molecular Instruments	NM_200050.1	
commercial assay, kit	<i>slc18a3a</i>	Molecular Instruments	NM_001077555.1	
commercial assay, kit	<i>hoxb5a</i>	Molecular Instruments	NM_131101.2	
commercial assay, kit	<i>hoxb5b</i>	Molecular Instruments	bc078285.1	

commercial assay, kit	<i>hoxd4a</i>	Molecular Instruments	NM_001126445	
commercial assay, kit	<i>hoxa5a</i>	Molecular Instruments	NM_131540.1	
commercial assay, kit	<i>twist1a</i>	Molecular Instruments	NM_130984.2	
commercial assay, kit	Single Cell 3' v2 Chemistry Kit for 10,000 cells	10x Genomics	CG00052	https://support.10xgenomics.com/single-cell-gene-expression/library-prep/doc/user-guide-chromium-single-cell-3-reagent-kits-user-guide-v2-chemistry
commercial assay, kit	NextSeq 500/550 Mid Output Kit v2.5 (150 Cycles)	illumina	20024904	https://www.illumina.com/products/by-type/sequencing-kits/cluster-gen-sequencing-reagents/nextseq-series-kits-v2-5.html
chemical compound, drug	-phenyl 2-thiourea (PTU)/E3 solution	Karlsson 741 et al., 2001	Sigma-Aldrich	P7629
chemical compound, drug	Tricaine	A5040	Sigma	
chemical compound, drug	Accumax buffer	A7089	Sigma-Aldrich	
chemical compound, drug	Hank's Buffer	55021C	Sigma-Aldrich	
chemical compound, drug	Phusion-HF	M0530S	New England Biolabs	
chemical compound, drug	Zero Blunt™	451245	Invitrogen	

	TOPO™ PCR Cloning Kit			
Software, algorithm	R v3.6.3	R-project	RRID:SCR_001905	https://www.r-project.org/
Software, algorithm	Seurat v3.1.1	Satija Lab	RRID:SCR_007322	https://github.com/satijalab/seurat
Software, algorithm	Fiji	PMID: 22743772	RRID:SCR_002285	https://imagej.net/Fiji
Software, algorithm	IMARIS v9.2	Bitplane	RRID:SCR_007370	Bitplane.com
Software, algorithm	PANTHER	GENEONTOLOGY Unifying Biology	RRID:SCR_004869	http://pantherdb.org
Software, algorithm	Cell Ranger v2.1.0	10x Genomics	RRID:SCR_017344	Zheng et al., 2017, https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/what-is-cell-ranger

806

807

808 *Animal Husbandry, Care, & Synchronous Embryo Collection*

809 Groups of at least 15 adult Tg(-4.9sox10:GFP)^{ba2Tg} (Carney et al., 2006) zebrafish (*Danio rerio*)
810 males and 15 females from different tank stocks were bred to generate synchronously staged
811 embryos across several clutches. All embryos were cultured in standard E3 media until 24 hours
812 post fertilization (hpf), then transferred to .003% 1-phenyl 2-thiourea (PTU)/E3 solution
813 (Karlsson et al., 2001), to arrest melanin formation and enable ease of GFP sorting. While it has
814 been suggested that high concentration (.03%) PTU incubation prior to 22 hpf may cause
815 organism-wide effects, .003% PTU application after 22 hpf has been shown to have no major
816 effects on NCC survival or pigment cell formation, as described (Bohnsack et al., 2011).
817 Embryos were manually sorted for GFP expression and synchronously staged at 24 hpf. Care
818 was taken such that embryos which exhibited developmental delay or other defects were

819 removed prior to collection. All work was performed under protocols approved by, and in
820 accordance with, the Rice University Institutional Animal Care and Use Committee (IACUC).
821

822 *Isolation of Tissue & Preparation of Single Cell Suspension*

823 100 embryos between 48-50 hpf and 100 larvae between 68-70 hpf were dechorionated
824 manually and then transferred to 1X sterile filtered PBS, supplemented with 0.4% Tricane
825 (Sigma, A5040) to anesthetize. Tissue anterior to the otic vesicle and tissue immediately
826 posterior to the anal vent was manually removed using fine forceps in 48-50 hpf embryos, while
827 tissue anterior to the otic vesicle was removed from 68-70 hpf larvae, as schematized in Figure
828 1. This was to capture as many posterior *sox10:GFP⁺* cells in the later time point as possible.
829 Remaining tissue segments were separated into nuclease-free tubes and kept on ice immediately
830 following dissection. Dissections proceeded over the course of 1 hour. To serve as control for
831 subsequent steps, similarly staged AB WT embryos were euthanized in tricaine and then
832 transferred to sterile 1X PBS. All following steps were conducted rapidly in parallel to
833 minimize damage to cells: Excess PBS was removed and tissue was digested in 37°C 1X
834 Accumax buffer (Sigma-Aldrich, A7089) for 30-45 minutes to generate a single cell suspension
835 for each sample. At 10 minute intervals, tissue was gently manually disrupted with a sterile
836 pipette tip. As soon as the tissue was fully suspended, the cell solutions were then transferred to
837 a fresh chilled sterile conical tube and diluted 1:5 in ice cold Hank's Buffer (1x HBSS; 2.5
838 mg/mL BSA; 10μM pH8 HEPES) to arrest the digestion. Cells were concentrated by
839 centrifugation at 200 rcf for 10 minutes at 4°C. Supernatant was discarded carefully and cell
840 pellets were resuspended in Hank's Buffer. Cell solution was passed through a 40 μm sterile
841 cell strainer to remove any remaining undigested tissue and then centrifuged as above.
842 Concentrated cells were resuspended in ice cold sterile 1X PBS and transferred to a tube
843 suitable for FACS kept on ice. The 48-50 hpf and 68-70 hpf experiments were performed on
844 completely separate dates and times using the above described procedures.

845

846 *Fluorescent Cell Sorting, & Single Cell Sequencing*

847 Fluorescent Assisted Cell Sorting (FACS) was performed under the guidance of the Cytometry
848 and Cell Sorting Core at Baylor College of Medicine (Houston, TX) using a BD FACSAria II
849 (BD Biosciences). Zebrafish cells sorted via GFP fluorescence excited by a 488 nm laser,
850 relying on an 85 μm nozzle for cell selection. Detection of GFP⁺ cells was calibrated against
851 GFP⁻ cells collected from AB wildtype embryos, as well as GFP⁺ cells collected from the

anterior portions of the *sox10*:GFP embryos. Optimal conditions for dissociated tissue inputs (number of embryos needed, etc.) and FACS gating was determined via pilot experiments prior to collection for subsequent scRNA-seq experiments. Sample preparation for scRNA-seq was performed by Advanced Technology Genomics Core (ATGC) at MD Anderson (Houston, TX). 4905 and 4669 FACS-isolated cells for the 48-50 and 68-70 hpf datasets were prepared on a 10X Genomics Chromium platform using 10X Single Cell 3' V2 chemistry kit for 10,000 cells. cDNA libraries were amplified and prepared according to the 10X Genomics recommended protocol, with details provided in **Figure 1-figure supplement 1C**. A 150 cycle Mid-Output flow cell was used for sequencing on an Illumina NextSeq500. Sequencing was aligned at MD Anderson ATGC to the DanioGRCz10 version of the zebrafish genome using the 10X Genomics Cell Ranger software (v2.1.0) (Zheng et al., 2017). Gene reads per cell were stored in a matrix format for further analysis.

Data Processing & Analysis

The 10x genomics sequencing data was then analyzed using Seurat (Satija et al., 2015, Stuart et al. 2019, Butler et al., 2018) v3.1.1 software package for R, v3.6.3 (R Core Team, 2020). The standard recommended workflow was followed for data processing. Briefly, for both the 48-50 hpf and 68-70 hpf datasets, cells which contained low (<200) or high (>2500) genes were removed from analysis. Gene expression was normalized using the NormilizeData command, opting for the LogNormalize method (Scale factor set at 10,000) and further centered using the ScaleData command. Variable features of the dataset were calculated with respect to groups of 2,000 genes at a time. Both datasets were evaluated considering the first 20 principle components (PC) as determined by the RunPCA command with a resolution of 1.2 for PCA, tSNE, and UMAP analyses. The appropriate PCs were selected based on a Jack Straw analysis with a significance of $P < 0.01$, as generated by the JackStraw command.

Clustering was performed using FindNeighbors and FindClusters in series. We identified 19 clusters in the 48-50 hpf dataset and 23 clusters in the 68-70 hpf dataset. Significant genes for each cluster were determined via a Wilcoxon Rank Sum test implemented by the FindAllMarkers command. From these expressed gene lists within each cluster, all cluster identities were manually curated via combinatorial expression analysis of published marker

genes in the literature, zfin.org and/or bioinformatics GO term analysis via the Panther Database.

Generation of the merged atlas was performed via the FindIntegrationAnchors workflow provided in the Standard Workflow found on the Seurat Integration and Label Transfer vignette. Clustering was performed for the atlas based on the first 20 PCs, consistent with the original datasets. Subsets of the atlas discounted any spuriously sorted cells for clarity. All features plots represent expression values derived from the RNA assay. Sub-clustering of the enteric clusters was performed by sub-setting clusters 5 and 12 from the 68-70 hpf dataset and reinitializing the Seurat workflow, as described above. Clusters were identified based on the first 6 PCs. Detection of cell cycle phase was conducted following the Cell cycle and scoring vignette. Genes used for identification of cell cycle phases can be found in the supplementary table (Figure 1-figure supplement 3). Pairwise *hox* analysis was conducted using tools from the Seurat package in R by assessing the number of cells which had expression for *hox* gene pairs queried with a log2 fold change greater than 0. Dendrogram Cluster trees were generated using Seurat's BuildClusterTree function.

Whole mount in situ Hybridization

cDNAs for *foxc1a*, *notch1a*, and *dla* were amplified via high fidelity Phusion-HF PCR (NEB) from 48 hpf AB WT cDNA libraries using primers in the Key resources table. PCR products were cloned using the Zero Blunt™ TOPO™ PCR Cloning Kit (Invitrogen), as per manufacturer protocols, and sequenced validated. Plasmids encoding *phox2bb*, *sox10*, *mmp2* were generously sourced as listed in the key resources table. Antisense digoxigenin (DIG)-labeled riboprobes were produced from cDNA templates of each gene. AB wild type embryos were treated and stained to visualize expression as previously described in (Jowett and Lettice, 1994). Following *in situ* reactions, embryos were post-fixed in 4% Paraformaldehyde (PFA) and mounted in 75% Glycerol for imaging. A Nikon Ni-Eclipse Motorized Fluorescent upright compound microscope with a 4X objective was used in combination with a DS-Fi3 color camera. Images were exported via Nikon Elements Image Analysis software.

Whole mount Hybridization Chain Reaction

HCR probes were purchased commercially (Molecular Instruments Inc., CA) and were targeted to specific genes based on their RefSeq ID (Key resources table). Whole mount HCR was

performed according to the manufacturer's instructions (v3.0, Choi et al., 2016, 2018) on *sox10*:GFP⁺ or AB embryos previously fixed at the appropriate stage in 4% PFA.

Confocal Imaging & Image Processing

Prior to imaging, embryos were embedded in 1% low melt agarose (Sigma) and were then imaged using an Olympus FV3000 Laser Scanning Confocal, with a UCPlanFLN 20×/0.70NA objective. Confocal images were acquired using lambda scanning to separate the Alexafluor 488/Alexafluor 514 or the Alexafluor 546/Alexafluor 594 channels. Final images were combined in the FlowView software and exported for analysis in either Fiji (Rueden et al., 2017; Schneider et al., 2012; Schindelin et al., 2012) or IMARIS image analysis software (Bitplane). Figures were prepared in Adobe Photoshop and Illustrator software programs, with some cartoons created via BioRender.com.

Data Availability

The raw sequence read files and processed cellular barcode, gene, and matrix files produced by Cell Ranger are available in the National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>), accession number: GSE152906. The atlas/associated processed Seurat objects are available on the University of California, Santa Cruz (UCSC) Cell Browser (<https://zebrafish-neural-crest-atlas.cells.ucsc.edu/>). Code written for Seurat data analysis is available on GitHub (<https://github.com/UribeLabRice>).

ACKNOWLEDGEMENTS

Funding for this project was provided by Rice University, Cancer Prevention & Research Institute of Texas (CPRIT) Recruitment of First-Time Tenure Track Faculty Members (CPRIT-RR170062) and the NSF CAREER Award (1942019) awarded to R.A.U., a Houston Livestock Show & Rodeo Research Award to J.A.M. and P.A.B., and a SDB Choose Development! Fellowship award to J.L.W. We acknowledge the Cytometry and Cell Sorting Core at Baylor College of Medicine, which is funded from the CPRIT Core Facility Support Award (CPRIT-RP180672), the NIH (P30 CA125123 and S10 RR024574), and the expert assistance of Joel M. Sederstrom for assistance with flow cytometry. Single cell library preparation, Illumina sequencing, and Cell Ranger alignment was facilitated by Advanced Technology Genomics Core at MD Anderson Cancer Research Center funded by CA016672(ATGC). IMARIS image analysis was performed using Rice University's Shared Equipment Authority (SEA) IMARIS workstation. We thank George Eisenhoffer and Oscar Ruiz (MD Anderson) for advice regarding flow cytometry and single-cell RNA-seq methodology. We thank Sarah Kucenas

(University of Virginia) for helpful advice on glial populations. We thank Robert Naja and Robyn Fenty for technical assistance.

Competing Interests: The authors claim no competing interests.

Citations

Ahn, D., and Ho, R.K. (2008). Tri-phasic expression of posterior Hox genes during development of pectoral fins in zebrafish: Implications for the evolution of vertebrate paired appendages. *Dev. Biol.* 322, 220–233.

Anderson, R.B., Stewart, A.L., and Young, H.M. (2006). Phenotypes of neural-crest-derived cells in vagal and sacral pathways. *Cell Tissue Res.* 323, 11–25.

Barlow, A.J. (1984). Neural Crest Cells in Enteric Nervous System Development and Disease. In *Neural Crest Cells*, (Elsevier Inc.), pp. 101–104.

Barsh, G.R., Isabella, A.J., and Moens, C.B. (2017). Vagus Motor Neuron Topographic Map Determined by Parallel Mechanisms of *hox5* Expression and Time of Axon Initiation. *Curr. Biol.* 27, 3812–3825.

Barske, L., Askary, A., Zuniga, E., Balczerski, B., Bump, P., Nichols, J.T., and Crump, J.G. (2016). Competition between Jagged-Notch and Endothelin1 Signaling Selectively Restricts Cartilage Formation in the Zebrafish Upper Face. *PLoS Genet.* 12, e1005967.

Becht, E., McInnes, L., Healy, J., Dutertre, C.A., Kwok, I.W.H., Ng, L.G., Ginhoux, F., and Newell, E.W. (2019). Dimensionality reduction for visualizing single-cell data using UMAP. *Nat. Biotechnol.* 37, 38–47.

Bergner, A.J., Stamp, L.A., Gonsalvez, D.G., Allison, M.B., Olson, D.P., Myers, M.G., Anderson, C.R., and Young, H.M. (2014). Birthdating of myenteric neuron subtypes in the small intestine of the mouse. *J. Comp. Neurol.* 522, 514–527.

Bertrand, C., Chatonnet, A., Takke, C., Yan, Y.L., Postlethwait, J., Toutant, J.P., and Cousin, X. (2001). Zebrafish acetylcholinesterase is encoded by a single gene localized on linkage group 7. Gene structure and polymorphism; molecular forms and expression pattern during development. *J. Biol. Chem.* 276, 464–474.

Bohnsack B.L., Gallina D., Kahana A. (2011). Phenothiourea Sensitizes Zebrafish Cranial Neural Crest and Extraocular Muscle Development to Changes in Retinoic Acid and IGF Signaling. *PLoS ONE* 6: e22991

986 Bolande, R.P. (1997). Neurocristopathy: Its Growth and Development in 20 Years. *Pediatr.*
987 *Pathol. Lab. Med.* *17*, 1–25.

988 Brosens, E., Burns, A.J., Brooks, A.S., Matera, I., Borrego, S., Ceccherini, I., Tam, P.K.,
989 García-Barceló, M.M., Thapar, N., Benninga, M.A., et al. (2016). Genetics of enteric
990 neuropathies. *Dev. Biol.* *417*, 198–208.

991 Butler, A., Hoffman, P., Smibert, P., Papalexi, E., and Satija, R. (2018). Integrating single-cell
992 transcriptomic data across different conditions, technologies, and species. *Nat. Biotechnol.* *36*,
993 411–420.

994 Carney, T.J., Dutton, K.A., Greenhill, E., Delfino-Machin, M., Dufourcq, P., Blader, P., and
995 Kelsh, R.N. (2006). A direct role for Sox10 in specification of neural crest-derived sensory
996 neurons. *Development* *113*, 4619–4630.

997 Cassar, S., Huang, X., and Cole, T. (2018). High-throughput Measurement of Gut Transit Time
998 Using Larval Zebrafish. *J. Vis. Exp.* *140*, e58497.

999 Cerdà, J., Conrad, M., Markl, J., Brand, M., and Herrmann, H. (1998). Zebrafish vimentin:
1000 Molecular characterisation, assembly properties and developmental expression. *Eur. J. Cell*
1001 *Biol.* *77*, 175–187.

1002 Choi, H.M.T., Calvert, C.R., Husain, N., Huss, D., Barsi, J.C., Deverman, B.E., Hunter, R.C.,
1003 Kato, M., Lee, S.M., Abelin, A.C.T., et al. (2016). Mapping a multiplexed zoo of mRNA
1004 expression. *Development* *143*, 3632–3637.

1005 Choi, H.M.T., Schwarzkopf, M., Fornace, M.E., Acharya, A., Artavanis, G., Stegmaier, J.,
1006 Cunha, A., and Pierce, N.A. (2018). Third-generation in situ hybridization chain reaction:
1007 multiplexed, quantitative, sensitive, versatile, robust. *Development* *145*, dev165753.

1008 Dash, S., and Trainor, P. (2020). The development, patterning and evolution of neural crest cell
1009 differentiation into cartilage and bone. *Bone* *137*, 115409.

1010 Delalande, J.M., Guyote, M.E., Smith, C.M., and Shepherd, I.T. (2008). Zebrafish *sipl*a and
1011 *sipl*b are essential for normal axial and neural patterning. *Dev. Dyn.* *237*, 1060–1069.

1012 Delfino-Machín, M., Madelaine, R., Busolin, G., Nikaido, M., Colanesi, S., Camargo-Sosa, K.,
1013 Law, E.W.P., Toppo, S., Blader, P., Tiso, N., et al. (2017). Sox10 contributes to the balance of
1014 fate choice in dorsal root ganglion progenitors. *PLoS One* *12*, e0172947.

1015 DiCello, J.J., Carbone, S.E., Saito, A., Rajasekhar, P., Ceredig, R.A., Pham, V., Valant, C.,
1016 Christopoulos, A., Veldhuis, N.A., Canals, M., et al. (2020). Mu and Delta Opioid Receptors
1017 Are Coexpressed and Functionally Interact in the Enteric Nervous System of the Mouse Colon.
1018 *CMGH* *9*, 465–483.

1019 Ding, H.L., Clouthier, D.E., and Artinger, K.B. (2013). Redundant roles of PRDM family
1020 members in zebrafish craniofacial development. *Dev. Dyn.* *242*, 67–79.

- 1021 Donica, C.L., Awwad, H.O., Thakker, D.R., and Standifer, K.M. (2013). Cellular mechanisms
1022 of nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor regulation and heterologous
1023 regulation by N/OFQ. *Mol. Pharmacol.* 83, 907–918.
- 1024 Le Douarin, N., and Kalcheim, C. (1999). *The Neural Crest* (Cambridge University Press).
- 1025 Le Douarin, N.M., and Teillet, M.A.M. (1974). Experimental analysis of the migration and
1026 differentiation of neuroblasts of the autonomic nervous system and of neurectodermal
1027 mesenchymal derivatives, using a biological cell marking technique. *Dev. Biol.* 41, 162–184.
- 1028 De Luca, A., and Coupart, L.M. (1996). *Insights into Opioid Action in the Intestinal Tract*
1029 (Elsevier Science Inc).
- 1030 Du, J., Miller, A.J., Widlund, H.R., Horstmann, M.A., Ramaswamy, S., and Fisher, D.E.
1031 (2003). MLANA/MART1 and SILV/PMEL17/GP100 are transcriptionally regulated by MITF
1032 in melanocytes and melanoma. *Am. J. Pathol.* 163, 333–343.
- 1033 Dutton, K.A., Pauliny, A., Lopes, S.S., Elworthy, S., Carney, T.J., Rauch, J., Geisler, R.,
1034 Haffter, P., and Kelsh, R.N. (2001). Zebrafish Colourless Encodes sox10 and Specifies Non-
1035 Ectomesenchymal Neural Crest Fates. *Development* 128, 4113–4125.
- 1036 Elworthy, S., Pinto, J.P., Pettifer, A., Cancela, M.L., and Kelsh, R.N. (2005). Phox2b function
1037 in the enteric nervous system is conserved in zebrafish and is sox10-dependent. *Mech. Dev.*
1038 122, 659–669.
- 1039 Epstein, M.L., Mikawa, T., Brown, A.M.C., and McFarlin, D.R. (1994). Mapping the origin of
1040 the avian enteric nervous system with a retroviral marker. *Dev. Dyn.* 201, 236–244.
- 1041 Escot, S., Blavet, C., Faure, E., Zaffran, S., Duband, J.L., and Fournier-Thibault, C. (2016).
1042 Disruption of CXCR4 signaling in pharyngeal neural crest cells causes DiGeorge syndrome-like
1043 malformations. *Development* 143, 582–588.
- 1044 Farnsworth, D.R., Saunders, L.M., and Miller, A.C. (2020). A single-cell transcriptome atlas for
1045 zebrafish development. *Dev. Biol.* 459, 100–108.
- 1046 Feregrino, C., Sacher, F., Parnas, O., and Tschopp, P. (2019). A single-cell transcriptomic atlas
1047 of the developing chicken limb. *BMC Genomics* 20, 401.
- 1048 Furness, J.B., Jones, C., Nurgali, K., and Clerc, N. (2004). Intrinsic primary afferent neurons
1049 and nerve circuits within the intestine. *Prog. Neurobiol.* 72, 143–164.
- 1050 Gandhi, S., Ezin, M., and Bronner, M.E. (2020). Reprogramming Axial Level Identity to
1051 Rescue Neural-Crest-Related Congenital Heart Defects. *Dev. Cell* 53, 300–315.e4.
- 1052 Ganz, J. (2018). Gut feelings: Studying enteric nervous system development, function, and
1053 disease in the zebrafish model system. *Dev. Dyn.* 247, 268–278.
- 1054 Gaudet, P., Livstone, M.S., Lewis, S.E., and Thomas, P.D. (2011). Phylogenetic-based
1055 propagation of functional annotations within the Gene Ontology consortium. *Brief. Bioinform.*
1056 12, 449–462.

1057 Gou, Y., Guo, J., Maulding, K., and Riley, B.B. (2018). *sox2* and *sox3* cooperate to regulate
1058 otic/epibranchial placode induction in zebrafish. *Dev. Biol.* *435*, 84–95.

1059 Graham, A., Begbie, J., and McGonnell, I. (2004). Significance of the Cranial Neural Crest.
1060 *Dev. Dyn.* *229*, 5–13.

1061 Green, S.A., Simoes-costa, M., Bronner, M.E., and Engineering, B. (2016). Evolution of
1062 vertebrates: a view from the crest. *Nature* *520*, 474–482.

1063 Hall, B.K., and Hörstadius, S. (1988). *The Neural Crest* (London, New York, Tokyo, Toronto:
1064 Oxford University Press).

1065 Hans, S., Irmscher, A., and Brand, M. (2013). Zebrafish *Foxi1* provides a neuronal ground state
1066 during inner ear induction preceding the *Dlx3b/4b*-regulated sensory lineage. *Development* *140*,
1067 1936–1945.

1068 Hao, M.M., and Young, H.M. (2009). Development of enteric neuron diversity. *J. Cell. Mol.*
1069 *Med.* *13*, 1193–1210.

1070 Harrison, C., Wabbersen, T., and Shepherd, I.T. (2014). In vivo visualization of the
1071 development of the enteric nervous system using a *Tg(-8.3bpHox2b: Kaede)* transgenic
1072 zebrafish. *Genesis* *52*, 985–990.

1073 Heanue, T.A., and Pachnis, V. (2008). Ret isoform function and marker gene expression in the
1074 enteric nervous system is conserved across diverse vertebrate species. *Mech. Dev.* *125*, 687–
1075 699.

1076 Heanue, T.A., Shepherd, I.T., and Burns, A.J. (2016). Enteric nervous system development in
1077 avian and zebrafish models. *Dev. Biol.* *417*, 129–138.

1078 Heffer, A., Marquart, G.D., Aquilina-Beck, A., Saleem, N., Burgess, H.A., and Dawid, I.B.
1079 (2017). Generation and characterization of *Kctd15* mutations in zebrafish. *PLoS One* *12*,
1080 e0189162.

1081 Higdon, C.W., Mitra, R.D., and Johnson, S.L. (2013). Gene Expression Analysis of Zebrafish
1082 Melanocytes, Iridophores, and Retinal Pigmented Epithelium Reveals Indicators of Biological
1083 Function and Developmental Origin. *PLoS One* *8*, e67801.

1084 Hockman, D., Chong-Morrison, V., Green, S.A., Gavriouchkina, D., Candido-Ferreira, I., Ling,
1085 I.T.C., Williams, R.M., Amemiya, C.T., Smith, J.J., Bronner, M.E., et al. (2019). A genome-
1086 wide assessment of the ancestral neural crest gene regulatory network. *Nat. Commun.* *10*,
1087 e4689.

1088 Holmqvist, B., Ellingsen, B., Forsell, J., Zhdanova, I., and Alm, P. (2004). The early ontogeny
1089 of neuronal nitric oxide synthase systems in the zebrafish. *J. Exp. Biol.* *207*, 923–935.

1090 Holzer, P. (2004). Opioids and opioid receptors in the enteric nervous system: From a problem
1091 in opioid analgesia to a possible new prokinetic therapy in humans. *Neurosci. Lett.* *361*, 192–
1092 195.

1093 Hong, E., Santhakumar, K., Akitake, C.A., Ahn, S.J., Thisse, C., Thisse, B., Wyart, C., Mangin,
1094 J.M., and Halpern, M.E. (2013). Cholinergic left-right asymmetry in the habenulo-
1095 interpeduncular pathway. *Proc. Natl. Acad. Sci. U. S. A.* *110*, 21171–21176.

1096 Hong, S.K., Tsang, M., and Dawid, I.B. (2008). The Mych gene is required for neural crest
1097 survival during zebrafish development. *PLoS One* *3*, e2029.

1098 Huang, V., Butler, A.A., and Lubin, F.D. (2019). Telencephalon transcriptome analysis of
1099 chronically stressed adult zebrafish. *Sci. Rep.* *9*, 1379.

1100 Hutchins, E.J., Kunttas, E., Piacentino, M.L., Howard, A.G.A., Bronner, M.E., and Uribe, R.A.
1101 (2018). Migration and diversification of the vagal neural crest. *Dev. Biol.* *444*, S98–S109.

1102 Janssens, E., Gaublomme, D., de Groef, L., Darras, V.M., Arckens, L., Delorme, N., Claes, F.,
1103 van Hove, I., and Moons, L. (2013). Matrix Metalloproteinase 14 in the Zebrafish: An Eye on
1104 Retinal and Retinotectal Development. *PLoS One* *8*, e52915.

1105 Jarinova, O., Hatch, G., Poitras, L., Prudhomme, C., Grzyb, M., Aubin, J., Bérubé-Simard, F.-
1106 A., Jeannotte, L., and Ekker, M. (2008). Functional resolution of duplicated *hoxb5* genes in
1107 teleosts. *Development* *135*, 3543–3553.

1108 Jowett, T., and Lettice, L. (1994). Whole-mount in situ hybridizations on zebrafish embryos
1109 using a mixture of digoxigenin- and fluorescein- labelled probes. *Trends Genet.* *10*, 73–74.

1110 Kague, E., Gallagher, M., Burke, S., Parsons, M., Franz-Odenaal, T., and Fisher, S. (2012).
1111 Skeletogenic Fate of Zebrafish Cranial and Trunk Neural Crest. *PLoS One* *7*, e47394.

1112 Kam, M.K.M., and Lui, V.C.H. (2015). Roles of *Hoxb5* in the development of vagal and trunk
1113 neural crest cells. *Dev. Growth Differ.* *57*, 158–168.

1114 Kam, M.K.M., Cheung, M.C.H., Zhu, J.J., Cheng, W.W.C., Sat, E.W.Y., Tam, P.K.H., and Lui,
1115 V.C.H. (2014). Perturbation of *Hoxb5* signaling in vagal and trunk neural crest cells causes
1116 apoptosis and neurocristopathies in mice. *Cell Death Differ.* *21*, 278–289.

1117 Karlsson, J., Von Hofsten, J., and Olsson, P.E. (2001). Generating transparent zebrafish: A
1118 refined method to improve detection of gene expression during embryonic development. *Mar.*
1119 *Biotechnol.* *3*, 522–527.

1120 Kelsh, R.N. (2004). Genetics and evolution of pigment patterns in fish. *Pigment Cell Res.* *17*,
1121 326–336.

1122 Kelsh, R.N., and Eisen, J.S. (2000). The zebrafish colourless gene regulates development of
1123 non-ectomesenchymal neural crest derivatives. *Development* *127*, 515–525.

1124 Knight, R.D., Nair, S., Nelson, S.S., Afshar, A., Javidan, Y., Geisler, R., Rauch, G.J., and
1125 Schilling, T.F. (2003). Lockjaw encodes a zebrafish *tfap2a* required for early neural crest
1126 development. *Development* *130*, 5755–5768.

1127 Kuo, B.R., and Erickson, C.A. (2011). Vagal neural crest cell migratory behavior: A transition
1128 between the cranial and trunk crest. *Dev. Dyn.* *240*, 2084–2100.

- 1129 Kwak, J., Park, O.K., Jung, Y.J., Hwang, B.J., Kwon, S.H., and Kee, Y. (2013). Live image
1130 profiling of neural crest lineages in zebrafish transgenic lines. *Mol. Cells* 35, 255–260.
- 1131 Lasrado, R., Boesmans, W., Kleinjung, J., Pin, C., Bell, D., Bhaw, L., McCallum, S., Zong, H.,
1132 Luo, L., Clevers, H., et al. (2017). Lineage-dependent Spatial and Functional Organization of
1133 the Mammalian Enteric Nervous System. *Science* (80-.). 356, 722–726.
- 1134 Lay, J., Carbone, S.E., Dicello, J.J., Bunnett, N.W., Canals, M., and Poole, D.P. (2016).
1135 Distribution and trafficking of the-opioid receptor in enteric neurons of the guinea pig. *Am J*
1136 *Physiol Gastrointest Liver Physiol* 311, 252–266.
- 1137 Leigh, N.R., Schupp, M.O., Li, K., Padmanabhan, V., Gastonguay, A., Wang, L., Chun, C.Z.,
1138 Wilkinson, G.A., and Ramchandran, R. (2013). Mmp17b Is Essential for Proper Neural Crest
1139 Cell Migration In Vivo. *PLoS One* 8, e76484.
- 1140 Le Lievre, C.S., and Le Douarin, N.M. (1975). Mesenchymal derivatives of the neural crest:
1141 analysis of chimaeric quail and chick embryos. *J. Embryol. Exp. Morphol.* 34, 125–154.
- 1142 Ling, I.T.C., and Sauka-Spengler, T. (2019). Early chromatin shaping predetermines
1143 multipotent vagal neural crest into neural, neuronal and mesenchymal lineages. *Nat. Cell Biol.*
1144 21, 1504–1517.
- 1145 Lister, J.A. (2002). Development of pigment cells in the zebrafish embryo. *Microsc. Res. Tech.*
1146 58, 435–441.
- 1147 Lister, J.A., Robertson, C.P., Lepage, T., Johnson, S.L., and Raible, D.W. (1999). Nacre
1148 Encodes a Zebrafish Microphthalmia-Related Protein That Regulates Neural-Crest-Derived
1149 Pigment Cell Fate. *Development* 126, 3757–3767.
- 1150 Lister, J.A., Lane, B.M., Nguyen, A., and Lunney, K. (2011). Embryonic expression of
1151 zebrafish Mitf family genes Tfe3b, Tfeb, and Tfec. *Dev. Dyn.* 240, 2529–2538.
- 1152 Lu, J.-K., Tsai, T.-C., Lee, H., Hsia, K., Lin, C.-H., and Lu, J.-H. (2019). Pectoral Fin
1153 Anomalies in tbx5a Knockdown Zebrafish Embryos Related to the Cascade Effect of N-
1154 Cadherin and Extracellular Matrix Formation. *J. Dev. Biol.* 7, 15.
- 1155 Ludwig, A., Rehberg, S., and Wegner, M. (2004). Melanocyte-specific expression of
1156 dopachrome tautomerase is dependent on synergistic gene activation by the Sox10 and Mitf
1157 transcription factors. *FEBS Lett.* 556, 236–244.
- 1158 Lumb, R., Buckberry, S., Secker, G., Lawrence, D., and Schwarz, Q. (2017). Transcriptome
1159 profiling reveals expression signatures of cranial neural crest cells arising from different axial
1160 levels. *BMC Dev. Biol.* 17, e5.
- 1161 Luo, R., An, M., Arduini, B.L., and Henion, P.D. (2001). Specific pan-neural crest expression
1162 of zebrafish crestin throughout embryonic development. *Dev. Dyn.* 220, 169–174.
- 1163 Martik, M.L., and Bronner, M.E. (2017). Regulatory Logic Underlying Diversification of the
1164 Neural Crest. *Trends Genet.* 33, 715–727.

- 1165 Matini, P., Manneschi, L.I., Mayer, B., and Fausone-Pellegrini, M.S. (1995). Nitric oxide
1166 producing neurons in the human colon: an immunohistochemical and histoenzymatical study.
1167 *Neurosci. Lett.* *193*, 17–20.
- 1168 McGraw, H.F., Nechiporuk, A., and Raible, D.W. (2008). Zebrafish Dorsal Root Ganglia
1169 Neural Precursor Cells Adopt a Glial Fate in the Absence of neurogenin1. *J. Neurosci.* *28*,
1170 12558–12569.
- 1171 McInnes, L., Healy, J., and Melville, J. (2018). UMAP: Uniform Manifold Approximation and
1172 Projection for Dimension Reduction. *ArXiv* 1802.03426v2.
- 1173 Memic, F., Knoflach, V., Morarach, K., Sadler, R., Laranjeira, C., Hjerling-Leffler, J.,
1174 Sundström, E., Pachnis, V., and Marklund, U. (2018). Transcription and Signaling Regulators
1175 in Developing Neuronal Subtypes of Mouse and Human Enteric Nervous System.
1176 *Gastroenterology* *154*, 624–636.
- 1177 Mi, H., Muruganujan, A., Huang, X., Ebert, D., Mills, C., Guo, X., and Thomas, P.D. (2019).
1178 Protocol Update for large-scale genome and gene function analysis with the PANTHER
1179 classification system (v.14.0). *Nat. Protoc.* *14*, 703–721.
- 1180 Minchin, J.E.N., and Hughes, S.M. (2008). Sequential actions of Pax3 and Pax7 drive
1181 xanthophore development in zebrafish neural crest. *Dev. Biol.* *317*, 508–522.
- 1182 Minoux, M., and Rijli, F.M. (2010). Molecular mechanisms of cranial neural crest cell
1183 migration and patterning in craniofacial development. *Development* *137*, 2605–2621.
- 1184 Morarach, K., Mikhailova, A., Knoflach, V., Memic, F., Kumar, R., Li, W., Ernfors, P., and
1185 Marklund, U. (2020). Diversification of molecularly defined myenteric neuron classes revealed
1186 by single cell RNA-sequencing. *Nat Neurosci.* <https://doi.org/10.1038/s41593-020-00736-x>
- 1187 Nagy, N., and Goldstein, A.M. (2017). Enteric Nervous System Development: A Crest Cell’s
1188 Journey From Neural Tube to Colon. *Semin. Cell Dev. Biol.* *66*, 94–106.
- 1189 Nakamura, T., Gehrke, A.R., Lemberg, J., Szymaszek, J., and Shubin, N.H. (2016). Digits and
1190 fin rays share common developmental histories. *Nature* *537*, 225–228.
- 1191 Nord, H., Dennhag, N., Muck, J., and Von Hofsten, J. (2016). Pax7 is required for
1192 establishment of the xanthophore lineage in zebrafish embryos. *Mol. Biol. Cell* *27*, 1853–1862.
- 1193 Olden, T., Akhtar, T., Beckman, S.A., and Wallace, K.N. (2008). Differentiation of the
1194 Zebrafish Enteric Nervous System and Intestinal Smooth Muscle. *Genesis* *46*, 484–498.
- 1195 van Otterloo, E., Li, W., Garnett, A., Cattell, M., Medeiros, D.M., and Cornell, R.A. (2012).
1196 Novel Tfap2-mediated control of soxE expression facilitated the evolutionary emergence of the
1197 neural crest. *Development* *139*, 720–730.
- 1198 Parichy, D.M., Ransom, D.G., Paw, B., Zon, L.I., and Johnson, S.L. (2000). An Orthologue of
1199 the Kit-Related Gene *Fms* Is Required for Development of Neural Crest-Derived Xanthophores
1200 and a Subpopulation of Adult Melanocytes in the Zebrafish, *Danio Rerio*. *Development* *127*,
1201 3031–3044.

1202 Parker, H.J., Pushel, I., and Krumlauf, R. (2018). Coupling the roles of Hox genes to regulatory
1203 networks patterning cranial neural crest. *Dev. Biol.* *444*, S67–S78.

1204 Parker, H.J., De Kumar, B., Green, S.A., Prummel, K.D., Hess, C., Kaufman, C.K., Mosimann,
1205 C., Wiedemann, L.M., Bronner, M.E., and Krumlauf, R. (2019). A Hox-TALE regulatory
1206 circuit for neural crest patterning is conserved across vertebrates. *Nat. Commun.* *10*, 1182.

1207 Petratou, K., Subkhankulova, T., Lister, J.A., Rocco, A., Schwetlick, H., and Kelsh, R.N.
1208 (2018). A Systems Biology Approach Uncovers the Core Gene Regulatory Network Governing
1209 Iridophore Fate Choice From the Neural Crest. *PLOS Genet.* *14*, e1007402.

1210 Petratou, K., Spencer, S.A., Kelsh, R.N., and Lister, J.A. (2019). The MITF paralog tfec is
1211 required in neural crest development for fate specification of the iridophore lineage from a
1212 multipotent pigment cell progenitor. *BioRxiv* 862011.

1213 Philippidou, P., and Dasen, J.S.S. (2013). Hox Genes: Choreographers in Neural Development,
1214 Architects of Circuit Organization. *Neuron* *80*, 12–34.

1215 Poon, K.L., Richardson, M., Lam, C.S., Khoo, H.E., and Korzh, V. (2003). Expression pattern
1216 of neuronal nitric acid oxide synthase in embryonic zebrafish. *Gene Expr. Patterns* *3*, 463–466.

1217 Qu, Z.D., Thacker, M., Castelucci, P., Bagyánszki, M., Epstein, M.L., and Furness, J.B. (2008).
1218 Immunohistochemical analysis of neuron types in the mouse small intestine. *Cell Tissue Res.*
1219 *334*, 147–161.

1220 Quigley, I.K., and Parichy, D.M. (2002). Pigment pattern formation in zebrafish: A model for
1221 developmental genetics and the evolution of form. *Microsc. Res. Tech.* *58*, 442–455.

1222 R Core Team (2020). R. R A Lang. *Environ. Stat. Comput. R Found. Stat. Comput.*

1223 Raffaeli, G., Cavallaro, G., Allegaert, K., Wildschut, E.D., Fumagalli, M., Agosti, M., Tibboel,
1224 D., and Mosca, F. (2017). Neonatal Abstinence Syndrome: Update on Diagnostic and
1225 Therapeutic Strategies. *Pharmacother. J. Hum. Pharmacol. Drug Ther.* *37*, 814–823.

1226 Rajan, S.G., Gallik, K.L., Monaghan, J.R., Uribe, R.A., Bronner, M.E., and Saxena, A. (2018).
1227 Tracking neural crest cell cycle progression in vivo. *Genesis* *56*, e23214.

1228 Rao, M., and Gershon, M.D. (2018). Enteric nervous system development: what could possibly
1229 go wrong? *Nat. Rev. Neurosci.* *19*, 552–565.

1230 Reedy, M. V., Faraco, C.D., and Erickson, C.A. (1998). Specification and migration of
1231 melanoblasts at the vagal level and in hyperpigmented silkie chickens. *Dev. Dyn.* *213*, 476–485.

1232 Rocha, M., Singh, N., Ahsan, K., Beiriger, A., and Prince, V.E. (2020). Neural crest
1233 development: insights from the zebrafish. *Dev. Dyn.* *249*, 88–111.

1234 Rodrigues, F.S.L.M., Doughton, G., Yang, B., and Kelsh, R.N. (2012). A novel transgenic line
1235 using the Cre-lox system to allow permanent lineage-labeling of the zebrafish neural crest.
1236 *Genesis* *50*, 750–757.

1237 Rueden, C.T., Schindelin, J., Hiner, M.C., DeZonia, B.E., Walter, A.E., Arena, E.T., and
1238 Eliceiri, K.W. (2017). ImageJ2: ImageJ for the next generation of scientific image data. *BMC*
1239 *Bioinformatics* *18*, 529.

1240 Roy-Carson, S., Natukunda, K., Chou, H., Pal, N., Farris, C., Schneider, S.Q., and Kuhlman,
1241 J.A. (2017). Defining the transcriptomic landscape of the developing enteric nervous system
1242 and its cellular environment. *BMC Genomics* *18*, 290.

1243 Ruzicka, L., Howe, D.G., Ramachandran, S., Toro, S., Van Slyke, C.E., Bradford, Y.M., Eagle,
1244 A., Fashena, D., Frazer, K., Kalita, P., et al. (2019). The Zebrafish Information Network: new
1245 support for non-coding genes, richer Gene Ontology annotations and the Alliance of Genome
1246 Resources. *Nucleic Acids Res.* *47*, D867-873.

1247 Satija, R., Farrell, J.A., Gennert, D., Schier, A.F., and Regev, A. (2015). Spatial reconstruction
1248 of single-cell gene expression data. *Nat. Biotechnol.* *33*, 495–502.

1249 Sauka-Spengler, T., and Bronner-Fraser, M. (2008). A gene regulatory network orchestrates
1250 neural crest formation. *Nat. Rev. Mol. Cell Biol.* *9*, 577–568.

1251 Saunders, L.M., Mishra, A.K., Aman, A.J., Lewis, V.M., Toomey, M.B., Packer, J.S., Qiu, X.,
1252 McFaline-Figueroa, J.L., Corbo, J.C., Trapnell, C., et al. (2019). Thyroid hormone regulates
1253 distinct paths to maturation in pigment cell lineages. *Elife* *8*, e45181.

1254 Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch,
1255 S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for
1256 biological-image analysis. *Nat. Methods* *9*, 676–682.

1257 Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of
1258 image analysis. *Nat. Methods* *9*, 671–675.

1259 Shepherd, I.T., Pietsch, J., Elworthy, S., Kelsh, R.N., and Raible, D.W. (2004). Roles for
1260 GFR α 1 receptors in zebrafish enteric nervous system development. *Development* *131*, 241–249.

1261 Simoes-Costa, M., and Bronner, M.E. (2016). Reprogramming of avian neural crest axial
1262 identity and cell fate. *Science* (80-.). *352*, 1570–1573.

1263 Simões-Costa, M., Tan-Cabugao, J., Antoshechkin, I., Sauka-Spengler, T., and Bronner, M.E.
1264 (2014). Transcriptome analysis reveals novel players in the cranial neural crest gene regulatory
1265 network. *Genome Res.* *24*, 281–290.

1266 Singleman, C., and Holtzman, N.G. (2014). Growth and maturation in the zebrafish, *Danio*
1267 *Rerio*: A staging tool for teaching and research. *Zebrafish* *11*, 396–406.

1268 Sobczak, M., Sałaga, M., Storr, M.A., and Fichna, J. (2014). Physiology, signaling, and
1269 pharmacology of opioid receptors and their ligands in the gastrointestinal tract: Current
1270 concepts and future perspectives. *J. Gastroenterol.* *49*, 24–45.

1271 Soldatov, R., Kaucka, M., Kastriti, M.E., Petersen, J., Chontorotzea, T., Englmaier, L.,
1272 Akkuratova, N., Yang, Y., Häring, M., Dyachuk, V., et al. (2019). Spatio-temporal structure of
1273 cell fate decisions in murine neural crest. *Science* (80-.). *364*, eaas9536.

- 1274 Sperber, S.M., and Dawid, I.B. (2008). *barx1* is necessary for ectomesenchyme proliferation
1275 and osteochondroprogenitor condensation in the zebrafish pharyngeal arches. *Dev. Biol.* *321*,
1276 101–110.
- 1277 Sperber, S.M., Saxena, V., Hatch, G., and Ekker, M. (2008). Zebrafish *dlx2a* Contributes to
1278 Hindbrain Neural Crest Survival, Is Necessary for Differentiation of Sensory Ganglia and
1279 Functions With *dlx1a* in Maturation of the Arch Cartilage Elements. *Dev. Biol.* *314*, 59–70.
- 1280 Stewart, R.A., Arduini, B.L., Berghmans, S., George, R.E., Kanki, J.P., Henion, P.D., and
1281 Look, A.T. (2006). Zebrafish *foxd3* Is Selectively Required for Neural Crest Specification,
1282 Migration and Survival. *Dev. Biol.* *292*, 174–188.
- 1283 Strausberg, R.L., Feingold, E.A., Grouse, L.H., Derge, J.G., Klausner, R.D., Collins, F.S.,
1284 Wagner, L., Shenmen, C.M., Schuler, G.D., Altschul, S.F., et al. (2002). Generation and initial
1285 analysis of more than 15,000 full-length human and mouse cDNA sequences. *Proc. Natl. Acad.*
1286 *Sci. U. S. A.* *99*, 16899–16903.
- 1287 Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W.M., Hao, Y.,
1288 Stoeckius, M., Smibert, P., and Satija, R. (2019). Comprehensive Integration of Single-Cell
1289 Data. *Cell* *177*, 1888-1902.e21.
- 1290 Tambalo, M., Mitter, R., and Wilkinson, D.G. (2020). A single cell transcriptome atlas of the
1291 developing zebrafish hindbrain. *Dev.* *147*, dev184143.
- 1292 Taylor, C.R., Montagne, W.A., Eisen, J.S., and Ganz, J. (2016). Molecular fingerprinting
1293 delineates progenitor populations in the developing zebrafish enteric nervous system. *Dev. Dyn.*
1294 *245*, 1081–1096.
- 1295 Theodore, L.N., Hagedorn, E.J., Cortes, M., Natsuhara, K., Liu, S.Y., Perlin, J.R., Yang, S.,
1296 Daily, M.L., Zon, L.I., and North, T.E. (2017). Distinct Roles for Matrix Metalloproteinases 2
1297 and 9 in Embryonic Hematopoietic Stem Cell Emergence, Migration, and Niche Colonization.
1298 *Stem Cell Reports* *8*, 1226–1241.
- 1299 Theveneau, E., and Mayor, R. (2012). Neural crest delamination and migration: From
1300 epithelium-to-mesenchyme transition to collective cell migration. *Dev. Biol.* *366*, 34–54.
- 1301 Thisse, B., and Thisse, C. (2004). Fast Release Clones: A High Throughput Expression
1302 Analysis. ZFIN Direct Data Submission. (<http://zfin.org>).
- 1303 Thisse, C., and Thisse, B. (2005). High Throughput Expression Analysis of ZF-Models
1304 Consortium Clones. ZFIN Direct Data Submission. <http://zfin.org>.
- 1305 Thisse, B., Pflumio, S., Fürthauer, M., Loppin, B., Heyer, V., Degraeve, A., Woehl, R., Lux, A.,
1306 Steffan, T., Charbonnier, X., et al. (2001). Expression of the zebrafish genome during
1307 embryogenesis. ZFIN Direct Data Submission. (<http://zfin.org>).
- 1308 Uribe, R.A., and Bronner, M.E. (2015). *Meis3* is required for neural crest invasion of the gut
1309 during zebrafish enteric nervous system development. *Mol. Biol. Cell* *26*, 3728–3740.

- 1310 Uyttebroek, L., Shepherd, I.T., Harrison, F., Hubens, G., Blust, R., Timmermans, J.P., and van
1311 Nassauw, L. (2010). Neurochemical coding of enteric neurons in adult and embryonic zebrafish
1312 (*Danio rerio*). *J. Comp. Neurol.* *518*, 4419–4438.
- 1313 Vega-Lopez, G.A., Cerrizuela, S., and Aybar, M.J. (2017). Trunk neural crest cells: formation,
1314 migration and beyond. *Int. J. Dev. Biol.* *61*, 5–15.
- 1315 Wagner, D.E., Weinreb, C., Collins, Z.M., Briggs, J.A., Megason, S.G., and Klein, A.M.
1316 (2018). Single-cell mapping of gene expression landscapes and lineage in the zebrafish embryo.
1317 *Science* (80-.). *360*, 981–987.
- 1318 Wang, H.H., Chen, H.S., Li, H.B., Zhang, H., Mei, L.Y., He, C.F., Wang, X.W., Men, M.C.,
1319 Jiang, L., Liao, X. Bin, et al. (2014). Identification and functional analysis of a novel mutation
1320 in the SOX10 gene associated with Waardenburg syndrome type IV. *Gene* *538*, 36–41.
- 1321 Wang, W. Der, Melville, D.B., Montero-Balaguer, M., Hatzopoulos, A.K., and Knapik, E.W.
1322 (2011). Tfp2a and Foxd3 regulate early steps in the development of the neural crest progenitor
1323 population. *Dev. Biol.* *360*, 173–185.
- 1324 Williams, A.L., and Bohnsack, B.L. (2015). Neural crest derivatives in ocular development:
1325 Discerning the eye of the storm. *Birth Defects Res. Part C Embryo Today Rev.* *105*, 87–95.
- 1326 Williams, R.M., Candido-Ferreira, I., Repapi, E., Gavriouchkina, D., Senanayake, U., Ling,
1327 I.T.C., Telenius, J., Taylor, S., Hughes, J., and Sauka-Spengler, T. (2019). Reconstruction of the
1328 Global Neural Crest Gene Regulatory Network In Vivo. *Dev. Cell* *51*, 522-576.e7.
- 1329 Wilson, Y.M., Richards, K.L., Ford-Perriss, M.L., Panthier, J.J., and Murphy, M. (2004).
1330 Neural crest cell lineage segregation in the mouse neural tube. *Development* *131*, 6153–6162.
- 1331 Wood, J.D., and Galligan, J.J. (2004). Function of opioids in the enteric nervous system.
1332 *Neurogastroenterol. Motil.* *16*, 17–28.
- 1333 Yelon, D., Brauch, T., Halpern, M.E., Ruvisnsky, I., Ho, R.K., Silver, L.M., and Stainier,
1334 D.Y.R. (2000). The bHLH transcription factor Hand2 plays parallel roles in zebrafish heart and
1335 pectoral fin development. *Development* *127*, 2573–2582.
- 1336 Yntema, C.L., and Hammond, W.S. (1954). The origin of intrinsic ganglia of trunk viscera from
1337 vagal neural crest in the chick embryo. *J. Comp. Neurol.* *101*, 515–541.
- 1338 Zheng, G.X.Y., Terry, J.M., Belgrader, P., Ryvkin, P., Bent, Z.W., Wilson, R., Ziraldo, S.B.,
1339 Wheeler, T.D., Mcdermott, G.P., Zhu, J., et al. (2017). Massively parallel digital transcriptional
1340 profiling of single cells. *Nat. Commun.* *8*, 14049.
- 1341 Zoli, M. (2000). Distribution of Cholinergic Neurons in the Mammalian Brain with Special
1342 Reference to their Relationship with Neuronal Nicotinic Acetylcholine Receptors. In *Neuronal*
1343 *Nicotinic Receptors. Handbook of Experimental Pharmacology*, F. Clementi, D. Fornasari, and
1344 C. Gotti, eds. (Berlin, Heidelberg: Springer), pp. 13–30.

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1347 **Figure Legends**

1348

1349 **Figure 1. Single-Cell profiling strategy and cell population composition of posterior**
1350 ***sox10*:GFP⁺ cells from the posterior zebrafish during the embryonic to larval stage**
1351 **transition.**

1352 (A) Confocal image of a *sox10*:GFP⁺ embryo at 48 hpf; Hb: Hindbrain; Sc: Spinal cord. A:
1353 Anterior, P: Posterior, D: Dorsal, V: Ventral. Scale bar: 50 μ M

1354 (B) Cartoon illustrations of a zebrafish embryo at 48-50 hpf and an early larval fish at 68-70 hpf
1355 depicted laterally to summarize the dissection workflow used to collect posterior *sox10*:GFP⁺
1356 cells.

1357 (C) Schematic of the 10X Genomics Chromium and data analysis pipeline.

1358 (D) tSNE plots showing the arrangement of Clusters 0-18 and where the major cell types
1359 identified among *sox10*:GFP⁺ cells arrange in the 48-50 hpf dataset.

1360 (E) tSNE plots showing the arrangement of Clusters 0-22 and where the major cell types
1361 identified among *sox10*:GFP⁺ cells arrange in the 68-70 hpf dataset.

1362 (F,G) Dot plots of the identifying gene markers for each major cell type classification in the 48-
1363 50 hpf and 68-70 hpf datasets, respectively. Dot size depicts the cell percentage for each marker
1364 within the dataset and the color summarizes the average expression levels for each gene.

1365

1366 **Figure 1- figure supplement 1. Statistics on generation and filtering of single cell**
1367 **transcriptomes at 48-50 hpf and 68-70 hpf.**

1368 (A,B) Fluorescence activated cell sorting plots highlighting the GFP⁺ cell population sorted at
1369 48-50 hpf (A) and 68-70 hpf (B).

1370 (C) Table of general statistics pertaining to the sequencing and alignment of reads from the Cell
1371 Ranger pipeline. Additional metrics provided were derived from the Seurat R package as
1372 described in the Materials & Methods section.

1373 (D,E) Plots showing the feature selection for both the 48-50 hpf (D) and 68-70 hpf (E) datasets.
1374 Cells were selected such that they had fewer than 2500 features to reduce spuriously sorted
1375 cells.

1376 (F,G) Top 2000 most variably expressed genes were identified and used for further downstream
1377 identification of significant principal components, as described in the Materials & Methods
1378 section.

1379 (H,I) Most significant principle components (top 20 for both datasets) were selected to be used
1380 for subsequent cluster identification and cell embedding in tSNE and UMAP spaces.

1381

1382 **Figure 1-figure supplement 2. Major cell type annotations among *sox10*:GFP⁺ cells.**

1383 (A,B) Heatmap summarizing the top 10 genes significantly expressed in each cluster, for 48-50
1384 and 68-70 hpf, respectively. Relative expression levels within each cluster is summarized within
1385 the color key, where yellow to magenta color indicates high to low gene expression levels.

(C,D) Heatmaps summarizing the top 30 genes significantly expressed among the major cell types identified among *sox10:GFP⁺* cells, for 48-50 and 68-70 hpf, respectively. Relative expression levels within each major cell type cluster is summarized within the color key, where yellow to magenta color indicates high to low gene expression levels.

(E,F) tSNE plots depicting the major cell type classification representative gene marker for each major cell type category, for 48-50 and 68-70 hpf, respectively. Relative expression levels are summarized within the color keys, where color intensity is proportional to expression level of each gene depicted.

1394

Figure 1- figure supplement 3. Major cell type categories and cell cycle distributions of the scRNA-seq datasets.

(A,B) tSNE plots summarizing the G1, S and G2/M phase cell cycle phase occupancies of the cells in the 48-50 and 68-70 hpf time points, respectively.

(C,E) A tSNE plot depicting the expression of *aurkb*, a G2/M phase marker, within the 48-50 and 68-70 hpf datasets, respectively. Relative expression levels are summarized within the color keys, where color intensity is proportional to expression level of each gene depicted.

(D,F) A tSNE plot depicting the expression of *mcm3*, a S phase marker, within the 48-50 and 68-70 hpf datasets, respectively. Relative expression levels are summarized within the color keys, where color intensity is proportional to expression level of each gene depicted.

(G) Bar graphs summarizing the cell cycle phase occupancies, as a fraction of cells within the total datasets for each time point.

(H) Bar graphs summarizing the major cell type categories, as a fraction of cells within the total datasets for each time point.

(I) Table summarizing the cell cycle genes used to demarcate cell cycle phase occupancy categories within the scRNA-seq datasets.

1411

Figure 1- figure supplement 4. Table summarizing the top identity markers used for major cell type and subtype cellular classifications for each cluster at 48-50 and 68-70 hpf.

1414

Figure 1- figure supplement 5. Identification of otic vesicle, muscle, and central nervous system (CNS) cellular populations

(A) Panel of tSNE feature plots at 48-50 hpf that identify combinatorial expression of otic vesicle (*otomp*, *cldna*, *cldn7b* and *epcam*), muscle (*ckmb*, *actc1b*, *tnnt3a* and *tpma*), or CNS (*slc32a1*, *gad1b*, *slc6a5*, *gata2a*) markers. Cluster of interest denoted by black arrows.

(B) Panel of tSNE feature plots at 68-70 hpf that identify combinatorial expression of otic vesicle markers (*otomp*, *cldna*, *cldn7b* and *epcam*) or muscle (*ckmb*, *actc1b*, *tnnt3a* and *tpma*). Cluster of interest denoted by black arrows.

Figure 1- figure supplement 6. Identification of fin bud and sensory neuronal progenitor cellular populations

1424

(A) Panel of tSNE feature plots of sensory neuronal progenitors at 48-50 hpf that show combinatorial expression of *neurod4*, *neurod1*, *vim*, and *ngfrb*. Cluster of interest denoted by black arrows.

(B) Panel of tSNE feature plots of fin bud makers at 48-50 hpf (top) and 68-70 hpf (bottom) that show combinatorial expression of *tbx5a*, *hand2*, *hoxd13a*, and *prrx1a*. Cluster of interest denoted by black arrows.

1431

Figure 1- source data 1. List of marker genes per cluster in the *sox10*:GFP scRNA-seq datasets.

Tables reporting the Seurat output for genes for each cluster at the 48-50 hpf (Tab 1) and 68-70 hpf (Tab 2) time points, including p-values (<0.01), average log-fold change (≥ 2.5), adjusted p-values (≤ 1.0), pct.1 summarizing proportion of cells expressing the individual gene in the cluster, pct.2 showing the proportion of cells expressing the individual gene in all other clusters in the dataset.

1439

Figure 2. Distinct pigment cell populations are present among *sox10*:GFP⁺ cells during embryonic to larval transition.

(A) Cartoon schematic depicting the model for neural crest delineation into pigment cell lineages and the genes that were used to identify each pigment cell population.

(B) Dot plot identifying melanophore markers within the 48-50 hpf dataset. Dot size depicts the cell percentage for each marker within the dataset and the color summarizes the average expression levels for each gene.

(C) tSNE plots depicting melanophore signature in the 48-50 hpf dataset. Relative expression levels are summarized within the color keys, where color intensity is proportional to expression level of each gene depicted.

(D) Dot plot showing distinct pigment chromatophore markers within the 68-70 hpf dataset. Dot size depicts the cell percentage for each marker within the dataset and the color summarizes the average expression levels for each gene. M: melanophore markers; X: xanthophore markers; I: iridophore markers.

(E-G) tSNE plots revealing the location of melanophores (E), xanthophores (F), and iridophores (G) in the 68-70 hpf dataset. Relative expression levels are summarized within the color keys, where color intensity is proportional to expression level of each gene depicted.

(H) HCR against *mitfa* and *tfec* at 48-50 hpf reveals *mitfa*⁺ melanophores (white arrowhead) and *mitfa*⁺/*tfec*⁺ pigment progenitors (red arrowhead). Cropped panels show individual fluorescent channels.

(I) HCR against *mitfa* and *tfec* at 68-70 hpf presents *mitfa*⁺ melanophores (white arrowhead), *tfec*⁺ iridophores (blue arrowhead), and *mitfa*⁺/*tfec*⁺ pigment progenitors (red arrowhead). Cropped panels show individual fluorescent channels.

(J) HCR against *mitfa* and *xdh* at 68-70 hpf shows *mitfa*⁺/*xdh*⁺ xanthophores (orange arrowhead). Cropped panels show individual fluorescent channels.

Scale bar in H-J: 50 μ m.

1466

Figure 2- source data 1. Melanophore populations, shared and unique genes at 68-70 hpf.
Table summarizing the genes (“elements”) exclusively found in Cluster 4, exclusively found in Cluster 18, and genes found in both Cluster 4 and 18 at the 68-70 hpf time point scRNA-seq dataset.

1471

Figure 3. Global analysis of mesenchyme cell signatures among *sox10*:GFP⁺ cells.

(A,B) A heatmap of signature mesenchyme identity genes within the major cell type classified cells at 48-50 and 68-70 hpf, respectively. Relative expression levels within each cluster is summarized within the color key, where red to blue color indicates high to low gene expression levels.

(C) A cluster tree depicting the relationship between general and chondrogenic mesenchyme cellular subtypes.

(D) Violin plots summarizing the expression levels for select mesenchyme identity markers within individual clusters at the 48-50 and 68-70 hpf time points, respectively. Data points depicted in each cluster represent single cells expressing each gene shown.

(E,L) tSNE plots depicting the expression of *prrx1b*, *barx1* and *twist1a* in the 48-50 and 68-70 hpf datasets, respectively. Relative expression levels are summarized within the color keys, where color intensity is proportional to expression level of each gene depicted.

(F-K) Whole mount HCR analysis reveals the spatiotemporal expression of *prrx1b* **(F)**, *barx1* **(G)**, *twist1a* **(H)**, *sox*:GFP **(J)** in 48 hpf embryos. **(I)** A merge of *barx1*, *prrx1b* and *twist1a* is shown. **(K)** A merge of *barx1*, *prrx1b*, *twist1a* and *sox10*:GFP is shown. White arrowheads denote expression in posterior pharyngeal arch, while yellow arrowheads highlight fin bud expression.

(M-R) Whole mount HCR analysis reveals the spatiotemporal expression of *prrx1b* **(M)**, *barx1* **(N)**, *twist1a* **(O)**, *sox*:GFP **(Q)** in 68 hpf embryos. **(P)** A merge of *barx1*, *prrx1b* and *twist1a* is shown. **(R)** A merge of *barx1*, *prrx1b*, *twist1a* and *sox10*:GFP is shown. White arrowheads denote expression in posterior pharyngeal arch, while yellow arrowheads highlight fin bud expression.

Ot: otic; Fb: Fin bud. Scale bar: 100 μm.

1496

Figure 3- figure supplement 1. Whole mount *in situ* hybridization of select ENCC, mesenchyme and neural markers at 48-50 hpf.

(A,A') The marker *sox10* is shown along the vagal region and within ENCC along the foregut in **(A'**; highlighted via arrowheads). Scale bar in **A**: 60 μM, in **A'**: 40 μM.

(B, B') Expression of *phox2bb* is shown within the hindbrain-axial level of the embryo, as well as within ENCC within the foregut **(B'**; highlighted via arrowheads). Scale bar in **A**: 60 μM, in **A'**: 40 μM.

(C,D) The mesenchyme markers *mmp2* **(C, highlighted via arrowheads)** and *foxc1a* **(D; highlighted via arrowheads)** are expressed within the posterior pharyngeal arches and the ventral mesenchyme.

(E,F) The neural markers *notch1a* **(E)** and *dla* **(F)** are expressed within the hindbrain and spinal cord (arrowheads). **(F)** *dla* expression is seen in the ENCC (arrow).

1509 Scale bar in C-F: 60 μ M

1510

1511 **Figure 4. Enteric neural crest cells and differentiating enteric neurons are present among**
 1512 **posterior *sox10*:GFP⁺ cell populations.**

1513 (A) tSNE feature plots reveal expression of core neural crest cell markers *sox10*, *foxd3*, *crestin*
 1514 and *tfap2a* mapping to the neural crest cell cluster (red arrow).

1515 (B) tSNE feature plots depict expression of the enteric neural crest cell markers *phox2bb*, *ret*,
 1516 *ngfrb* and *gfra1a* within the neural crest cell cluster (red arrow).

1517 Relative expression levels are summarized within the color keys in (A) and (B), where color
 1518 intensity is proportional to expression level of each gene depicted.

1519 (C) A heatmap reveals expression levels of enteric neural crest cell markers across the 8 major
 1520 cell populations captured in the 48-50 hpf data set (color key denotes cells types represented in
 1521 color bar on top of heatmap). Neural crest cell cluster highlighted in black rectangle. Relative
 1522 expression levels within each major cell type cluster is summarized within the color key, where
 1523 yellow to magenta color indicates high to low gene expression levels.

1524 (D) Dot plot of expanded list of neural crest (green line) and enteric neural crest (purple line)
 1525 cell markers across each major cell type within 48-50 hpf data set. Dot size depicts the cell
 1526 percentage for each marker within the data set and the color summarizes the average expression
 1527 levels for each gene.

1528 (E,F) Whole mount HCR analysis of 48 hpf embryos reveals co-expression of enteric neural
 1529 crest cell markers within the developing gut (dashed outline). Top panels depict merged images
 1530 of color channels for each HCR probe. Lower panels represent grey-scale images of each
 1531 separated channel corresponding to the magnified region of foregut (grey rectangle). Arrows
 1532 depict regions where all markers are found to be co-expressed. Hb: Hindbrain, Sc: Spinal cord,
 1533 pLLg: posterior Lateral Line ganglia, LL: Lateral Line. A: Anterior, P: Posterior, D: Dorsal, V:
 1534 Ventral. Scale bar: 50 μ M.

1535 (G) tSNE feature plots reveal expression levels of enteric neuron markers *elavl3*, *phox2bb*,
 1536 *gfra1a*, *nos1*, *vipb* and *ret*, within a common region of a neuronal cluster (red arrow). Relative
 1537 expression levels are summarized within the color keys, where color intensity is proportional to
 1538 expression level of each gene depicted.

1539 (H) Dot plot depicts expression levels of pan-neuronal and enteric neuron specific markers
 1540 across individual clusters generated within the original 68-70 hpf tSNE. Pan-neuronal markers
 1541 found throughout clusters 5 and 12, with enteric neuron markers most prominently expressed
 1542 within cluster 12. Dot size depicts the cell percentage for each marker within the data set and
 1543 the color summarizes the average expression levels for each gene.

1544 (I) Whole mount HCR analysis depicts differentiating enteric neurons within the foregut region
 1545 at 69 hpf co-expressing *nos1*, *phox2bb*, *vipb*, and *elavl3* (yellow arrow). Anterior: Left,
 1546 Posterior: Right. Scale bar: 50 μ M.

1547

1548 **Figure 5. Differentiating enteric neurons captured during key transitional stage of**
 1549 **subtype diversification within 68-70 hpf *sox10*:GFP⁺ larval cells.**

1550 (A) tSNE plot reveals 5 distinct sub-clusters following the subset analysis and re-clustering of
 1551 clusters 5 and 12 from the 68-70 hpf data set.
 1552 (B) Dot plot depicts expression levels of enteric neuron markers across resulting Sub-clusters.
 1553 Each marker was expressed at low levels in Sub-cluster 1 and were found to be expressed at
 1554 higher levels within Sub-cluster 3.
 1555 (C) tSNE feature plots further depict the expression of enteric neuron markers by illustrating the
 1556 levels and localization of expression within the Sub-cluster architecture. Feature plots
 1557 supplement dot plot and demonstrate the prominent expression of enteric neuron markers within
 1558 Sub-cluster 3, which appears to emanate from Sub-cluster 1.
 1559 (D,E) Violin and feature plots reveal expression levels of acetylcholine-associated and
 1560 excitatory neuron markers reported to distinguish enteric IPANs. These markers were found in a
 1561 discrete pocket of cells forming the distal-most region of Sub-cluster 3 (red arrow). Violin data
 1562 points depicted in each Sub-cluster represent single cells expressing each gene shown.
 1563 (F) Graphical model summarizes expression patterns observed in 68-70 hpf data set and HCR
 1564 validation. Common enteric neuroblast capable of diverging into subsequent lineages, IPAN,
 1565 inhibitory neuron, and interneuron through lineage restricted gene expression. *pbx3b* promotes
 1566 assumption of IPAN role through loss of *nos1* and *vipb* and begins expressing *calb2a*, *ache* and
 1567 *slc18a3a*.
 1568 (G) Whole mount HCR analysis reveals co-expression of IPAN markers, *pbx3b* and *calb2a*, and
 1569 inhibitory neurochemical markers, *vipb* and *nos1* (white arrows), within the foregut (dashed
 1570 white line) at 68 hpf. Vesicular acetylcholine transferase, *slc18a3a*, was not observed in tandem
 1571 with *pbx3b* but was co-expressed with *calb2a*, *vipb*, and *nos1* (yellow arrow). Scale bar: 50 μ M.
 1572 (H) Feature plots reveal expression of opioid receptors, *opr1l* and *opr1b* within the
 1573 differentiated enteric neuron Sub-Cluster 3.
 1574 (I-N) Whole mount HCR analysis validates expression of *opr1l* in combination with *vipb* and
 1575 *phox2bb* (yellow arrows) in enteric neurons localized to the foregut region of a 68 hpf embryo.
 1576 Scale bar: 10 μ M.

1577

1578 **Figure 5- figure supplement 1. Enteric neuron subtype diversification gene expression**
 1579 **patterns seen in enteric neuron Sub-Clusters related to Figure 5D,E**

1580 Panel of tSNE feature plots magnified and cropped to focus on progressively differentiating
 1581 enteric neurons (highlighted by *etv1* expression). Subtype diversification and IPAN emergence
 1582 depicted via combinatorial gene expression (*etv1*, *ntng1aa*, *pbx3b*, *slc18a3a*, *calb2a*, and *ache*)
 1583 localized to the distal tip of Sub-cluster 3. Inhibitory neuron markers, *nos1*, *vip*, and galanin
 1584 (*galn*) were present within the pocket of diverging enteric subtypes.

1585

1586 **Figure 5- figure supplement 2. Enteric and sympathetic neuron markers distinguished**
 1587 **among common autonomic neuron precursors.**

1588 UMAP analysis of Sub-Clusters depicts enteric and sympathetic neurons delineating from a
 1589 common pool of autonomic neurons.

1590 (A) UMAP plot generated following re-clustering of Clusters 5 and 12 from the 68-70 hpf data
 1591 set.

(B) Dot plot depicts expression levels of general autonomic neuron makers (*ascl1a*, *hand2*, *phox2a*, *phox2bb*), sympathetic neuron markers (*th*, *dbh*, *lmo1*, *insmla*) and enteric neuron markers (*vipb*, *etv1*, *nos1*, *gfal1a*) within Sub-Clusters 0-4.

(C) Dendrogram denotes similarity of Sub-Clusters based on average gene expression of each cell within the Sub-Clusters, which reveals transcriptomic distinction of enteric neuron Sub-Cluster 3.

(D-F). Feature plots highlight the expression of autonomic, sympathetic and enteric neuron gene markers within UMAP Sub-Clusters.

1600

Figure 5- source data 1. List of marker genes per Sub-Cluster, following subset and re-clustering of enteric Cluster 5 and 12 at 68-70 hpf.

Table reporting the Seurat output for genes for each Sub-Cluster (0-4), including p-values (<0.01), average log-fold change (.25), adjusted p-values (1.0), pct.1 summarizing proportion of cells expressing the individual gene in the sub-cluster, pct.2 showing the proportion of cells expressing the individual gene in all other sub-clusters in the sub-data set.

Figure 5- source data 2. List of enriched pathways within enteric neuron Sub-Cluster 3 and genes present in specific opioid proenkephalin pathway identified following PANTHER Overrepresentation Test.

Source Data table contains two sheets. Enriched Gene Pathways sheet lists the enriched pathways identified following the use of the statistical test, Fisher's exact test that compares the number of genes in a given pathway within the reference *Danio rerio* list to the number of genes in a given pathway within the enteric neuron Sub-Cluster 3 list. This analysis produced 43 statistically overrepresented pathways. Column B: Number of genes within the given pathway present with *Danio rerio* reference list of 25888 genes. Column C: Number of genes within the given pathway present within Sub-Cluster 3 gene list. Column D: Number of pathway genes expected to be present within the Sub-Cluster 3 gene list based on the percentage of pathway genes present in the *Danio rerio* reference list. Column E: Denotes that more pathway genes were present in Sub-Cluster 3 gene list than expected. Column F: Fold enrichment of Sub-Cluster 3 pathway genes comparative to reference list. Column G: P-values calculated following Fisher's exact test comparing expected number of pathway genes to number of genes pathway genes in Sub-Cluster 3. Column H: P-value following Benjamini-Hochberg false discovery rate (FDR) correction. Opioid proenkephalin pathway sheet lists genes associated with this pathway that are present within the enteric neuron Sub-Cluster 3 gene list, notably, opioid receptor *oprd1b*.

1626

Figure 6. Integrated atlas of posterior *sox10*:GFP⁺ cell types spanning the embryonic to larval transition.

(A) Global UMAP embedding demonstrating the clustering of cell types across 48-50 hpf and 68-70 hpf. Cell labels were transferred from the original curation to the new atlas after its creation, allowing for unbiased assessment of cell type organization.

1632 (B) Previously identified mesenchyme clusters form a large discernible cluster marked by
1633 *prrx1b*, *twist1a*, *foxc1a*, and *snaila*, which was separated into both chondrogenic and general
1634 mesenchyme, as denoted by its differential expression of *barx1* and *dlx2a*. Importantly, nearly
1635 every 48-50 hpf cell type nests with a cluster at 68-70 hpf.

1636 (C) Pigment cells clusters reflect differentiation paths described in Figure 4A. Melanophores at
1637 48-50 hpf group near to the 68-70 hpf melanophore cluster, bipotent pigment progenitors
1638 bridges both the iridophores and melanophores. Xanthophores cluster separately, reflecting their
1639 distinct lineage of origin at this developmental window.

1640 (D) Detailed analysis of the larger neural/neuronal cluster shows clear progression of cell fates
1641 from progenitor to differentiating glia or neuron. We confirm the presence of a clear enteric
1642 neuronal population, which is distinct from other subtypes at this dataset.

1643

1644 **Figure 6- figure supplement 1. Annotated *sox10*:GFP⁺ atlas labeled by cell types.**

1645 (A) UMAP labeling the 27 new clusters formed after the generation of the Atlas.

1646 (B) Following label transfer integration, major cell type classifications group together into
1647 distinct clusters.

1648 (C) High resolution visualization of both clustering of original cluster labels as well as their
1649 position within the UMAP. Cell categories segregate in the dendrogram largely as expected
1650 from the UMAP visualization.

1651 (D) Additional markers shown by UMAP for validation of the neural/neuronal clusters.

1652

1653 **Figure 6- figure supplement 2. Differential expression among pigment, mesenchyme and
1654 neural/neuronal subsets of the *sox10*:GFP⁺ atlas**

1655 (A) UMAP visualization of cells labeled by source identity (either 48-50 hpf or 68-70 hpf)
1656 following integration. All 48-50 hpf cells (pink) approximately map to a major cluster found at
1657 68-70 hpf (aqua).

1658 (B-D) Subset of the pigment clusters (B), the mesenchyme clusters (C), and the neural/neuronal
1659 clusters (D) highlighting several differentially expressed genes between the cells derived from
1660 each timepoint.

1661

1662 **Figure 6 - source data 1. List of marker genes per major cell type identity in the
1663 *sox10*:GFP⁺ merged atlas.**

1664 Tables reporting the Seurat output for genes for each major cell type identity including p-values
1665 (<0.01), average log-fold change (≥.25), adjusted p-values (≤1.0), pct.1 summarizing proportion
1666 of cells expressing the individual gene in the cluster identity category, pct.2 showing the
1667 proportion of cells expressing the individual gene in all cluster identity categories in the atlas
1668 dataset.

1669

1670 **Figure 7. Description of Hox genes expressed across cell lineages within the *sox10*:GFP⁺
1671 atlas.**

1672 (A) Dot plot shows both the mean expression (color) as well as percent of cells (size) per
1673 lineage for zebrafish *hox* genes in the first 8 paralogy groups (PG). The full list of *hox* gene

expression profiles per lineage can be found in Figure 7 - figure supplement 1. Discrete *hox* profiles discern specific cell types, which is particularly evident in the enteric neuronal cluster. (B) Clustering of atlas lineages based *hox* expression profiles groups highlights robust core neural signature, which distinguishes the neural lineages from the remainder of the clades. Neural and glial progenitors formed an intermediate clade between the low-*hox* expressing lineages and the main neural branch. Additionally, the fin bud mesenchyme, which also has a highly distinctive *hox* profile, also forms a distinct clade. Subtle variations in *hox* expression by remaining lineages further are further reflected in the remaining portion of the dendrogram, however these are far less distinct.

(C-D) Pairwise comparison of the fraction of cells in either the autonomic neural progenitor lineage (C) or the enteric neurons (D) for the first eight paralogy groups. Intersection of the gene pairs reflect the fraction of cells with expression for both genes with a log2 Fold change values > 0, with the identical gene intersections along the primary diagonal representing the total number of cells which express that gene in the lineage. Enteric neural *hox* signature was not only specific to this cell population, but also was abundantly coexpressed.

(E) Summary panel describing the specific autonomic and enteric *hox* signatures detected. We found a common *hox* expression profile, which we refer to as the core signature, that is then modified across the specific lineages.

(F-M) In situ validation of the chief enteric neural *hox* signature via hybridization chain reaction. *phox2bb* (F-J) labels enterically fated neurons at the level of the midgut in larval stage embryos fixed at 70-72hpf. White arrows highlight specific cells of interest. Key *hox* signature constituents *hoxb5a* (G) and *hoxb5b* (H) or *hoxd4a* (K) and *hoxa5a* (L) were found to be co-expressed within *phox2bb* expressing cells (White Arrows). Scale bars in (I,M):50 μ m.

Figure 7 - figure supplement 1. Comprehensive overview of *hox* expression profiles within the atlas.

(A) Similar to Figure 7A, a comprehensive dot plot shows both the mean expression (color) as well as percent of cells per lineage (size) for zebrafish all *hox* genes. Discrete *hox* profiles discern specific cell types, which is particularly evident in the enteric neuronal cluster, as well as the fin bud mesenchyme.

(B-I) Hindbrain expression patterns for enteric *hox* profiles. Autonomic neural marker *phox2bb* clearly marks the midbrain and hindbrain (hb) domains, as well as the pharyngeal arches (pa). Enterically fated cells can also clearly be seen along the tract of the gut (dash region), starting the foregut (fg). Anterior domains for *hoxb5a* (C), *hoxb5b* (D), *hoxd4a* (G), and *hoxa5a* (H) are represented by a vertical line. Overlapping regions can be approximated by the merged images (E, I). Scale in (B, F): 50 μ m; dorsoventral-rostrocaudal axis defined in (F).

(J) UMAP of the atlas demonstrating pervasive *hox* expression throughout the dataset. Expression was defined as having a log2 fold change > 0. Approximately 85% of cells contained expression for at least one *hox* gene.

(K-R) Feature plots representing the expression of selected *hox* genes within the atlas UMAP. *hoxd4a* (K), *hoxa5a* (L), *hoxb5a* (M), *hoxb5b* (N) all localize in varying degrees to the enteric neural signature, though *hoxd4a* also marks the mesenchymal clusters, as seen (A). *hoxa4a* (O)

1716 highlights its pervasive expression among the clusters. *hoxa13b* (**P**) and *hoxa11b* (**Q**) are key
1717 markers denoting the finbud mesenchyme. Alternative neural populations are further identified
1718 by their *hoxa9a* (**R**) expression profile, contrasting with the enteric populations.

Figure 1

Figure 1 - figure supplement 1

Figure 1-figure supplement 3

Figure 1-Figure supplement 4

Major Cell Type		Classifier			# of Cells in Cluster		Citations	
Time Point	Cluster ID #	Subtype	Identifier genes					
48-50HPF	cl0	Neuronal	Autonomic Neuronal Progenitor	phox2bb, tfap2a/b, elavl3/4, phox2a, tuba2, ncam1a, draxin, snca, fez1, cdh2	201		Farnsworth et al., 2020; Soldatov et al., 2019	
48-50HPF	cl1	Mesenchyme	Mesenchymal Progenitor	prrx1a, snai1a, twist1a, foxc1b, mcm5, pcna, mcm7, cdh11, meox1	160		Soldatov et al., 2019	
48-50HPF	cl2	Mesenchyme	Chondrogenic Mesenchyme Proliferative 1	dlx2a, prrx1a/b, twist1a/b, barx1, cdk1, ccna2	154		Soldatov et al., 2019; Sperber et al., 2008; Sperber and Dawid, 2008; Ding et al., 2013; Barske et al., 2016	
48-50HPF	cl3	Mesenchyme	Chondrogenic Mesenchyme Proliferative 2	barx1, dlx2a, mcm6, prrx1b, chaf1a, pcna, hand2	129		Soldatov et al., 2019; Sperber et al., 2008; Sperber and Dawid, 2008; Ding et al., 2013; Barske et al., 2016	
48-50HPF	cl4	Mesenchyme	Chondrogenic Mesenchyme Stem-like	prrx1a, dlx2a, barx1, twist1a, rpa1, fen1, rpa2, uhrf1, id3, chaf1a	108		Soldatov et al., 2019; Sperber et al., 2008; Sperber and Dawid, 2008; Ding et al., 2013; Barske et al., 2016	
48-50HPF	cl5	Neural Crest	Migratory Neural Crest/ Enteric Neural Crest	sox10, foxd3, crestin, tfap2a, mmp17b, pcdh10a, phox2bb, ret, ngrfb, gfra1a, foxc1a, twist1a, tagln2, aldh1a2, rdh10a, meis3, cyp26a1, hand2, tpm4a, actb1	93		Dutton et al., 2001; Luo et al., 2001; Knight et al., 2003; Stewart et al., 2006	
48-50HPF	cl6	Mesenchyme	Mesenchyme Differentiating 1	gad1b, gad2, slc6a5, slc32a1, gata2a, irx1b, elavl3, elavl4, tuba2, phox2bb	88		Soldatov et al., 2019	
48-50HPF	cl7	Neuronal	Central Nervous System neuron	tyrp1a/b, mitfa, dct, pmela, slc45a2, sox10, mlpha, tspan10, oca2	86		Farnsworth et al. 2020	
48-50HPF	cl8	Pigment	Melanophore	dlx2a, barx1, foxc1a, dlx4a, id2a, snai1a/b, twist3	81		Du et al., 2003; Lister et al., 1999; Ludwig et al., 2004; Quigley and Parichy, 2002	
48-50HPF	cl9	Mesenchyme	Chondrogenic Mesenchyme Migratory	cdh11, foxc1a/b, prrx1a, meox1, pdgfra, col2a1a, col9a1a, col9a2, sparc, colec12, reck	77		Soldatov et al., 2019; Sperber et al., 2008; Sperber and Dawid, 2008; Ding et al., 2013; Barske et al., 2016	
48-50HPF	cl10	Mesenchyme	Mesenchyme Differentiating 2	cxcl12a, cxcl12b, rac1, reck, foxc1a, twist1a, prrx1a, meox1, snai2	74		Soldatov et al., 2019	
48-50HPF	cl11	Mesenchyme	Mesenchymal Migratory	prrx1b, twist1b, foxc1a, barx1, cyp26a1, rdh10a, meis3, aldh1a2, mcm5, mcm6	74		Soldatov et al., 2019	
48-50HPF	cl12	Mesenchyme	Chondrogenic Mesenchyme Proliferative 3	notch1a, dla/b, olig4, elavl3, phox2ba, neurod4, scrt2, phox2bb, ncam1a	61		Soldatov et al., 2019	
48-50HPF	cl13	Neural	Neural Progenitor	tbx5a, hand2, hoxa13a/b, prrx1a/b, foxc1b, hoxd13a, pcna	61		Farnsworth et al., 2020	
48-50HPF	cl14	Mesenchyme	Fin Bud	sox10, olig1/2, fabp7a, notch1a, sox3, nkx2.2a, pou3f1, plp1a, gfap, s100b	58		Yelon et al., 2000; Lu et al., 2019; Nakamura et al., 2016; Feregrino et al., 2019	
48-50HPF	cl15	Glial	Peripheral Glial Progenitor	sox10, cdh11, otomp, cldnb, cldn7b, cldnh, epcam, aldh1a3, vamp8	44		Soldatov et al., 2019; Carney et al. 2006	
48-50HPF	cl16	Otic	Otic Epithelium	ngfrb, neurod1, neurod4, vim, neurog1, grfra1a, six1a/b, elavl4, isl2a/b, pou4f1	23		Thisse et al., 2004; Thisse et al., 2005	
48-50HPF	cl17	Neuronal	Sensory Neuronal Progenitor	ckmb, actc1b, tnn2, tnnt3a, pvalb2, tnnt3b, ak1, tpma, desma	23		Soldatov et al., 2019	
48-50HPF	cl18	Muscle	Muscle		13		Thisse et al., 2004	
68-70HPF	cl0	Mesenchyme	Chondrogenic Differentiating Mesenchyme 1	barx1, sparc, prrx1a/b, twist1a/b, pdgfra, col1a2, col6a1, col5a1	281		Soldatov et al., 2019; Sperber et al., 2008; Sperber and Dawid, 2008; Ding et al., 2013; Barske et al., 2016	
68-70HPF	cl1	Mesenchyme	Fin Bud	tbx5a, hand2, prrx1a/b, hoxd13a, pcna, foxc1a/b, meox1, hoxa13a/b	254		Yelon et al., 2000; Lu et al., 2019; Nakamura et al., 2016; Feregrino et al., 2019	
68-70HPF	cl2	Mesenchyme	Mesenchymal Migratory	meox1, prrx1a/b, foxc1b, snai1a, cdh11, rac1a, myl9b, twist1a/b	229		Soldatov et al., 2019	
68-70HPF	cl3	Neural	Neural Progenitor	fabp7a, foxd3, sox10, phox2bb, her4.2, mdka, her4.1, ccnd2a, ngrfb, tuba8l3	228		Farnsworth et al. 2020	
68-70HPF	cl4	Pigment	Melanophore	mitfa, pmela, tyrp1a/b, dct, oca2, mlpha, tspan10, tfap2e	213		Du et al., 2003; Lister et al., 1999; Ludwig et al., 2004; Quigley and Parichy, 2002	
68-70HPF	cl5	Neuronal	Sympatho-enteric Progenitor	asc1a, phox2bb, phox2a, hand2, insm1a/b, dpyl3, ret, ngrfb, gfra1a, elavl3/4	202		Thisse et al., 2004; Heanue and Pachnis, 2008	
68-70HPF	cl6	Mesenchyme	Chondrogenic Differentiating Mesenchyme 2	barx1, prrx1a, foxc1a/b, twist1a, col1a2, col5a1, cdh11, dlx5a, sparc, meox1	191		Soldatov et al., 2019	
68-70HPF	cl7	Mesenchyme	Fin Bud	tbx5a, hand2, hoxa13a/b, prrx1a/b, hoxd13a, pcna, sox10	178		Yelon et al., 2000; Lu et al., 2019; Nakamura et al., 2016; Feregrino et al., 2019	
68-70HPF	cl8	Mesenchyme	Chondrogenic Proliferative Mesenchyme	prrx1a/b, foxc1a, twist1a/b, barx1, col2a1a, pcna, cdc6, ccnb2	98		Soldatov et al., 2019	
68-70HPF	cl9	Muscle	Pigmented Muscle	tnnt3b, mitfa, tyrp1a, tpma, ckmb, gch2, dct, tnn2, myl1,	93		Thisse et al., 2004; Thisse et al., 2005	
68-70HPF	cl10	Neural	Neural Progenitor	sox10, foxd3, asc1a, aurkb, ncam1b, ccna2, tpx2, phox2bb, ret, elavl3/4	76		Farnsworth et al. 2020	
68-70HPF	cl11	Otic	Otic Epithelium	cldne, vamp8, cldnb, epcam, cldn7b, cldnh, cdh1	70		Thisse et al., 2004, Thisse et al., 2001	
68-70HPF	cl12	Neuronal	Enteric Neuron	elavl3/4, phox2bb, gfra1a, ret, fgf13b, vip, vipb, nos1, ngrfb, hoxb5b	44		Shepherd et al., 2004; Heanue and Pachnis, 2008; Uyttebroek et al., 2010; Taylor et al., 2016; Gaudet et al., 2011	
68-70HPF	cl13	Pigment	Pigment Progenitor	sox10, tfec, tfap2e, mitfa, zeb2a, cdh1, mlpha, pax3a, trpm1b, gpx3	43		Saunders et al., 2019	
68-70HPF	cl14	Glial	Schwann Cells	sox10, mbpa/b, pou3f1, egr2b, fabp7a, erbb3a, plp1a, cx27.5, gldn, mag	42		Farnsworth et al. 2020	
68-70HPF	cl15	Pigment	Xanthophore	xdh, pax7a/b, gch2, aox5	39		Nord et al., 2016; Parichy et al., 2000; Saunders et al., 2019; Minchin and Hughes, 2008; Lister et al., 1999	
68-70HPF	cl16	Pigment	Iridiophore	atic, gpnmb, tfec, pnp4a	32		Higdon et al., 2013; Lister et al., 2011; Petratou et al., 2018, 2019	
68-70HPF	cl17	Mesenchyme	Differentiating Mesenchyme 1	twist1a, prrx1a, colec12, sparc, mmp2, msx2b, col5a1, col1a1a, pdgfra, tnc	25		Soldatov et al., 2019	
68-70HPF	cl18	Pigment	Proliferative Melanophores	mitfa, pmela, tyrp1a/b, aurkb, pcna, cenph, mlpha, dct, gpr143, tfap2e	19		Du et al., 2003; Lister et al., 1999; Ludwig et al., 2004; Quigley and Parichy, 2002	
68-70HPF	cl19	Muscle	Muscle	ckmb, tpma, tnn2, tnnt3b, actc1b, cavin4a, myom1a, neb, flnca, desma,	18		Thisse et al., 2004	
68-70HPF	cl20	Mesenchyme	Chondrogenic Mesenchyme Migratory	barx1, twist1a, foxc1a/b, snai1a, dlx3b, mmp2, sparc, snai2, dlx2a, sox9a	16		Soldatov et al., 2019	
68-70HPF	cl21	Unidentified/Spurious	Unidentified/Spurious	anxa2b, cldnc, faah2b, vil1, fabp1b.1, plac8.1	13			
68-70HPF	cl22	Mesenchyme	Differentiating Mesenchyme 2	rac2, sparc, col18a1, col2a1b, col1a1a, lama5, thy1	6		Soldatov et al., 2019	

Figure 1 Figure supplement 5

A

B

68-70 hpf

Figure 1-Figure supplement 6

A Sensory Nerons

B Fin Bud

Figure 2

Figure 3-figure supplement 1
Enteric Neural Crest

Figure 4

Figure 5

Figure 5 - figure supplement 1

Figure 5 - figure supplement 2

Figure 7

Figure 7 - Figure Supplement 1