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Molecular fingerprinting delineates progenitor populations in the developing zebrafish enteric nervous system

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Abstract

Background—To understand the basis of nervous system development, we must learn how multipotent progenitors generate diverse neuronal and glial lineages. We addressed this issue in the zebrafish enteric nervous system (ENS), a complex neuronal and glial network that regulates essential intestinal functions. Little is currently known about how ENS progenitor subpopulations generate enteric neuronal and glial diversity.

Results—We identified temporally and spatially dependent progenitor subpopulations based on coexpression of three genes essential for normal ENS development: *phox2bb*, *sox10*, and *ret*. Our data suggest that combinatorial expression of these genes delineates three major ENS progenitor subpopulations, (1) *phox2bb*+/*ret*−/*sox10*−, (2) *phox2bb*+/*ret*+/*sox10*−, and (3) *phox2bb*+/*ret*+/*sox10*+, that reflect temporal progression of progenitor maturation during migration. We also found that differentiating zebrafish neurons maintain *phox2bb* and *ret* expression, and lose *sox10* expression.

Conclusion—Our data show that zebrafish enteric progenitors constitute a heterogeneous population at both early and late stages of ENS development and suggest that marker gene expression is indicative of a progenitor's fate. We propose that a progenitor's expression profile reveals its developmental state: "younger" wave front progenitors express all three genes, whereas more mature progenitors behind the wave front selectively lose *sox10* and/or *ret* expression, which may indicate developmental restriction.

Keywords

development; neural crest; enteric neuron; enteric glia; gene expression

Introduction

A fundamental question in developmental neurobiology is how a complex nervous system arises from a multipotent progenitor pool. Distinguishing individual progenitor subgroups is key to elucidating their different lineages. In this study, we addressed the question of progenitor subpopulations in the zebrafish enteric nervous system (ENS). The ENS is a

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neural crest-derived complex network of neurons and glia that innervates and regulates intestinal functions, such as motility, secretion, and homeostasis (Furness, 2006).

Progenitor subpopulations have been best studied in the central nervous system. These subpopulations are distinguished from one another by distinct gene expression patterns that are often temporally or spatially dependent, for example in the fruit fly central nervous system, the mouse telencephalon, or the mouse retina (Guillemot, 2005; Flames et al., 2007; Trimarchi et al., 2008; Hafler et al., 2012; Kohwi and Doe, 2013). In each of these examples, differential gene expression patterns identify pools of progenitors with distinct spatial distributions that are linked to the types of progeny they generate (Flames et al., 2007; Hafler et al., 2012; Kohwi and Doe, 2013). Progenitor heterogeneity has also been observed on a molecular level in the peripheral nervous system. During early cranial neural crest migration in the chick embryo, so-called trailblazer cells at the migratory front have a distinct molecular signature compared to the trailing cell populations (McLennan et al., 2015). Clearly, characterization of gene expression profiles in progenitor populations has greatly enhanced our understanding of different progenitor populations, and paved the way for elucidating their progeny. In the case of the ENS, understanding neural and glial lineages requires learning when and how enteric neural and glial progenitors differ from one another during development.

Enteric progenitors are typically identified by expression of genes thought to regulate specific aspects of ENS development. These markers include *Sox10*, *Phox2b*, and *Ret* whose expression in enteric progenitors has been described in mouse, chicken, and zebrafish (Schiltz et al., 1999; Young et al., 1999; Young et al., 2002; Young et al., 2003; Shepherd et al., 2004; Elworthy et al., 2005; Nagy et al., 2012). Loss of function of any one of these genes results in a severely depleted or completely absent ENS and is associated with Hirschsprung disease in humans, which is characterized by aganglionosis of distal intestine (Schuchardt et al., 1994; Herbarth et al., 1998; Pingault et al., 1998; Pattyn et al., 1999; Garcia-Barcelo et al., 2003; Emison et al., 2010), indicating that each marker plays an essential role in ENS development.

Sox10 is a member of the SOX family of transcription factors that is expressed in delaminating neural crest cells, and consequently enteric progenitors (Southard-Smith et al., 1998). Sox10 is required for migration of neural crest cells to the intestine, as enteric progenitors fail to enter the intestine in both mouse and zebrafish *Sox10* mutants (Kapur, 1999; Elworthy et al., 2005). Additionally, expression of *Sox10* maintains the undifferentiated state of enteric progenitors (Paratore et al., 2002; Kim et al., 2003; Bondurand et al., 2006), and is required for *Ret* and *Phox2b* expression (Lang and Epstein, 2003; Elworthy et al., 2005).

Phox2b is a homeodomain transcription factor expressed in all enteric progenitors as well as differentiating neurons during ENS development (Pattyn et al., 1997; Young et al., 2003; Elworthy et al., 2005). *Phox2b* is necessary for activating *Ret* expression in enteric progenitors (Leon et al., 2009), as suggested by inhibited migration through the intestine and apoptosis of enteric progenitors in Phox2b$^{-/-}$ mice, which essentially mimics the ENS phenotype of Ret$^{-/-}$ mice, in addition to absence of Ret+ enteric cells (Pattyn et al., 1999).

Ret, a tyrosine kinase, acts in concert with a family of four receptors, each of which specifically binds one of four neurotrophic factors, including glial derived neurotrophic factor (GDNF) (Takahashi and Cooper, 1987; Durbec et al., 1996a; Jing et al., 1996; Kotzbauer et al., 1996; Trupp et al., 1996; Baloh et al., 1997; Buj-Bello et al., 1997; Klein et al., 1997; Baloh et al., 1998; Milbrandt et al., 1998; Worby et al., 1998; Masure et al., 2000). Rat cell culture studies suggest that GDNF promotes proliferation, survival, and differentiation of enteric progenitors (Taraviras et al., 1999). Inhibited migration through the intestine in $Ret^{-/-}$ mice also suggests that GDNF signaling through Ret facilitates progenitor migration along the intestine (Durbec et al., 1996b). Ret signaling during zebrafish ENS development appears conserved, because blockade of GDNF signaling by knockdown of *ret* eliminates migration and proliferation of enteric progenitors (Shepherd et al., 2004). Thus, following *ret* knockdown, *phox2bb* expressing progenitors reach the intestine but fail to populate it resulting in fewer enteric neurons, especially posteriorly (Shepherd et al., 2004; Heanue and Pachnis, 2008).

Ret, Sox10, and Phox2b are involved in early and late developmental processes in the ENS, which makes them good candidates for investigating heterogeneity among enteric progenitors. In mouse, coexpression analyses of these markers suggest that early enteric progenitors and those at the migratory wave front constitute homogenous cells coexpressing Ret, Sox10, and Phox2b (Young et al., 1999; Young et al., 2002; Young et al., 2003). Later in development, enteric progenitors are thought to differentially lose expression of these markers resulting in heterogeneity (Young et al., 1999; Young et al., 2003; Anderson et al., 2006). Phox2b, Sox10, and Ret also all play later roles in ENS development: in mouse, Phox2b and Ret promote neuronal differentiation (Chalazonitis et al., 1998; Pattyn et al., 1999; Taraviras et al., 1999; Young et al., 1999; Young et al., 2002; Young et al., 2003), whereas Sox10 is maintained in differentiated enteric glia (Young et al., 2003). Although the functions of these genes have been studied extensively during ENS development, descriptions of their temporal expression patterns during the course of ENS development are limited, especially in non-mammalian model species, and patterns of colocalization among all three markers remain to be elucidated. Thus, a comprehensive spatio-temporal analysis of progenitor marker coexpression patterns is still necessary for a complete understanding of ENS progenitor development.

In this study, we analyzed the spatial and temporal colocalization patterns of zebrafish orthologs of the enteric progenitor markers, *phox2bb*, *ret*, and *sox10*. We characterized expression of these markers at three discrete anterior-posterior levels of the intestine at four different stages of development. In addition, we investigated the degree to which *phox2bb*, *ret*, and *sox10* colocalize with a marker for differentiating neurons. Our data suggest that temporally and spatially dependent combinatorial expression patterns of *phox2bb, sox10*, and *ret* delineate three major enteric progenitor subpopulations that fluctuate in size during zebrafish ENS development. Additionally, as shown in mouse (Young et al, 2003), we found that *phox2bb* and *ret* expression is maintained in differentiating neurons, whereas *sox10* expression is lost. From these results we conclude that zebrafish enteric progenitors are heterogeneous at early and late time points during enteric progenitor migration along the intestine. Enteric progenitor heterogeneity (Corpening et al., 2008), as well as the coexpression patterns of *phox2bb*, *sox10*, and *ret* that we describe here are parallel to

findings from mouse (Young et al., 1999; Young et al., 2002; Young et al., 2003; Anderson et al., 2006). Establishing these parallels in zebrafish and mouse ENS progenitor development lays essential ground work for future high throughput, lineage tracing studies using live imaging for which the zebrafish model is especially well suited because embryos are transparent and develop rapidly.

Results

phox2bb driven GFP is highly colocalized with *phox2bb* mRNA

To facilitate our investigation of marker gene coexpression in ENS progenitors, we utilized a fluorescent transgene strategy and examined zebrafish intestines at specific locations and times during development (Fig. 1). To use *phox2bb:*EGFP as a tracer to recognize enteric progenitors, it is crucial that the transgene accurately represent *phox2bb* mRNA expression in enteric progenitors. To demonstrate fidelity of transgene expression, we determined whether *phox2bb*:EGFP colocalized with *phox2bb* mRNA at three different anterior-posterior levels of the intestine at 54 and 60 hours post fertilization (hpf) (Fig. 1B and C). We found 83–98% colocalization of *phox2bb* mRNA and *phox2bb*:EGFP (Fig. 2A–D, Table 1). The remaining progenitors are comprised of GFP+/probe− and GFP−/probe+ cells, which might reflect dynamic mRNA expression patterns, and thus differ from more consistent protein levels. Although it is possible that the larger GFP−/probe+ group in the anterior intestine represents a small yet unique population of progenitors, small subpopulations are comprised of too few cells (either barely above or equal to the standard error of the mean value), and therefore we cannot draw conclusions about their significance (Table 1, Fig. 2B and D). Thus, we focused on the largest progenitor populations and their fluctuations. From the results described above, we conclude that *phox2bb*:EGFP expression is a sufficiently accurate measure of *phox2bb* mRNA levels in enteric progenitors, meaning that we can use *phox2bb:*EGFP transgenic fish to examine coexpression of enteric progenitor markers. Therefore, we refer to *phox2bb*:EGFP expression as *phox2bb* and use expression of this transgene to illustrate coexpression patterns between *phox2bb*, *ret*, and *sox10*.

Differential expression of *sox10* and *ret* reveals two progenitor subpopulations

Function of *phox2bb*, *sox10*, and *ret* are each necessary for normal ENS formation, and thus all cells are expected to express each gene at some point in development. Mouse data suggest that progenitors selectively lose expression of these genes as ENS development progresses (Young et al., 1999; Young et al., 2003; Anderson et al., 2006) and that differentiation of progenitors occurs in an anterior to posterior wave along the intestine (Rothman and Gershon, 1982). Thus, we expect that coexpression of these genes would also show spatio-temporal dynamics in zebrafish enteric progenitors. We hypothesized that enteric progenitors are distributed among subpopulations characterized by particular combinations of the three enteric progenitor markers, and that the distributions change over time and along the length of the intestine. We analyzed the spatial distribution of marker gene coexpression patterns at two time points, 54 and 60 hpf. At these two time points progenitors have migrated an appreciable distance, thus allowing analysis of discrete levels along the length of the intestine (Fig. 1B and C). Additionally, since neuronal differentiation

has been shown to start in zebrafish at 54 hpf (Olden et al., 2008), the two time points capture a mixture of undifferentiated and differentiating progenitor cells. We first describe the degree to which *sox10* and *ret* each separately colocalize with *phox2bb*, because temporally and spatially dependent trends are far easier to visualize than in the triple colocalization analysis. Consistent with our hypothesis, we found that neither *ret* nor *sox10* colocalized entirely with *phox2bb*, and that the prevalence of particular expression profiles was spatially and temporally dependent (Fig. 3A–H).

At 54 hpf the degree of *ret* and *phox2bb* colocalization varied between 58–77% depending on the region of the intestine (58% anterior, 74% mid, 77% posterior; Fig. 3B, Table 2). The number of double positive cells was higher in the mid and posterior regions relative to the anterior region (Fig. 3B). The remaining cells were primarily *phox2bb*+/*ret*− (42% anterior, 23% mid, 20% posterior; Fig. 3B). At 60 hpf the *phox2bb*+/*ret*+ subpopulation made up 74–79% of progenitors (75% anterior, 74% mid, 79% posterior; Fig. 3D, Table 2). Again, the remaining cells were predominantly *phox2bb*+/*ret*− (22% anterior, 18% mid, 16% posterior; Fig. 3D). These results demonstrate two main progenitor subpopulations present at both 54 and 60 hpf; one subpopulation is *phox2bb*+/*ret*+ and the other is *phox2bb*+/*ret*−. The size of the two subpopulations in the mid and posterior regions was relatively consistent from 54 to 60 hpf. Distribution between the two subpopulations in the mid and posterior level was consistent from 54 to 60 hpf, however, we observed a decrease in the number of *phox2bb*+/*ret*− cells from 54 to 60 hpf in the anterior intestine (compare Fig. 3B and D).

Our colocalization analysis of *sox10* and *phox2bb* also revealed two main subpopulations present at 54 and 60 hpf; a *phox2bb*+/*sox10*− subpopulation and a *phox2bb*+/*sox10*+ subpopulation (Fig. 3E–H). At 54 hpf the double positive subpopulation made up 16–51% of enteric progenitors in each region (16% anterior, 42% mid, 51% posterior; Fig. 3F, Table 3). The remaining progenitors were primarily *phox2bb*+/*sox10*− (81% anterior, 56% mid, 48% posterior; Fig. 3F). As seen with the *phox2bb*+/*ret*+ population, there were more double positive cells posteriorly than anteriorly. We also observed a temporal shift in the number of *phox2bb*+/*sox10*+ cells from 54 to 60 hpf; there were slightly more double positive cells at 60 hpf relative to 54 hpf (compare Fig. 3F and H, Table 3).

We also observed small subpopulations at 54 and 60 that were positive for either *ret* or *sox10*, but did not express *phox2bb* (Fig. 3B,D,F,H, Table 2, 3). However, these small subpopulations generally did not exceed the standard error of the mean value, and therefore we could not draw conclusions about their significance.

In summary, *ret* and *sox10* are differentially expressed in enteric progenitor subpopulations at 54 and 60 hpf. From these data, we conclude that there are at least three major enteric progenitor subpopulations characterized by the following combinations of marker genes at both 54 and 60 hpf: 1) *phox2bb+*, 2) *phox2bb+*/*ret+*, and 3) *phox2bb+*/*sox10+*. We also conclude that the spatial distributions of these subpopulations are relatively consistent from 54 to 60 hpf. Apart from the slight increase in *phox2bb*+/*ret*+ cells in the anterior from 54 to 60 hpf, we consistently observed a high degree of colocalization between *ret* and *phox2bb* along the length of the intestine. We also saw more *phox2bb/sox10* colocalization at the migratory wave front relative to the anterior intestine.

Coexpression of *ret* and *sox10* with *phox2bb* reveals a total of three enteric progenitor subpopulations

The double colocalization analysis enabled temporal and spatial pattern recognition. However, to determine whether *ret* and *sox10* colocalization patterns distinguished additional subpopulations, we performed coexpression analysis of all three genes. We analyzed coexpression of *phox2bb* with *ret* and *sox10* at 54 and 60 hpf. This analysis revealed three major subpopulations characterized by the following combinations of marker genes: 1) *phox2bb*+/*ret*−/*sox10*−, 2) *phox2bb*+/*ret*+/*sox10*−, and 3) *phox2bb*+/*ret*+/*sox10*+ (Fig. 4A–D, Table 4). These results suggest that at 54 and 60 hpf, *sox10* is always coexpressed with both *ret* and *phox2bb*, whereas *ret*, while typically coexpressed with *phox2bb*, can be expressed in the absence of *sox10* (Fig. 4B and D). Finally, *phox2bb* is expressed independently of *ret* and *sox10* in a noteworthy population of cells only at 60 hpf, suggesting that *phox2bb* can be expressed in the absence of the other two markers. The proportion of triple positive cells was higher posteriorly than anteriorly (Fig. 4B and D). This spatial trend was exhibited at both 54 hpf (23% anterior, 42% mid, 60% posterior) and 60 hpf (27% anterior, 42% mid, 58% posterior; Fig. 4B and D, Table 4). In contrast, the *phox2bb*+/*ret*+/*sox10*− subpopulation was more prominent anteriorly at both 54 hpf and 60 hpf (Fig. 4B and D, Table 4). The *phox2bb*+/*ret*−/*sox10*− population being negligibly small at 54 hpf was most prominent in the anterior and the mid-region of the intestine at 60 hpf (Fig. 4D, Table 4). From these results, we conclude that there are three major enteric progenitor subpopulations that exhibit marked trends in spatial distribution, but do not noticeably change from 54 to 60 hpf except for the *phox2bb*+/*ret*−/*sox10*− population. The three subpopulations are characterized by the following marker combinations: *phox2bb*+/*ret*−/*sox10*−, *phox2bb*+/*ret*+/*sox10*−, *phox2bb*+/*ret*+/*sox10*+ (Table 4).

A small subpopulation of differentiating neurons expresses *phox2bb* and *ret*

To determine whether marker gene expression indicates that progenitors are becoming developmentally restricted, we investigated whether cells that are fated to become enteric neurons express specific markers. To address this issue, we labeled with an antibody to the pan-neuronal marker Elavl (Marusich et al., 1994), and probed for either *ret* or *sox10* mRNA. A small portion of cells colocalized *phox2bb*+ and Elavl at 54 and 60 hpf in the anterior and mid regions of the intestine (Fig. 5A–H), consistent with previously observed *phox2bb* mRNA/Elavl coexpression at 55 hpf (Elworthy et al., 2005). Most of the Elavl-expressing cells were double positive for *ret* and *phox2bb* at both 54 and 60 hpf, and were most abundant in the anterior and midregion (Fig. 5A–D, Table 5), which is consistent with an anterior-posterior wave of neuronal differentiation observed in mouse and zebrafish (Rothman and Gershon, 1982; Olden et al., 2008). As expected, we saw a greater number of Elavl+ cells overall at 60 hpf due to increased neuronal differentiation at later stages of development. We also investigated expression patterns at 84 hpf, when enteric neurogenesis is well underway (Fig. 1D) (Olden et al., 2008). We see a similar pattern compared to 54 and 60 hpf (Fig. 6, Table 7, 8). As expected, most *phox2bb*+ cells are also Elavl+ and many of these cells are also *ret*+ (Fig. 6A and B, Table 7). In the anterior and midregion, approximately 40% of *phox2bb*+ cells are *ret*+/Elavl+ (Fig. 6B, Table 7). In the anterior, 40% of the remaining cells are *phox2bb*+/Elavl+ and 17% are only *phox2bb*+; in the midregion 32% are *phox2bb*+, 18% are *phox2bb*+/Elavl+, and 9% are *phox2bb*+/*ret*+ (Fig.

6B, Table 7). There is a clear decrease in triple positive cells when moving from the anterior and midregions to the posterior (Fig. 6B, Table 7). Only 15% of cells are *phox2bb*+/*ret*+/Elavl+ in the posterior. In this region the majority of cells are either only *phox2bb*+ (44%) or *phox2bb*+/Elavl+ (37%) (Fig. 6B, Table 7). Finally, we did not see any Elavl and *sox10* mRNA colocalization at 54, 60, or 84 hpf (Fig. 5E–H, Fig. 6C and D, Table 6, 8), consistent with *sox10* being a known enteric glial marker in mammals (Young et al., 2003).

Interestingly, very few *sox10*+ cells were observed at 84 hpf (Fig. 6C and D, Table 8). Some Elavl− cells appeared to express lower levels of *phox2bb*, however, only a small fraction of these cells were also *sox10*+ (data not shown).

In summary, a proportion of differentiating enteric neurons express *phox2bb* and *ret* at 54, 60, and 84 hpf, whereas *sox10* expression is absent from differentiating neurons at all three time points.

Marker expression reveals that enteric progenitor subpopulations are present early in ENS development

To learn whether undifferentiated zebrafish enteric progenitors express all three enteric progenitor markers as they have been suggested to do in mouse (Young et al., 2003), we investigated expression patterns at 36 hpf. This is the earliest time point at which enteric progenitors migrating along the intestine are clearly identifiable in zebrafish ENS development, and is also well before neuronal differentiation has begun (Olden et al., 2008). We analyzed expression of *ret* and *sox10* at the enteric progenitor migratory wave front (Fig. 1A). We found that neither *sox10* nor *ret* colocalized completely with *phox2bb* at 36 hpf (Fig. 7A–D, Table 9). *phox2bb* and *ret* exhibited a relatively high degree of colocalization, about 68% of cells were double positive (Fig. 7B, Table 9). As seen at 54 and 60 hpf, we observed a close to even split between *phox2bb*+/*sox10*− and *phox2bb*+/*sox10+* cells (Fig. 7D, Table 9). In contrast to studies in mouse, these results suggest that undifferentiated zebrafish progenitors do not all colocalize *phox2bb*, *ret*, and *sox10*; instead zebrafish enteric progenitors appear to be a heterogeneous population of cells even at the beginning of their migration along the intestine.

Discussion

We investigated whether *phox2bb*, *sox10*, and *ret* are coexpressed in zebrafish enteric progenitors during early and late stages of their migration along the intestine. These genes are known to be expressed in enteric progenitors, and each of them have essential roles in ENS development, as loss of function of *Sox10*, *Phox2b*, or *Ret* results in a severely depleted or absent ENS in all vertebrates examined to date, including humans (Schuchardt et al., 1994; Herbarth et al., 1998; Pingault et al., 1998; Pattyn et al., 1999; Garcia-Barcelo et al., 2003; Emison et al., 2010). Here we describe a comprehensive spatio-temporal analysis of coexpression of these marker genes during zebrafish ENS development (Fig. 8). We present two key findings: (1) zebrafish enteric progenitors constitute a heterogeneous, highly dynamic population of cells at early and late time points during enteric progenitor migration along the intestine; (2) marker gene expression profile is indicative of a zebrafish

progenitor's fate. Based on our observations, we propose that the expression profile of a progenitor is indicative of its developmental state.

Heterogeneity of zebrafish enteric progenitors

Considering the important roles each gene plays in enteric progenitor development, one might predict that all enteric progenitors would express *phox2bb*, *ret*, and *sox10* throughout ENS development. Finding that this is the case would imply that enteric progenitors are homogenous according to *phox2bb*, *ret*, and *sox10* coexpression. An alternative hypothesis is that enteric progenitors express all three genes, however not simultaneously or at all times, but instead in a temporally and spatially dependent manner, resulting in heterogeneity of progenitors. In mouse, early in development expression of the three genes of interest has shown that enteric progenitors constitute a homogenous group of cells (Young et al., 2003). As development progresses, enteric progenitor expression profiles differ from one another, and these cells become heterogeneous (Young et al., 1999; Young et al., 2003; Anderson et al., 2006; Corpening et al., 2008). Our data suggest that zebrafish enteric progenitors constitute a heterogeneous population of cells as early in development as we can recognize them, just as they begin their migration along the intestine, and that this heterogeneity extends through later developmental stages. However, it remains to be investigated whether prior to 36 hpf enteric progenitors are homogenous with respect to *phox2bb*, *ret*, and *sox10* expression. At later time points, when migrating progenitors are close to the posterior end of the intestine and neuronal differentiation has begun in the anterior intestine, we also find enteric progenitor subpopulations with distinct expression profiles. Based on mRNA and *phox2bb*:EGFP transgene expression patterns, we observed three major enteric progenitor subpopulations: *phox2bb*+/*ret*−/*sox10*−, *phox2bb*+/*ret*+/*sox10*−, and *phox2bb*+/*ret*+/*sox10*+. The distribution among these subpopulations fluctuates over time and along the length of the intestine. Colocalization was somewhat variable between embryos. This variation may be due to fluctuations of mRNA expression in enteric progenitors, or might reflect the dynamic nature of progenitor gene expression, which would magnify any slight age differences between embryos. These subpopulations are thus highly dynamic, and therefore, should be thought of as progenitor states rather than distinct progenitor pools that are present at the onset of ENS development.

Our findings have important implications for choice of progenitor markers, because our results reveal that *ret* and *sox10* are not continuously expressed by all zebrafish progenitors at early or late stages of their migration along the intestine. This is especially relevant for ENS research in zebrafish, where *in situ* hybridization remains a useful tool for studying gene expression, since there is a lack of zebrafish-specific antibodies. It is important to note that the expression profiles reported here may reflect dynamic mRNA expression patterns, which might differ from protein levels. Thus, the use of mRNA probes rather than antibodies is a limitation of this study. However, the clear parallels between zebrafish data presented here and previously reported mouse data (Young et al., 1999; Young et al., 2003; Anderson et al., 2006) suggest that the discrepancy between mRNA and protein levels has only limited influence on our final conclusions.

Progenitor heterogeneity has been described during central nervous system development in other model systems, for example in the embryonic mouse telencephalon and during fruit fly nervous system development (Puelles et al., 2000; Guillemot, 2005; Flames et al., 2007; Kohwi and Doe, 2013). It is now clear from these and other studies that gene expression profiles of progenitors are instrumental in determining what types of progeny they generate (Guillemot, 2005; Kohwi and Doe, 2013). In the ENS, previous studies in mouse (Young et al., 1999; Young et al., 2003) and our work in zebrafish suggest that enteric progenitors also exhibit heterogeneity in their expression profiles, which may be correlated to the offspring they generate. In addition, heterogeneity among enteric progenitors has been shown based on proliferative characteristics, as the first two progenitor cells at the migratory wave front show higher proliferation than the following cohort of progenitors (Harrison et al., 2014). Future genetic live lineage tracing, more easily performed in zebrafish than mouse, will help determine whether the progenitor subpopulations we have identified in zebrafish contribute to the generation of different neuronal or glial subtypes.

Enteric subpopulations distinguished by combinatorial marker gene expression may generate distinct types of progeny

The functions of Phox2b, Sox10, and Ret are associated with a progenitor like state. Sox10 is necessary to maintain multipotency (Paratore et al., 2002; Kim et al., 2003; Bondurand et al., 2006; Bondurand and Sham, 2013)), it has been suggested that Phox2b is required for Ret expression (Pattyn et al., 1999), and Ret facilitates migration and proliferation (Durbec et al., 1996b; Taraviras et al., 1999; Shepherd et al., 2004; Heanue and Pachnis, 2008). Not only are these markers expressed early in enteric progenitors to maintain multipotency, migration, and proliferation, but they also play later roles in ENS development in regulating differentiation into enteric neuronal and glial cells. In mouse, Phox2b and Ret promote neuronal differentiation (Chalazonitis et al., 1998; Pattyn et al., 1999; Taraviras et al., 1999; Young et al., 1999; Young et al., 2003), whereas Sox10 is maintained in differentiated enteric glia (Young et al., 2003). Therefore, the differential expression of these genes might be indicative of a progenitor cell's developmental state as well as its fate. One might expect less restricted progenitors to express all three genes simultaneously to maintain multipotency and the ability to migrate and proliferate. Cells at the migratory wave front are both highly migratory and highly proliferative, suggesting that these cells require functions of all three progenitor markers. Consistent with this idea, our data indicate that progenitors in close proximity to the wave front are more likely to express all three markers at both 54 and 60 hpf. One might also predict that as progenitors start to differentiate into enteric neurons and glia, the number of cells expressing all three markers would decrease. In zebrafish, neurogenesis starts at around 54 hpf in the anterior intestine (Olden et al., 2008). Consistent with our prediction, we find that at 54 hpf and 60 hpf the largest subpopulation in the anterior intestine is *phox2bb*+/*ret*+/sox10− and the number of triple positive cells is markedly lower. The smaller number of triple positive cells in the anterior intestine may be a result of progenitor cells becoming fate-restricted. This idea is reflected by substantial colocalization between *phox2bb*, *ret*, and Elavl in the anterior intestine at 54, 60, and 84 hpf, suggesting that these progenitors are fated to generate neuronal progeny. It would be interesting to investigate whether these cells are still proliferative and/or migratory, as maintained expression of *ret* would imply that these cells are neuronal progenitors that are

still capable of migration and proliferation. In the anterior and mid region, we also observed a small *phox2bb*+/ret− subpopulation at 54 hpf. At 84 hpf, a substantial portion of the *phox2bb*+/Elavl+ cells in the anterior and midregion are also *ret*−. However, we also found that this population is very small at 60 hpf. These cells might represent a subgroup of neurons that does not maintain *ret* expression. Alternatively, *ret* might become downregulated when cells are no longer migratory, and then become upregulated again as the cells approach neuronal differentiation. If this is the case, we would expect to see more *ret*− cells in the anterior intestine at the very beginning of neuronal differentiation. The slight increase in *phox2bb*+/*ret*− cells anteriorly at 54 hpf relative to later into differentiation at 60 hpf is consistent with this proposed pattern. Additionally, a large proportion of *phox2bb*+/Elavl− cells in the posterior are *ret*− at 84 hpf, a late developmental time point at which one would expect progenitors to have finished their anterior to posterior migration.

Because Sox10 is a glial marker in mouse later in ENS development (Young et al., 2003), absence of *Sox10* expression is likely indicative of a neuronal fate (Young et al., 2002). Consistent with what is seen in mouse, we observed that *sox10* was not colocalized in Elavl + neuronal progenitors in zebrafish. Considering that *sox10* is thought to be maintained only in enteric glial cells (Young et al., 2003), the size of the *sox10*+ population at 54, and 60 hpf (approximately 20% at both time points) suggests that considerably fewer enteric progenitors are restricted to glial differentiation compared to neuronal differentiation at these time points (Table 6). This pattern is maintained at 84 hpf. At this time point we see few *sox10*+/*phox2bb*+ cells (about 18% in the anterior). Interestingly, we see some Elavl− cells that exhibit lower levels of *phox2bb* expression than most of the Elavl+ cells (data not shown). We speculate that these may be glial cells, since mouse data suggests Phox2bb expression is lower in glia relative to neurons (Corpening et al., 2008). This would suggest that there may even be two populations of enteric glia cells, one that is *sox10*+/*phox2bb*^{high} and one that is *sox10*−/*phox2bb*^{low}. Little is known about development of enteric glial cells in zebrafish, but it has been suggested that gliogenesis starts around 3 days post fertilization (dpf) (Hagstrom and Olsson, 2010). Therefore, the triple positive anterior subpopulation at 54 and 60 hpf likely consists of enteric progenitors that have not started to differentiate, but remain proliferative and migratory. The data described thus far suggest that enteric progenitors exist on a developmental spectrum according to their temporally and spatially dependent gene expression profiles.

Zebrafish enteric progenitor expression profiles parallel those of mouse

Interestingly, the most prominent *phox2b*, *ret*, and *sox10* colocalization patterns are comparable to the expression patterns seen during mouse ENS development. We have shown that generally *ret* colocalizes with *phox2bb*, however there is a small population of *phox2bb*+/*ret*− cells present, which is most prominent in the anterior intestine. Similarly, at later stages in mouse ENS development, between E12.5 and E16.5, the majority of Phox2b+ progenitors also express Ret, however a small number of Phox2b+ cells are Ret− (Young et al., 2003). Young et al. (2003) suggest that the Phox2b+/Ret− population is glial fated, since Ret has been shown to be downregulated in glial cells (Chalazonitis et al., 1998; Taraviras et al., 1999). However, one would expect to see Sox10 expression in such a population, and we have not observed a *phox2bb*+/*ret*−/*sox10*+ progenitor subpopulation at 54 or 60 hpf.

Instead, our results show that *sox10* is almost always coexpressed with *ret* and the number of *sox10*+/*ret*− cells is negligible. This result suggests that at 54 and 60 hpf, glial fated cells have not yet lost *ret* expression. Therefore, if *ret* expression is indeed lost in zebrafish enteric glia, then the gene must be turned off relatively late in the cell's development, or possibly only at the level of translation. We also observed a large *phox2bb*+/*ret*+/Elavl+ population of cells, which is in agreement with the idea that Ret promotes neuronal differentiation, and parallels the observation in mouse that Phox2b and Ret are coexpressed with PGP9.5, a pan-neuronal marker (Young et al., 2003). Finally, it has been shown in mouse that the intestine contains Ret+/Sox10+ and Ret+/Sox10− cells during development. We also observe these two expression profiles in zebrafish enteric progenitors at both 54 and 60 hpf. These parallels between mouse and zebrafish enteric progenitors point toward conserved progenitor expression profiles. Thus, zebrafish can be used to increase our general understanding of progenitor populations in the ENS by conducting high-throughput, lineage tracing studies using *in vivo* imaging techniques.

Conclusion

In summary, we have shown that zebrafish enteric progenitors make up a heterogeneous group of cells according to their gene expression patterns at early and late developmental stages during their migration along the intestine, which mirrors ENS data already established in mouse. In this study, we looked at three known progenitor marker genes, however, there are many more markers that could delineate subpopulations with greater specificity. We propose that the spatial and temporal distribution of marker coexpression describes four different types of enteric progenitors (Fig. 8): (1) actively migrating triple positive undifferentiated progenitors (*phox2bb*+/*ret*+/*sox10*+) at the migratory wave front, (2) potentially glial fated triple positive progenitors (*phox2bb*+/*ret*+/*sox10*+) in the anterior and mid regions of the intestine, (3) undifferentiated triple positive progenitors, and (4) double positive neuronally fated progenitors (*phox2bb*+/*ret*+/*sox10*-) in the anterior and mid regions of the intestine. The relative consistency among the subpopulations from 54 to 60 hpf suggests that each subgroup is replenished over time; as cells progress developmentally their expression patterns change, so that cells replace those that have entered another subpopulation maintaining the overall distribution across subgroups. By 84 hpf the more progenitor-like cells, those not expressing Elavl and expressing at least two of the three markers, are fewer and most prominent in the posterior end of the intestine, which suggests that subpopulations are eventually reduced as the ENS approaches maturation. Determining how other important markers colocalize with the three major subpopulations we have described might reveal progenitor subpopulations that are important for specifying individual neuronal or glial subtypes.

Experimental Procedures

Zebrafish

Tg(*phox2b*:EGFP)^{w37} (Nechiporuk et al., 2007) zebrafish were maintained in a laboratory breeding colony according to established protocols (Westerfield, 2000). After generation of the transgenic line, the *phox2b* gene was renamed to *phox2bb* (http://zfin.org/cgi-bin/

webdriver?MIval=aa-ZDB_home.apg), hence we refer to this transgenic line as Tg(*phox2bb*:EGFP). Embryos were allowed to develop at 28.5°C and staged by hours post fertilization according to morphological criteria (Kimmel et al., 1995). All experiments were carried out in accordance with animal welfare laws, guidelines and policies and were approved by the University of Oregon Institutional Animal Care and Use Committee.

Tissue Preparation

Tg(*phox2bb*:EGFP) embryos were fixed overnight in 4% paraformaldehyde/1 × Phosphate Buffered Saline (PBS) at 4°C, and then washed 3 × 10 minutes with 1 × PBS, incubated in 30% sucrose for 1 hour, and sectioned into 16μm cryosections. Sections were stored at −20°C.

Fluorescent RNA *in situ* hybridization and GFP immunohistochemistry

RNA probe generation was essentially performed as previously described (Ganz et al., 2012). For RNA *in situ* hybridization, sections were rehydrated for 15 minutes in phosphate-buffered saline (PBS) after defrosting at room temperature (RT), then incubated in 3% H_2O_2 for 30 minutes, rinsed in PBS, and washed in 0.3% TritonX (PBSTx) for 10 minutes. Subsequently, sections were incubated in antisense probe (200ng) in hybridization buffer overnight at 60°C. Information on the antisense *in situ* riboprobes is in the following references *phox2bb* (Elworthy et al., 2005) [NM_001014818.1, 352–2164 bp], *ret* (Bisgrove et al., 1997) [NM_181662.2, 110–3427 bp], and *sox10* (Honjo et al., 2008) [NM_131875.1, 805–2759 bp]. After hybridization, sections were washed 4 × 20 minutes in 50% formamide/1 × SSC at 62°C, followed by 4 × 15 minutes washes in 1 × MAB with 0.1% Tween-20 (MABTw). After incubating sections in 1% blocking buffer (Perkin Elmer, FP1136) for 1 hour at RT, they were incubated overnight at 4°C with anti-DIG antibody (Jackson ImmunoResearch, 200–002156, 1:1000 diluted in 1% Perkin Elmer (PE) blocking reagent). Following 6 × 10 minutes washes in MABTw, sections were incubated in HRP anti-mouse secondary antibody (Invitrogen, T20912, 1:1000) for 4 hours at RT. Sections were washed 6 × 10 minutes in MABTw, incubated in amplification reagent (Perkin Elmer, FP1050) for 10 minutes, and then incubated at RT for 25 minutes in cyanine 3 substrate (Perkin Elmer, FP1170, 1:1000 in amplification buffer). Sections were subsequently washed 6 × 10 minutes in PBSTx, incubated in 3% H_2O_2 for 1 hour at RT, and then incubated overnight at 4°C in HRP anti-DNP primary antibody (Perkin Elmer, FP1129, 1:1000) and mouse anti-GFP primary antibody (Living Color Clontech, 632381, 1:1000). Next, sections were washed 6 × 10 minutes in MABTw, rinsed for 10 minutes in amplification buffer, and then incubated for 25 minutes at RT in tyramide DNP (Perkin Elmer, FP1133, 1:50 in amplification buffer). After washing 6 × 10 minutes in MABTw, sections were blocked with 1% PE blocking buffer for 1 hour at RT, and then incubated overnight at 4°C in HRP anti-DNP (1:1000) alongside secondary anti-mouse IgG2a 488 (Life Technologies, A21131, 1:750). Following overnight incubation, sections were washed 6 × 10 minutes in MABTw. After washing sections in amplification buffer for 10 minutes, sections were incubated for 25 minutes at RT in cyanine 5 substrate (Perkin Elmer, FP1171 1:1000 in amplification buffer). Finally, sections were washed 6 × 10 minutes in PBS and mounted in 50% glycerol/PBS.

Immunohistochemistry

Immunohistochemistry for the pan-neuronal marker Elavl was performed following RNA *in situ* hybridization. Briefly, following overnight incubation at 4°C in primary antibody (Molecular probes A21271, 1:10,000), sections were washed 3 × 15 minutes in PBSTx, and incubated overnight at 4°C in secondary antibody (Life Technologies, A21146, 1:1000). Finally, sections were washed 3 × 15 minutes in PBSTx and mounted in 50% glycerol/PBS.

Image Acquisition

All images were acquired on a Zeiss Pascal confocal microscope (Carl Zeiss Microscopy, LLC, Thornwood, New York, USA) using a 40X water immersion objective. Image brightness and contrast was adjusted using Photoshop CS5 (Version 12.0, Adobe Systems, Inc., San Jose, CA, USA).

Experimental strategy

Use of sections of *phox2bb*:EGFP transgenic embryos facilitated accurate cell counts, because the cytoplasmic GFP fills cell bodies thus revealing average progenitor cell size and shape throughout colocalization analysis. At 36 hpf, migrating progenitors were imaged in four alternate sections at the migratory wave front (w) (Fig. 1A). At 54 and 60 hpf, all observations of marker gene colocalization were made in anterior, mid, and posterior intestine (Fig. 1B and C). The anterior-most region was defined by the most anterior appearance of GFP-positive progenitors around the intestinal epithelium and the posterior-most region by the most posterior section containing GFP-positive enteric progenitors. The three sections in between these points defined the mid region. Three alternating sections were imaged in each region. 36 hpf was chosen as an early time point for analysis because progenitors have just started to migrate along the intestine at this stage. 54 hpf and 60 hpf were chosen as relatively late time points, as enteric progenitors have migrated approximately one third (54 hpf) and mid-way (60 hpf) along the intestine at these stages, thus allowing for spatially distinct analysis time points. Additionally, neuronal differentiation starts at 54 hpf in the ENS (Olden et al., 2008), therefore progression from 54 hpf to 60 hpf to 84 hpf captures increased neuronal differentiation. Note that in Figs. 2–6 the dotted line between the measured values does not show the continuous percentage of coexpression between the anterior, mid, and posterior values, but is drawn to facilitate visualization of coexpression trends.

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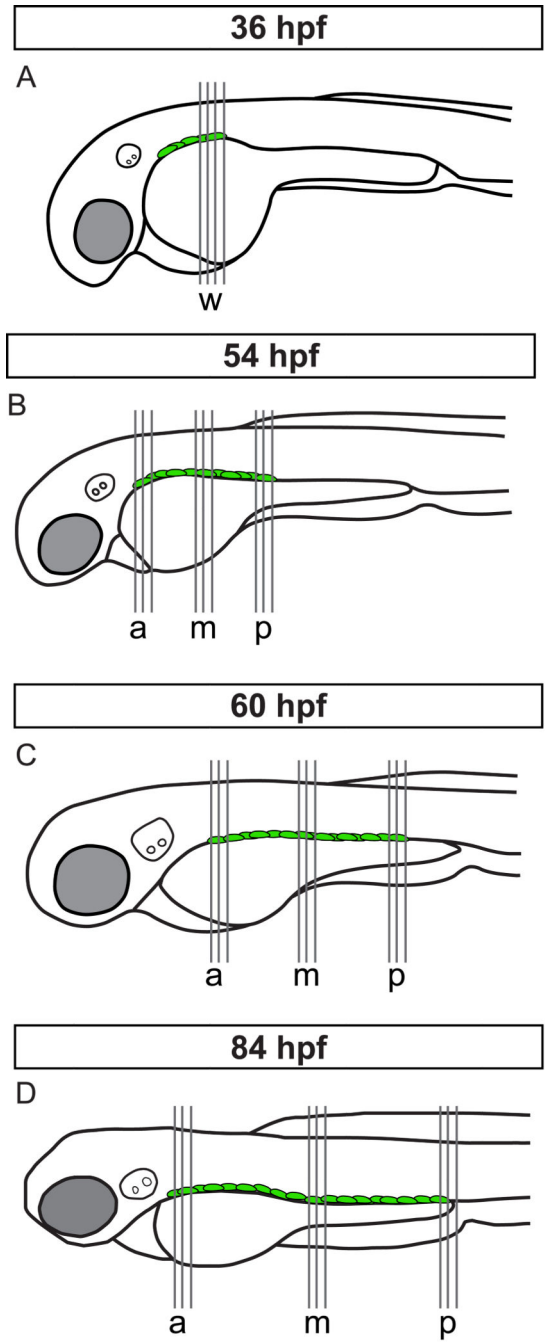

Figure 1. Experimental setup

(A) At 36 hpf, we imaged migrating progenitors in four alternate sections at the wave front (w). **(B)** At 54, **(C)** 60 hpf, and **(D)** 84 hpf we analyzed three regions, an anterior region (a) defined by the most anterior appearance of *phox2bb*:EGFP+ progenitor cells around the intestinal epithelium; a posterior region (p) defined to be the last section with *phox2bb*:EGFP+ enteric progenitors, and a mid region (m) defined as the middle three sections between a and p. Schematic side-views of zebrafish embryos at the ages indicated.

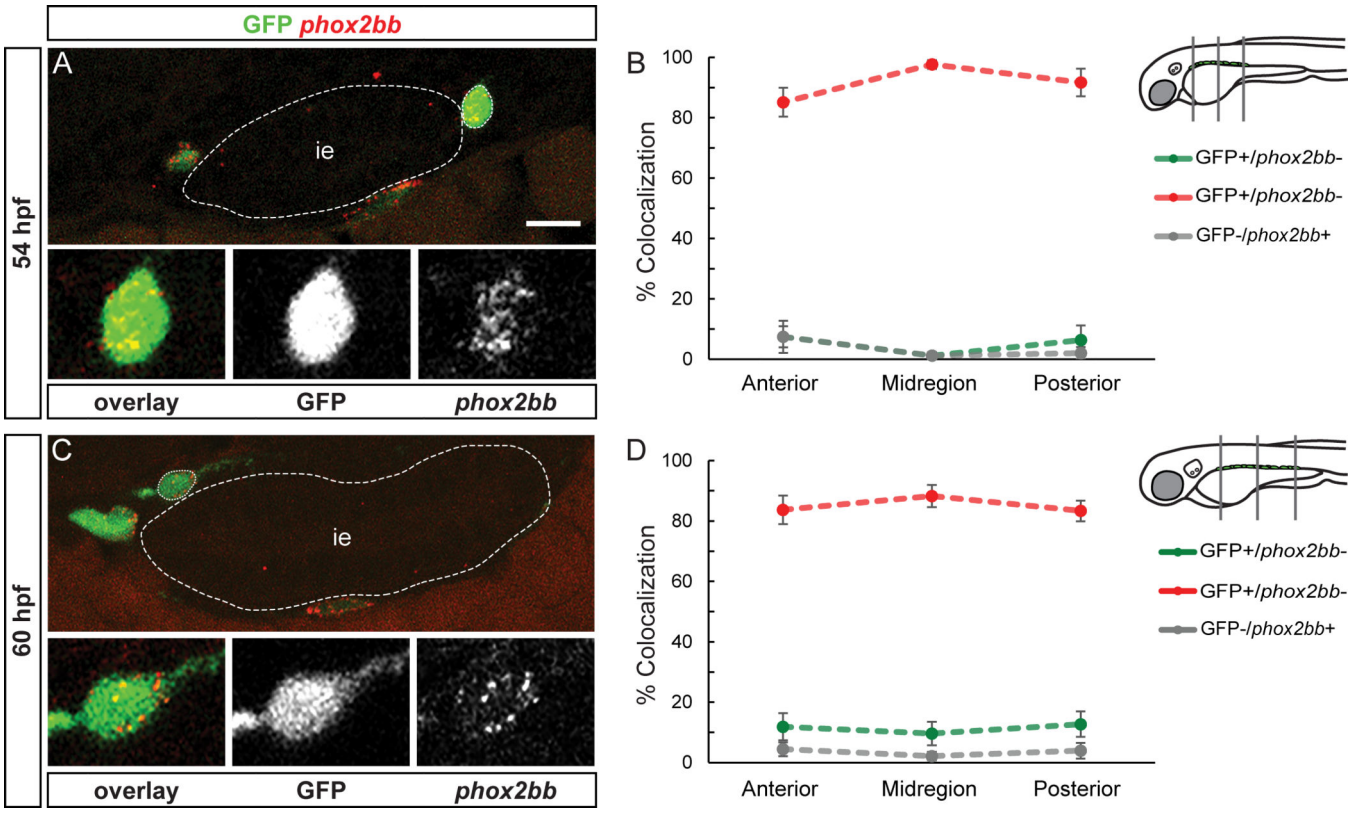

Figure 2. *phox2bb*:EGFP exhibits a high degree of colocalization with *phox2bb* mRNA

(A) and **(C)** show confocal images of cross-sections through the trunk. **(A)** At 54 and **(C)** 60 hpf *phox2bb* mRNA (red) largely colocalizes with *phox2bb*:EGFP (green). Insets show enlargements of outlined cells, overlay, *phox2bb*:EGFP, *phox2bb* mRNA (from left to right). **(B)** At 54 and **(D)** 60 hpf quantification in percent of *phox2bb*:EGFP and *phox2bb* mRNA colocalization in the three regions analyzed. Note that the dotted line between the measured values in **(B)** and **(D)** does not show the continuous percentage of coexpression between the anterior, mid and posterior values, but is drawn to facilitate visualization of coexpression trends. ie – intestinal epithelium Scale bar = 10μm.

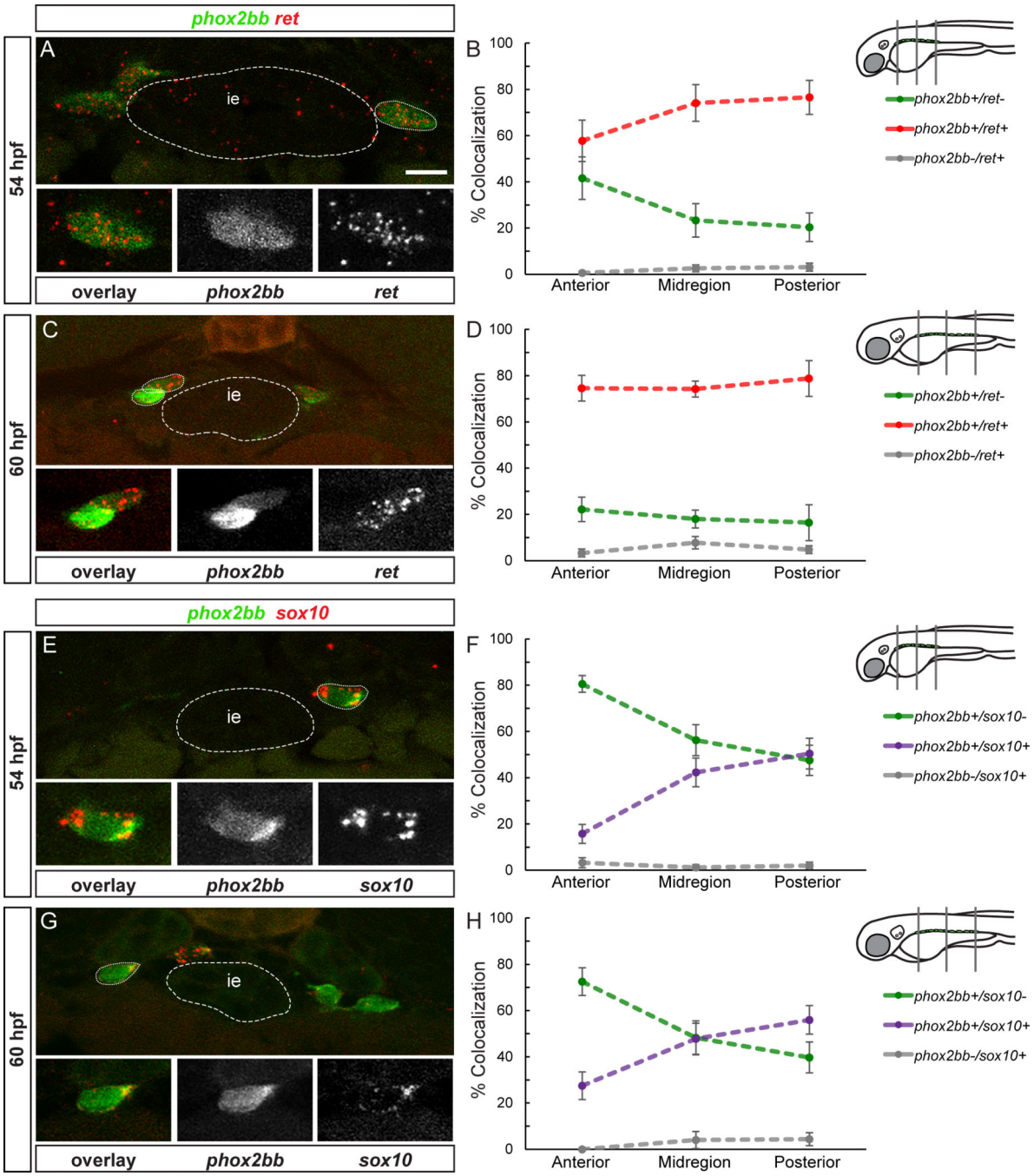

Figure 3. *sox10* and *ret* are differentially expressed in two different progenitor subpopulations

(A), (C), (E) and **(G)** show confocal images of cross-sections through the trunk. **(A)** At 54 and **(C)** 60 hpf *ret* mRNA (red) colocalizes with *phox2bb* (green). Insets show enlargements of outlined cells, overlay, *phox2bb*, *ret* mRNA (from left to right). Note that *phox2bb* refers to *phox2bb*:EGFP expression as described in the text. **(B)** At 54 and **(D)** 60 hpf quantification in percent of *phox2bb* and *ret* colocalization in the three regions analyzed. **(E)** At 54 and **(G)** 60 hpf *sox10* mRNA (red) partially colocalizes with *phox2bb* (green). Insets show enlargement of outlined cell, overlay, *phox2bb*, *sox10* mRNA (from left to right). **(F)**

At 54 and **(H)** 60 hpf quantification in percent of *phox2bb* and *sox10* mRNA colocalization in the three regions analyzed. The dotted line between the measured values does not show the continuous percentage of coexpression between the anterior, mid and posterior values, but is drawn to facilitate visualization of coexpression trends. ie – intestinal epithelium Scale bar = 10μm.

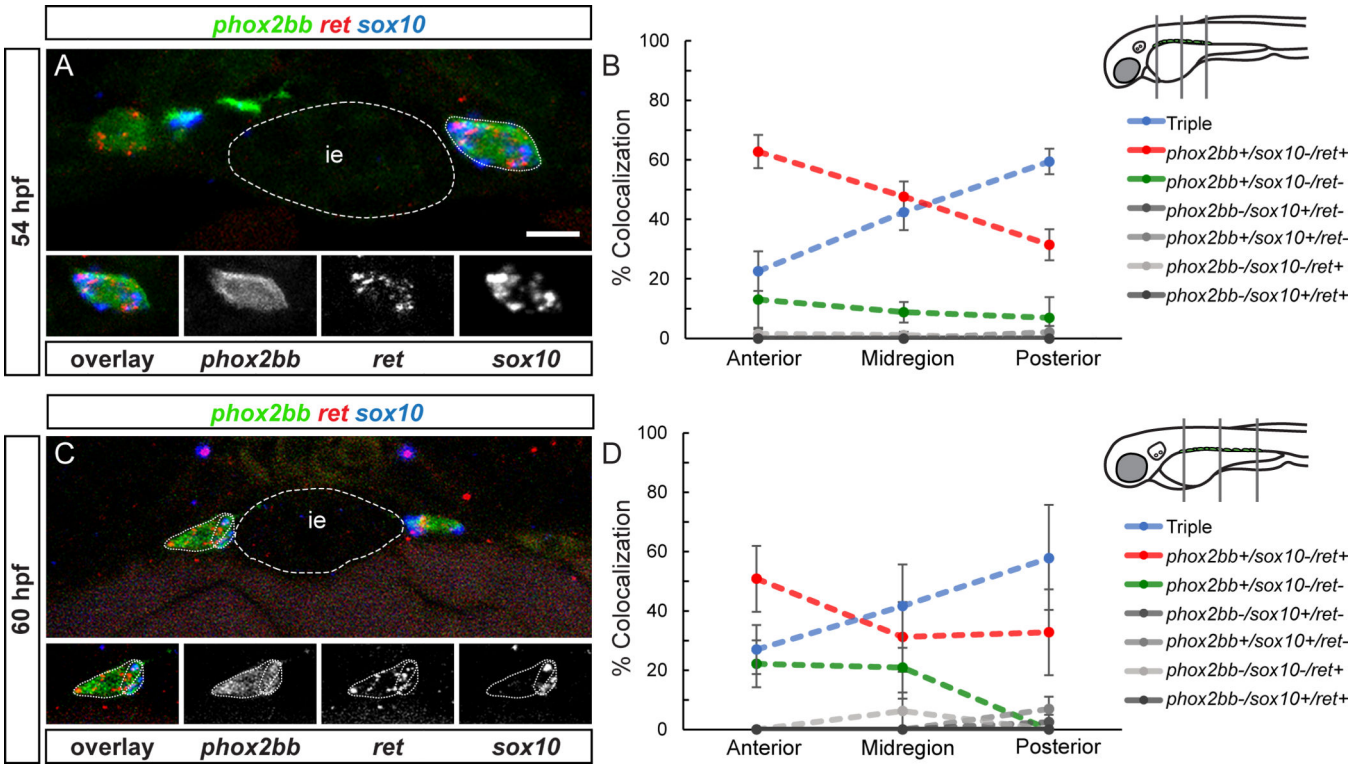

Figure 4. Colocalization of *ret* and *sox10* with *phox2bb* reveals a total of three enteric progenitor subpopulations

(A) and **(C)** show confocal images of cross-sections through the trunk. **(A)** At 54 and **(C)** 60 hpf *phox2bb* (green), *ret* mRNA (red) and *sox10* mRNA (blue) colocalize in subpopulations. Insets show close-up of outlined cell(s), overlay, *phox2bb*, *ret* mRNA, *sox10* mRNA (from left to right). Note that *phox2bb* refers to *phox2bb*:EGFP expression as described in the text. **(B)** At 54 and **(D)** 60 hpf quantification in percent of *phox2bb*, *ret* and *sox10* colocalization in the three regions analyzed. The dotted line between the measured values does not show the continuous percentage of coexpression between the anterior, mid and posterior values, but is drawn to facilitate visualization of coexpression trends. ie – intestinal epithelium Scale bar = 10μm.

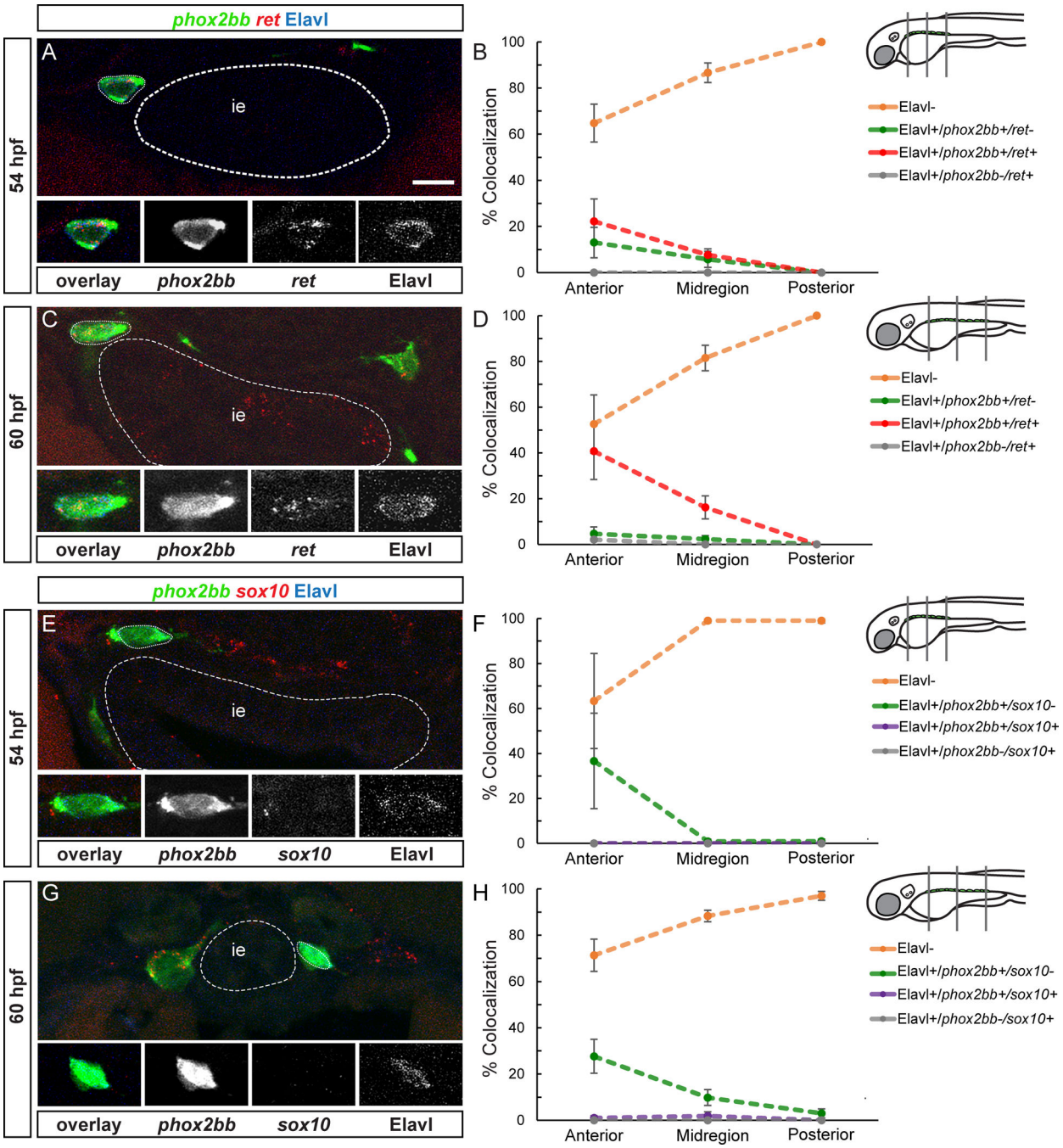

Figure 5. A small population of differentiating neurons express *phox2bb* and *ret*

(A), (C), (E) and **(G)** show confocal images of cross-sections through the trunk. **(A)** At 54 and **(C)** 60 hpf *ret* mRNA (red) colocalizes with Elavl (blue) and *phox2bb* (green). Insets show close-ups of outlined cell, overlay, *phox2bb*, *ret* mRNA, Elavl (from left to right). Note that *phox2bb* refers to *phox2bb*:EGFP expression as described in the text. **(B)** At 54 and **(D)** 60 hpf quantification in percent of *phox2bb*, *ret* and Elavl colocalization in the three regions analyzed. **(E)** At 54 and **(G)** 60 hpf *sox10* mRNA (red) does not colocalize with Elavl positive (blue), *phox2bb* positive cells. Insets show close-up of outlined cell, overlay,

phox2bb, *sox10* mRNA, Elavl (from left to right). **(F)** At 54 and **(H)** 60 hpf quantification in percent of *phox2bb*, *sox10* and Elavl colocalization in the three regions analyzed. The dotted line between the measured values does not show the continuous percentage of coexpression between the anterior, mid and posterior values, but is drawn to facilitate visualization of coexpression trends. ie – intestinal epithelium Scale bar = 10μm.

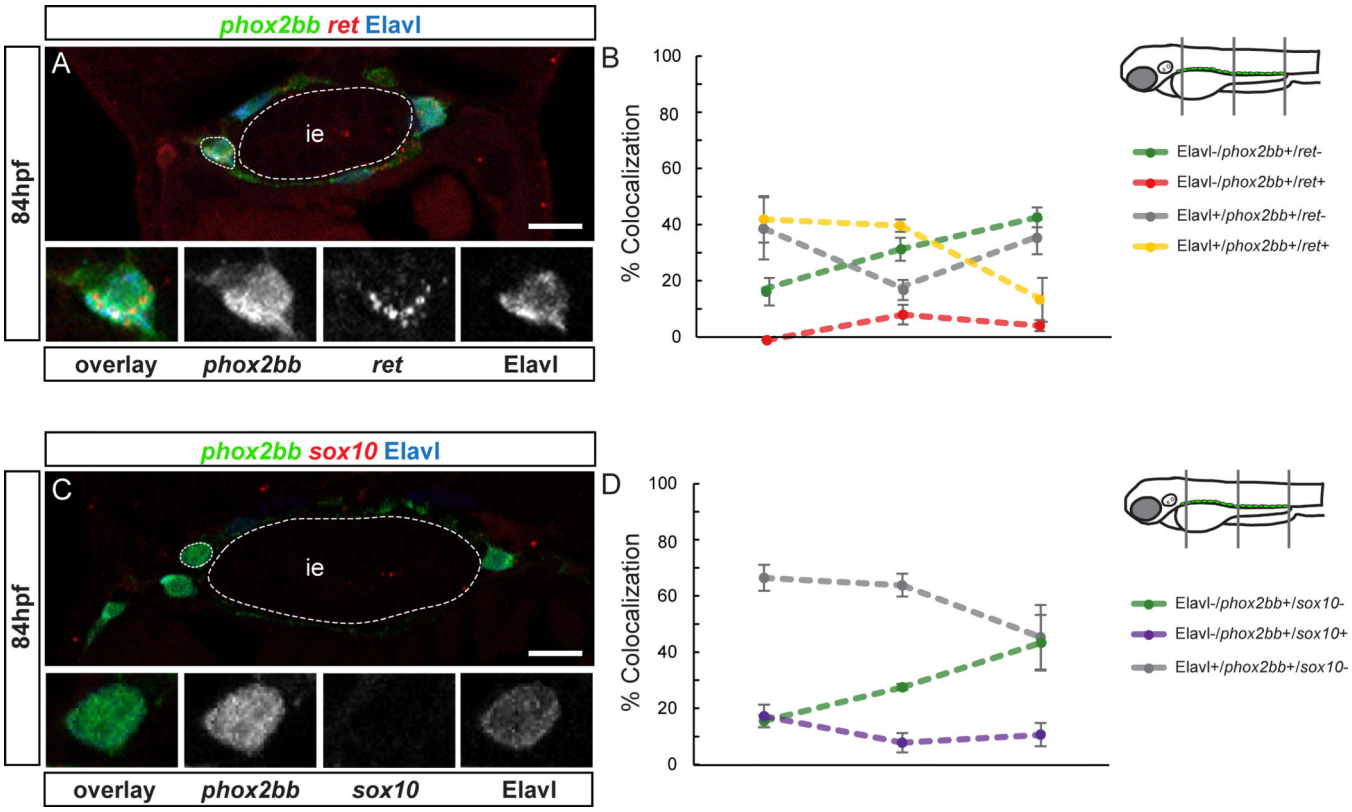

Figure 6. A small population of differentiating neurons express *phox2bb* and *ret* at later stages of neuronal differentiation

(A) and **(C)** show confocal images of cross-sections through the trunk. **(A)** At 84 hpf *ret* mRNA (red) colocalizes with Elavl (blue) and *phox2bb* (green). Insets show close-ups of outlined cell, overlay, *phox2bb*, *ret* mRNA, Elavl (from left to right). Note that *phox2bb* refers to *phox2bb*:EGFP expression as described in the text. **(B)** At 84 hpf quantification in percent of *phox2bb*, *ret* and Elavl colocalization in the three regions analyzed. **(C)** At 84 hpf *sox10* mRNA (red) does not colocalize with Elavl positive (blue), *phox2bb* positive (green) cells. Insets show close-up of outlined cell, overlay, *phox2bb*, *sox10* mRNA, Elavl (from left to right). **(D)** At 84 hpf quantification in percent of *phox2bb*, *sox10* and Elavl colocalization in the three regions analyzed. The dotted line between the measured values does not show the continuous percentage of coexpression between the anterior, mid and posterior values, but is drawn to facilitate visualization of coexpression trends. ie – intestinal epithelium Scale bar = 10μm.

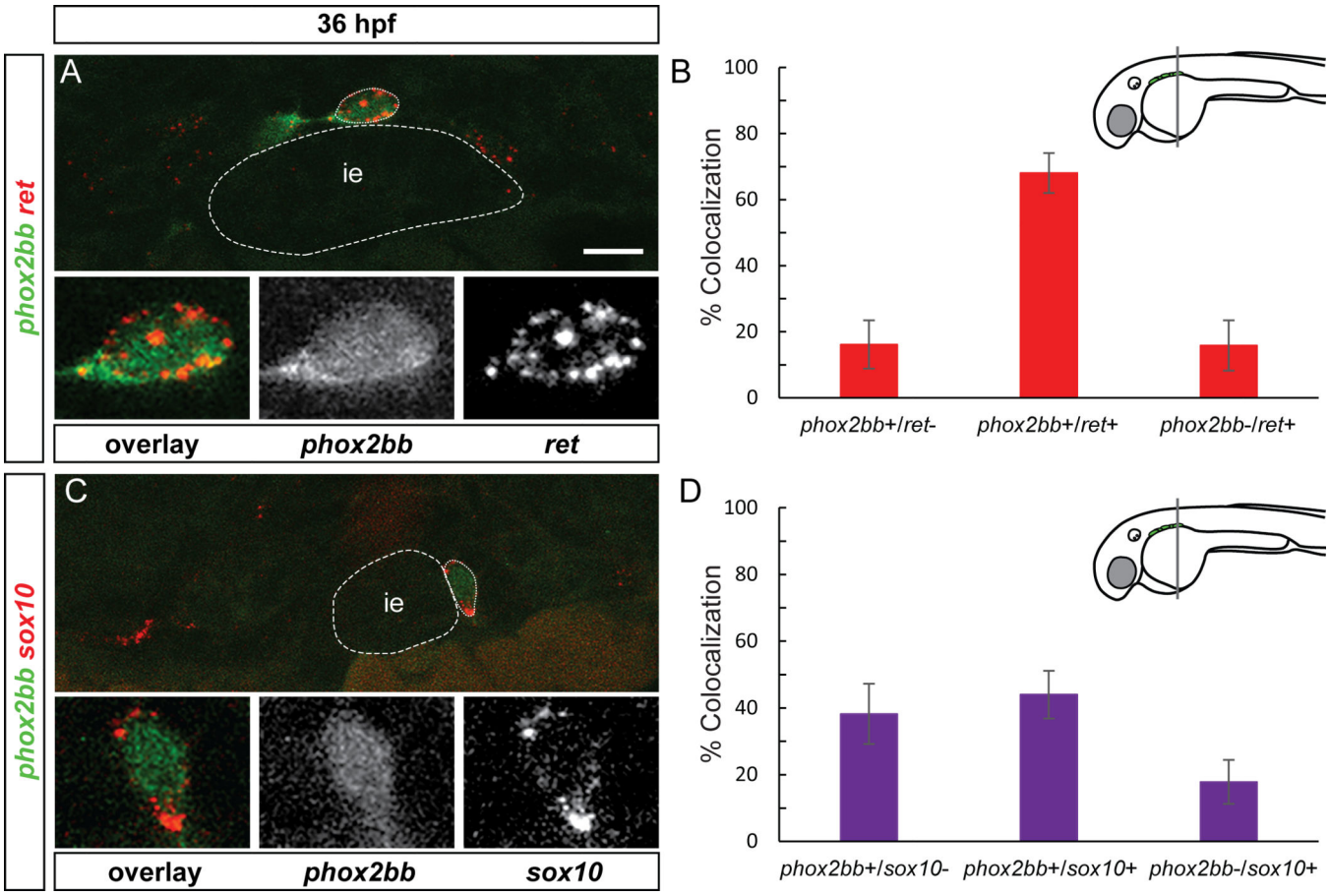

Figure 7. Enteric progenitor subpopulations are present at 36 hpf

(A) and **(C)** show confocal images of cross-sections through the trunk. At 36 hpf **(A)** *ret* mRNA (red) partially colocalizes with *phox2bb* (green). Insets show close-up of outlined cell, overlay, *phox2bb*, *ret* mRNA (from left to right). Note that *phox2bb* refers to *phox2bb*:EGFP expression as described in the text. **(B)** Quantification in percent of *phox2bb* and *ret* colocalization at the wave front. **(C)** *sox10* mRNA (red) partially colocalizes with *phox2bb* (green). Insets show close-up of outlined cell, overlay, *phox2bb*, *sox10* mRNA (from left to right). **(D)** Quantification in percent of *phox2bb* and *sox10* colocalization at the wave front. ie – intestinal epithelium Scale bar = 10μm.

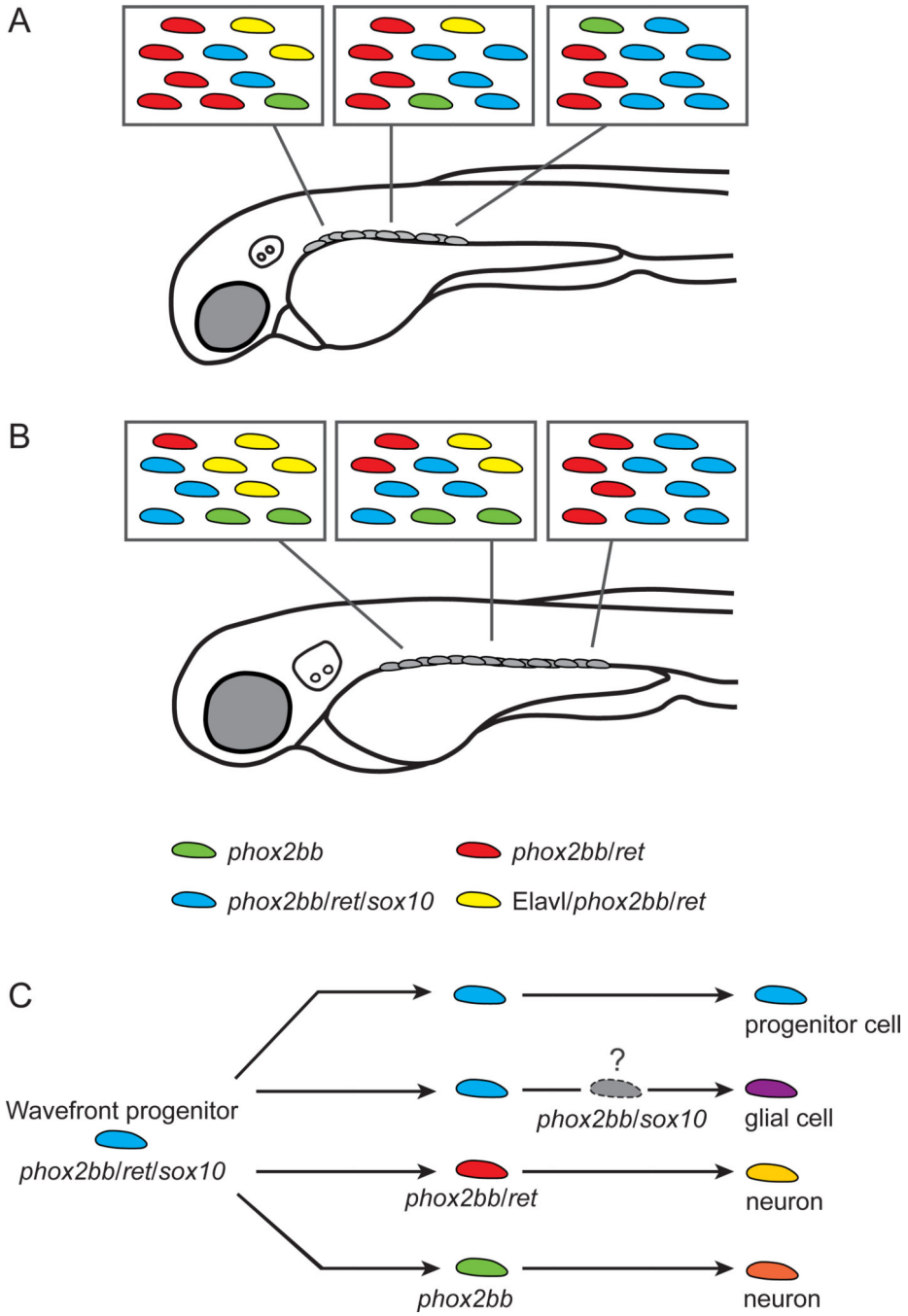

Figure 8. Summary of proposed enteric subpopulations during zebrafish ENS development

(A) At 54 and **(B)** 60 hpf enteric progenitors constitute a heterogeneous population of cells based on spatio-temporally dynamic expression of *phox2bb*, *ret*, and *sox10*. Elavl+ differentiating neurons express *ret* and *phox2bb*, but do not express *sox10*. **(C)** Proposed model for types of enteric progenitor subpopulations. Expression profiles may be indicative of a progenitor's developmental state and its fate. Note that the *phox2bb* and the *phox2bb/ret*

positive progenitor populations are likely to give rise to two different suites of neuronal subtypes.

Table 1

Percent colocalization of *phox2bb*:EGFP and *phox2bb* mRNA in enteric progenitors at 54 and 60 hpf

	54 hpf			60 hpf		
	Anterior	**Midregion**	**Posterior**	**Anterior**	**Midregion**	**Posterior**
GFP+/*probe*−	7.4 ± 5.3	1.2 ± 1.2	6.3 ± 4.8	11.9 ± 4.5	9.6 ± 3.9	12.7 ± 4.2
GFP+/*probe*+	85.1 ± 4.8	97.7 ± 1.4	91.7 ± 4.6	83.7 ± 4.7	88.3 ± 3.7	83.4 ± 3.5
GFP−/probe+	7.4 ± 3.5	1.2 ± 1.2	2.0 ± 2.0	4.4 ± 2.3	2.1 ± 1.4	3.9 ± 2.6

Variation corresponds to plus or minus the standard error of the mean (54 hpf: n=4; 60 hpf n=9). Probe refers to *phox2bb* mRNA.

Table 2

Percent colocalization of *phox2bb* and *ret* in enteric progenitors at 54 and 60 hpf

	54 hpf			60 hpf		
	Anterior	**Midregion**	**Posterior**	**Anterior**	**Midregion**	**Posterior**
phox2bb+/*ret*−	41.6 ± 9.2	23.3 ± 7.2	20.4 ± 6.2	22.2 ± 5.3	18.0 ± 3.9	16.4 ± 7.7
phox2bb+/*ret*+	57.8 ± 9.0	74.1 ± 7.9	76.5 ± 7.3	74.6 ± 5.6	74.2 ± 3.4	78.8 ± 7.7
phox2bb−/*ret*+	0.6 ± 0.6	2.6 ± 1.5	3.1 ± 1.7	3.3 ± 1.7	7.8 ± 2.7	4.8 ± 1.6

Variation corresponds to plus or minus the standard error of the mean (54 hpf: n=8; 60 hpf n=10). Note that *phox2bb* refers to *phox2bb*:EGFP expression.

Table 3

Percent colocalization of *phox2bb* and *sox10* in enteric progenitors at 54 and 60 hpf

	54 hpf			60 hpf		
	Anterior	**Midregion**	**Posterior**	**Anterior**	**Midregion**	**Posterior**
phox2bb+/*sox10*–	80.5 ± 3.6	56.2 ± 6.7	47.5 ± 6.6	72.5 ± 6.0	48.2 ± 7.3	39.7 ± 6.7
phox2bb+/*sox10*+	15.7 ± 4.1	42.3 ± 6.2	50.5 ± 6.6	27.5 ± 6.0	47.8 ± 6.7	55.6 ± 6.1
phox2bb–/*sox10*+	3.2 ± 2.2	1.2 ± 1.2	2.0 ± 1.5	0	4.0 ± 3.7	4.3 ± 2.8

Variation corresponds to plus or minus the standard error of the mean (54 hpf: n=10; 60 hpf n=13). Note that *phox2bb* refers to *phox2bb*:EGFP expression.

Table 4

Percent colocalization of *phox2bb*, *ret*, and *sox10* in enteric progenitors at 54 and 60 hpf

	54 hpf			60 hpf		
	Anterior	**Midregion**	**Posterior**	**Anterior**	**Midregion**	**Posterior**
phox2bb+/ret−/sox10−	13.0 ± 9.5	8.8 ± 3.4	6.9 ± 6.9	22.2 ± 7.9	20.9 ± 10.5	0
phox2bb+/ret+/sox10−	62.8 ± 5.6	47.7 ± 5.1	31.5 ± 5.2	50.9 ± 11.1	31.2 ± 11.7	32.8 ± 14.5
phox2bb−/ret+/sox10−	1.6 ± 1.6	1.1 ± 1.1	0	0	6.5 ± 6.5	0
phox2bb+/ret−/sox10+	0	0	2.1 ± 2.1	0	0	6.9 ± 4.2
phox2bb−/ret−/sox10+	0	0	0	0	0	2.5 ± 2.5
phox2bb−/sox10+/ret+	0	0	0	0	0	0
phox2bb+/ret+/sox10+	22.6 ± 6.6	42.4 ± 6.0	59.5 ± 4.3	27.0 ± 8.2	41.6 ± 14.1	57.8 ± 17.8

Variation corresponds to plus or minus the standard error of the mean (54 hpf: n=4; 60 hpf n=4). Note that *phox2bb* refers to *phox2bb*:EGFP expression.

Table 5

Percent colocalization of *phox2bb*, *ret*, and Elavl at 54 and 60 hpf

	54 hpf			60 hpf		
	Anterior	**Midregion**	**Posterior**	**Anterior**	**Midregion**	**Posterior**
Elavl−	64.8 ± 8.2	86.7 ± 4.2	100.0 ± 0.0	52.6 ± 12.8	81.5 ± 5.6	100.0 ± 0.0
Elavl+/*phox2bb*+/*ret*−	13.0 ± 6.6	5.7 ± 3.5	0	4.6 ± 3.0	2.3 ± 1.5	0
Elavl+/*phox2bb*+/*ret*+	22.2 ± 9.8	7.6 ± 2.6	0	40.7 ± 12.3	16.2 ± 5.1	0

Variation corresponds to plus or minus the standard error of the mean (54 hpf: n=5; 60 hpf n=6). Note that *phox2bb* refers to *phox2bb*:EGFP expression.

Table 6

Percent colocalization of *phox2bb*, *sox10*, and Elavl at 54 and 60 hpf

	54 hpf			60 hpf		
	Anterior	**Midregion**	**Posterior**	**Anterior**	**Midregion**	**Posterior**
Elavl−	63.3 ± 21.2	99.1 ± 0.9	99.1 ± 1.0	71.3 ± 7.0	88.3 ± 2.5	97.0 ± 1.9
Elavl+/*phox2bb*+/*sox10*−	36.7 ± 21.2	0.9 ± 0.9	1.0 ± 1.0	27.7 ± 7.3	9.8 ± 3.4	3.0 ± 1.9
Elavl+/*phox2bb*+/*sox10*+	0	0	0	1.0 ± 1.0	1.8 ± 1.8	0

Variation corresponds to plus or minus the standard error of the mean (54 hpf: n=5; 60 hpf n=5). Note that *phox2bb* refers to *phox2bb*:EGFP expression.

Table 7

Percent colocalization of *phox2bb*, *ret*, and Elavl at 84 hpf

	84 hpf		
	Anterior	**Midregion**	**Posterior**
phox2bb+/*ret*−/Elavl−	17.6 ± 5.6	32.0 ± 4.6	43.6 ± 4.0
phox2bb+/*ret*+/Elavl−	0.0 ± 0.0	9.3 ± 4.0	5.3 ± 2.3
phox2bb+/*ret*−/Elavl+	39.6 ± 12.6	18.2 ± 4.1	36.5 ± 7.0
phox2bb+/*ret*+/Elavl+	42.8 ± 9.4	40.5 ± 2.5	14.7 ± 8.9

Variation corresponds to plus or minus the standard error of the mean (n=4). Note that *phox2bb* refers to *phox2bb*:EGFP expression.

Table 8

Percent colocalization of *phox2bb*, *sox10*, and Elavl at 84 hpf

	84 hpf		
	Anterior	**Midregion**	**Posterior**
phox2bb+/*sox10*−/Elavl−	15.7 ± 2.1	27.8 ± 1.1	43.7 ± 9.9
phox2bb+/*sox10*+/Elavl−	17.5 ± 4.1	8.0 ± 3.5	10.7 ± 4.2
phox2bb+/*sox10*−/Elavl+	66.9 ± 4.7	64.1 ± 4.1	45.6 ± 11.6

Variation corresponds to plus or minus the standard error of the mean (n=4). Note that *phox2bb* refers to *phox2bb*:EGFP expression.

Table 9

Percent colocalization of *phox2bb*, *sox10*, or *ret* at 36 hpf

	ret	***sox10***
	wave front	**wave front**
phox2bb+/probe−	16.4 ± 7.3	38.2 ± 9.1
phox2bb+/probe+	68.0 ± 6.0	44.0 ± 7.2
phox2bb−/probe+	15.8 ± 7.6	17.8 ± 6.6

Variation corresponds to plus or minus the standard error of the mean (*ret* n=6; *sox10* n=6). Probe refers to either *ret* mRNA or *sox10* mRNA. Note that *phox2bb* refers to *phox2bb*:EGFP expression.