# Phox2b function in the enteric nervous system is conserved in zebrafish and is *sox10*-dependent

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## Abstract

Zebrafish lacking functional *sox10* have defects in non-ectomesenchymal neural crest derivatives including the enteric nervous system (ENS) and as such provide an animal model for human Waardenburg Syndrome IV. Here, we characterize zebrafish *phox2b* as a functionally conserved marker of the developing ENS. We show that morpholino-mediated knockdown of Phox2b generates fish modeling Hirschsprung disease. Using markers, including *phox2b*, we investigate the ontogeny of the *sox10* ENS phenotype. As previously shown for melanophore development, ENS progenitor fate specification fails in these mutant fish. However, in addition, we trace back the *sox10* mutant ENS defect to an even earlier time point, finding that most neural crest cells fail to migrate ventrally to the gut primordium.
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## 1. Introduction

A diverse range of cell-types including pigment cells, craniofacial cartilage and most of the peripheral nervous system are derived from the neural crest. This mixed population of progenitors originates from the dorsal neural tube and includes cells showing the multipotency and self-renewal capacity of stem cells (Le Douarin and Kalcheim, 1999; Stemple and Anderson, 1992). Neural crest cells undertake extensive migration to their final locations and undergo proliferation and progressive fate restriction as they do so (Baroffio et al., 1991; Weston, 1991). The enteric nervous system (ENS) is a very complex neural crest derivative with an extensive network of diverse neurons and glia (Gershon, 1997). In amniotes ENS progenitors have a dual origin from neural crest cells of both the vagal and sacral axial levels and undergo the most extensive migration of any neural crest cell population in order to populate the whole gut (Taraviras and Pachnis, 1999). In a first (pre-enteric) phase of ENS development, vagal neural crest cells migrate ventrally to the anterior most region of the gut primordium (Taraviras and Pachnis, 1999). In the second (enteric) phase, these cells then migrate caudally down the developing gut, contributing most of the ENS along the whole gut from foregut to hindgut. Sacral neural crest cells migrate ventrally to the hindgut, and then migrate rostrally, making a small contribution, predominantly in the hindgut. In zebrafish, ENS progenitors first appear in the vagal region and then display a rostrocaudal gradient of ENS progenitor migration and differentiation (Dutton et al., 2001; Kelsh and Eisen, 2000; Shepherd et al., 2001). It is not known whether there is a sacral contribution in fish.

In humans, defects in neural crest derivatives are known collectively as neurocristopathies (Bolande, 1974). Of these, Hirschsprung's disease (HSCR), characterized by megacolon due to terminal aganglionosis of the gut, is one

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of the most common, with an estimated incidence of 1:5000 live births (Badner et al., 1990). The genetics of HSCR is complex, but a major contribution comes from genes involved in RET receptor tyrosine kinase and endothelin receptor-B (EDRNB) signaling (Amiel et al., 1996; Angrist et al., 1995; Auricchio et al., 1996; Hofstra et al., 2000; Kusafuka et al., 1997). In many cases, HSCR forms part of a syndrome, with diverse-associated features. Where HSCR is associated with sensorineural deafness and pigmentation defects, it is categorized as Waardenburg Syndrome IV (WS4; Waardenburg–Shah syndrome). The identification of candidate genes for HSCR and WS involved mapping of the human disease loci and characterization of mouse mutants showing defects in ENS development (Angrist et al., 1993; Dow et al., 1994; Farrer et al., 1992; Foy et al., 1990; Luo et al., 1993; Lyonnet et al., 1993; Pattyn et al., 1999; Van de Putte et al., 2003). This work has revealed distinct roles for these genes in neural crest development. *Ret* mutant mice lack the ENS below the esophagus. Ret and Ednrb together stimulate proliferation of ENS progenitors (Barlow et al., 2003) with Ret signaling being critical for their rostrocaudal migration (Natarajan et al., 2002; Schuchardt et al., 1994; Young et al., 2001). Morpholino antisense knockdown studies in zebrafish have shown the evolutionary conservation of Ret signaling function in ENS development (Shepherd et al., 2001, 2004).

Heterozygous mutations of the Sry-related HMG-domain transcription factor SOX10 are associated with WS4 (Kuhlbrodt et al., 1998b; Pingault et al., 1998a). Similar phenotypes are seen in mouse heterozygous for presumed null alleles (Herbarth et al., 1998; Lane and Liu, 1984; Pingault et al., 1998b; Southard-Smith et al., 1998, 1999). Homozygotes for zebrafish *sox10* (also known as *colourless*, *cls*) or mouse *Sox10* mutations have a complete absence of the ENS, glia and pigment cells and a strong reduction of sensory neurons, although craniofacial derivatives are unaffected (Dutton et al., 2001; Herbarth et al., 1998; Kelsh and Eisen, 2000; Pingault et al., 1998b; Southard-Smith et al., 1998).

The role of Sox10 has been analyzed in several NC lineages. Work in mice has shown that peripheral glia require *Sox10* for initial specification and also later to regulate the expression of terminal differentiation genes (Britsch et al., 2001; Paratore et al., 2001; Sonnenberg-Riethmacher et al., 2001). Strong *Sox10* expression persists in glia both in mice and zebrafish (Dutton et al., 2001; Kelsh et al., 2000). A failure of sympathetic neuron specification has been noted in mouse *Sox10* mutants, where *Mash1* and *Phox2b* induction failed in autonomic precursors. However, in contrast to glia, sympathetic neuronal differentiation requires subsequent *Sox10* downregulation (Kim et al., 2003). In this lineage, Sox10 acts to maintain NC cells in an undifferentiated stem cell state. In the melanocyte lineage, Sox10 is required solely to induce expression of the master regulator transcription factor, Mitf (Nacre[mitfa] in zebrafish) (Bondurand et al., 2000; Dutton et al., 2001;

Elworthy et al., 2003; Lee et al., 2000; Potterf et al., 2000, 2001; Verastegui et al., 2000). Melanocyte fate specification occurs in the premigratory NC and subsequently *sox10* expression is downregulated in this lineage (Dutton et al., 2001). Work in *Xenopus* has suggested that *Sox10* has a role in specification of neural crest from the ectoderm (Honore et al., 2003). In contrast in zebrafish or mouse *sox10* mutants there is no reduction in premigratory NC cells (Dutton et al., 2001; Kelsh and Eisen, 2000; Britsch et al., 2001). It is conceivable that other Group E *Sox* genes (e.g. *sox9b* in zebrafish; Chiang et al., 2001; Li et al., 2002) are functionally redundant with *sox10* during neural crest specification in fish and mammals.

The basis for the defect in the ENS of *sox10* homozygous mutants is poorly understood. *Sox10* is expressed in both pre-enteric and enteric phases of ENS development (Dutton et al., 2001; Herbarth et al., 1998; Kuhlbrodt et al., 1998a; Southard-Smith et al., 1998). In zebrafish, both Hu-expressing enteric neurons and *ret*-positive progenitors are severely reduced in 3–5 days post fertilization (dpf) *sox10* larvae, as are GFAP-expressing enteric glia (Kelsh and Eisen, 2000). Although the enteric defect has been traced back to 60 h post fertilization (hpf) using *sox10* as a marker of enteric precursors (Dutton et al., 2001), the time and location when ENS development fails has not been defined. In mouse, enteric ganglia are completely absent from *Sox10^{Dom/Dom}* mice and early enteric neuron differentiation markers are absent from the gut primordium at embryonic day (e)12.5, consistent with the absence of *sox10* expression at e14.5 (Herbarth et al., 1998; Kapur, 1999). These studies, in conjunction with observations of increased cell death on the ventral medial migration pathway at e10, led Kapur to suggest that ENS development fails prior to gut colonization, probably during early neural crest cell migration (Kapur, 1999).

In mouse, Phox2b is a critical transcription factor required for autonomic neuron specification (Lo et al., 1999; Pattyn et al., 1999). In *Phox2b* null homozygotes, ENS progenitors reach the anterior gut primordium, but fail to migrate caudally or to express early enteric neuronal markers, and subsequently die by apoptosis between e10 and e13.5 (Pattyn et al., 1999). At least in sympathetic neuron progenitors in mice, *Sox10* is required for the expression of *Phox2b* (Kim et al., 2003). Furthermore, *Phox2b* is required for *Ret* expression in the ENS (Pattyn et al., 1999). In principle, therefore, the ENS defects in *Sox10* mutant embryos might be attributable to absence of enteric neuron fate specification resulting from a failure of transcriptional activation of *Phox2b*. Here we use molecular markers to determine when ENS defects are first apparent in *sox10* mutant zebrafish. We clone a zebrafish *phox2b* orthologue, show that its function in enteric neuron development is conserved and then demonstrate that *phox2b* expression is entirely absent in ENS progenitors in *sox10* mutant zebrafish. Thus, enteric neuron specification fails in zebrafish *sox10* mutants. Additionally, we provide

evidence that most ENS progenitors in *sox10* mutants fail to migrate to the gut primordium, suggesting that ENS development is defective at a very early stage, prior to enteric neuron specification.

## 2. Results

### 2.1. Isolation of zebrafish Phox2b

We required molecular markers to characterize the earliest aspects of the ENS defect in *sox10* mutant embryos. Established zebrafish ENS markers include the pan-neuronal marker Hu, and also *ret* and *sox10*. To provide a possible additional marker, we isolated a zebrafish *Phox2b* homologue. Sequence comparisons with mammalian *Phox2* genes identify this zebrafish sequence as an orthologue of *Phox2b* and not *Phox2a* (Fig. 1A), which we name *phox2b*. Using radiation hybrid mapping with the LN54 panel we showed that *phox2b* is located on Linkage Group 14, 12.67 cR from Z6847 (Fig. 1B). We note an interesting synteny involving *phox2b* and four other zebrafish genes on Linkage Group 14 and their human homologues on human

Chromosome 4 (Fig. 1B). This synteny provides further support that *phox2b* is a true zebrafish orthologue of *PMX2B*.

### 2.2. Zebrafish phox2b is expressed in the CNS and epibranchial ganglia

In situ hybridization analysis showed that *phox2b* was first expressed in the developing CNS in a pattern identical to that described by Guo et al., (1999a) for an unpublished zebrafish *phox2b* gene (data not shown). We assume that the *phox2b* described here corresponds to the same gene. In situ hybridization at 60 hpf revealed prominent *phox2b* expression in the epibranchial ganglia at the proximal end of the arches, consistent with previous descriptions in chick (Begbie et al., 2002) (Fig. 2A). Wild-type embryos also showed a few *phox2b*-expressing cells scattered along the arches. The identity of these cells is unclear; based on their spatiotemporal pattern they seem unlikely to correspond to the arch-associated catecholaminergic cells identified by (Guo et al., 1999b). We asked whether these cells are also seen in *sox10* mutants. Interestingly, epibranchial *phox2b*
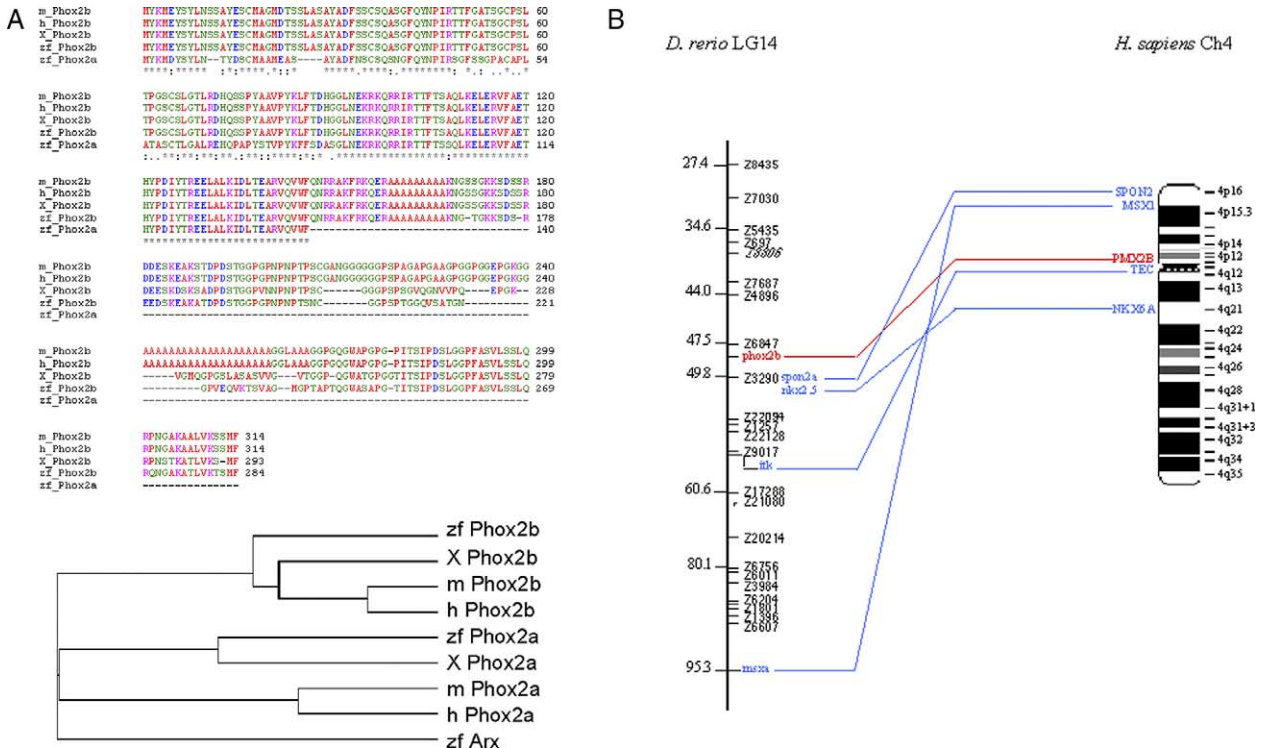


Fig. 1. Identification and mapping of a zebrafish *phox2b* orthologue. (A) Alignment and cladogram of vertebrate Phox protein sequences generated using CLUSTALW. zf_Phox2b is zebrafish Phox2b gi_56900853; X_Phox2b is *Xenopus* Phox2b gi_38425329; m_Phox2b is mouse Phox2b gi_2632151; h_Phox2b is human Phox2b gi_12707580; zf_Phox2a is zebrafish Phox2a (Guo et al., 1999a); X_Phox2a is *Xenopus* Phox2a gi_38425325; m_Phox2a is mouse Phox2a gi_6679399; h_Phox2a is human Phox2a gi_27371330, zf_Arx is zebrafish Arx gi_18858285. Residues AVFPMILW are shown red, DE blue, RK magenta, STYHCNGQ green and others gray. Residues identical in all sequences are marked *, conserved substitutions: and semi-conserved substitutions. (B) Zebrafish *phox2b* (red) maps to Linkage Group 14, 37.11 cR from z6847 marker. We note a synteny between zebrafish LG14 and human chromosome 4, as indicated by lines between syntenic homologues. The human chromosome figure was adapted from the NCBI website (http://www.ncbi.nlm.nih.gov/mapview/maps. cgi?ORG=human&CHR=4) with permission.
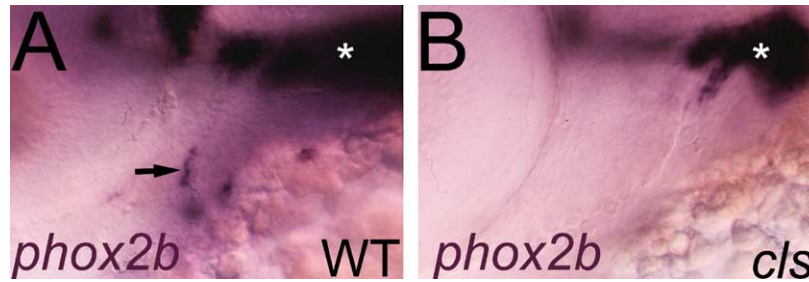
Fig. 2. Expression of *phox2b* in the zebrafish branchial arches. Lateral views of whole-mount *phox2b* mRNA in situ hybridization in 60 hpf embryos shows strong expression in epibranchial ganglia (asterisk) in both wild-types (A) and *sox10* mutant embryos (B). *phox2b* expression was also seen in isolated cells associated with the more ventral branchial arches (arrow, A), but these were absent in *sox10* mutants (B).

expression, but not that in the scattered arch-associated cells, was detectable in *sox10* mutants (Fig. 2B).

### 2.3. Zebrafish phox2b is expressed in the developing ENS

In mice *Phox2b* is a valuable marker of the developing ENS. To confirm and extend our initial observations of *phox2b* in wild-type ENS progenitors (Shepherd et al., 2004), we compared in detail the expression pattern of *phox2b* with that of established zebrafish ENS markers. We used whole-mount immunofluorescence with an anti-Hu antibody to define the time of neuronal differentiation in the developing ENS, focusing on the hindgut region (i.e. posterior trunk). Consistent with our previous observations in sectioned and whole-mount material (Kelsh and Eisen, 2000; Shepherd et al., 2004), numerous Hu-expressing neurons were present at 120 hpf, fewer at 100 hpf and none at 75 hpf (Fig. 3F–K). Enteric precursors can be shown to be present in the hindgut at this and earlier stages using *ret* and *sox10* mRNA in situ hybridization (Bisgrove et al., 1997; Dutton et al., 2001; Marcos-Gutierrez et al., 1997). As previously reported, at 60 hpf *ret* and *sox10* expressing enteric precursors in the hindgut formed two parallel rows lateral to the developing gut tube (Fig. 3A,C,E). Strikingly, at this stage *phox2b* is expressed in this same pattern (Fig. 3B,D). Thus, we conclude that *phox2b* expression in the developing ENS precursors is evolutionarily conserved in zebrafish. Careful comparison of the distal extent of marker expressing cells in the hindgut at 60 hpf demonstrated that *sox10* expression consistently extended almost to the anus whereas *phox2b* and *ret* expression only extended to the level of somite 11. Just like in other vertebrates, there is a rostro-caudal gradient of ENS migration and differentiation (Bisgrove et al., 1997; Kelsh and Eisen, 2000; Marcos-Gutierrez et al., 1997; Shepherd et al., 2004). Hence, we interpret our in situ results as indicating that *sox10* is expressed somewhat earlier in ENS progenitors than either *ret* or *phox2b*, whilst Hu is expressed considerably later.

We asked whether *phox2b* was expressed in differentiating neurons by combining whole-mount immunofluorescence with anti-Hu antibody and mRNA in situ hybridization with *phox2b* at 55 hpf (Fig. 4). At this stage Hu expression was not detectable in the hindgut ENS, so we focused on anterior trunk enteric neurons. We saw that approximately 30% (11 out of 30 *phox2b*-expressing cells in 5 different embryos) of trunk *phox2b*-expressing enteric cells were also expressing Hu. Hu-expressing neurons were predominantly seen only in anterior regions, whereas *phox2b*-expressing cells extended throughout the trunk. Thus, many of the *phox2b*-expressing cells that lack Hu are likely to be developmentally slightly younger. We conclude that *phox2b* is expressed in enteric neuron precursors and early differentiating neurons.

### 2.4. Phox2b is required for zebrafish ENS development

*Phox2b* knockout mice lack enteric neurons (Pattyn et al., 1999). We used morpholino knockdown to test whether zebrafish ENS development similarly requires *phox2b* (Fig. 5). We tested two non-overlapping oligonucleotide sequences targeting Phox2b translation. Injection of 4.6 ng of either morpholino resulted in 40–70% of embryos showing a consistent phenotype of a small head and eyes with brain necrosis concentrated in ventral regions (data not shown). This morphant phenotype was consistent between the two non-overlapping morpholinos and is therefore likely to reflect the role of *phox2b* in brain development. However, these embryos also showed growth retardation, making interpretation of any ENS phenotypes difficult. A dose of 1.2–2.3 ng per embryo gave some embryos with this severe phenotype, but many were overtly morphologically normal. Consequently, we focused on analyzing the ENS in embryos that lacked overt morphological changes at 5 dpf. Immunofluorescent detection of Hu antigen in these embryos revealed that Phox2b knockdown using either morpholino caused a marked reduction in the number of enteric neurons in comparison with mock-injected or uninjected control siblings (Fig. 5; data not shown). Importantly, gut morphology was indistinguishable in experimental and control groups. In control embryos counts of Hu-positive cells revealed a higher density of
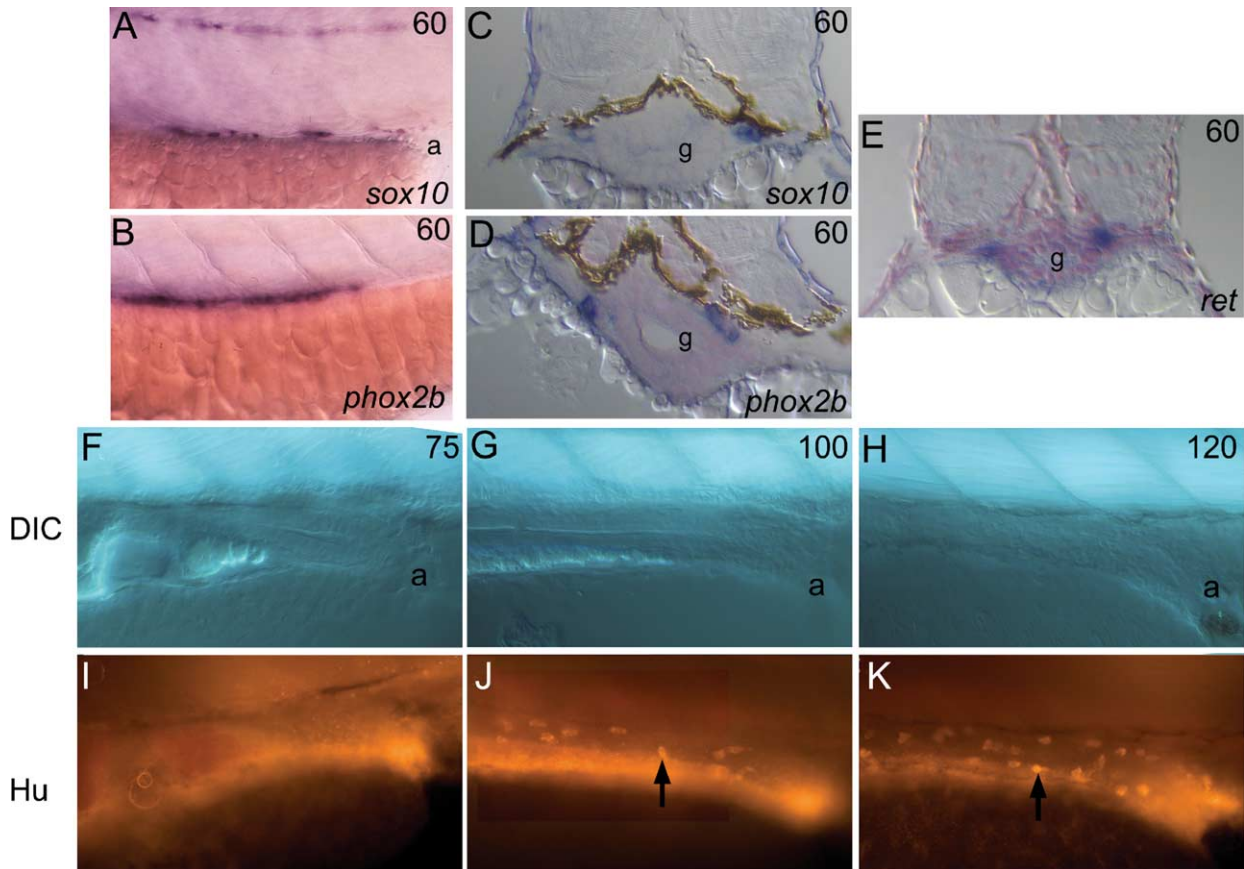
Fig. 3. Late embryonic development of the zebrafish enteric nervous system. Lateral views of whole-mount (A, B, F–K) and transverse sections (C–E) of mRNA in situ hybridizations (A–E) or immunofluorescent detection (I–K) and corresponding DIC images (F–H) of markers of ENS progenitors in hindgut region. From 60 hpf, *sox10* (A, C), *phox2b* (B, D) and *ret* (E) expression is prominent in two chains of ENS progenitors migrating posteriorly along the gut primordium (g). Note that *sox10* expression at this stage is seen in cells right up to the anus (a), whereas *phox2b* (and *ret*, data not shown) is not expressed this far posteriorly (A, B). In contrast, expression of Hu antigen is not seen in the hindgut at 75 hpf (I), but is seen in increasing numbers of cells at 100 (J) and 120 hpf (K).

neurons in the proximal than the distal gut. Either morpholino caused a significant reduction in neuron number both in the proximal and distal gut (Fig. 6; data not shown). In some individual embryos enteric neurons were absent from the distal hindgut (Fig. 5). Thus, in zebrafish, just as in mouse, enteric neuron development requires *phox2b* function.

## 2.5. sox10 is required for neural crest cell migration to the gut primordium

To investigate the origins of the *sox10* mutant ENS defect we needed to compare the expression patterns of ENS markers at incrementally earlier times of development. Previously the ENS defects in *sox10* mutant zebrafish have
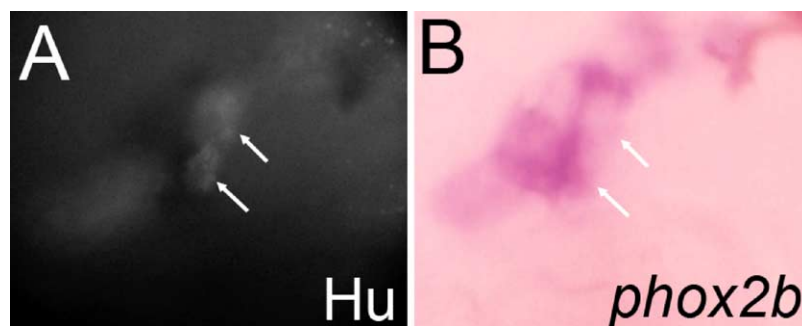


Fig. 4. Expression of *phox2b* in enteric neurons and non-neuronal cells. (A, B) Dorsolateral views of ventral trunk to show ENS progenitors in 55 hpf embryos. Combined immunofluorescent detection of Hu antigen (A) and mRNA in situ hybridization for *phox2b* (purple, B) reveals double-labeled early differentiating neurons (arrows) containing both markers. Other cells express only *phox2b* and are presumably undifferentiated progenitors.
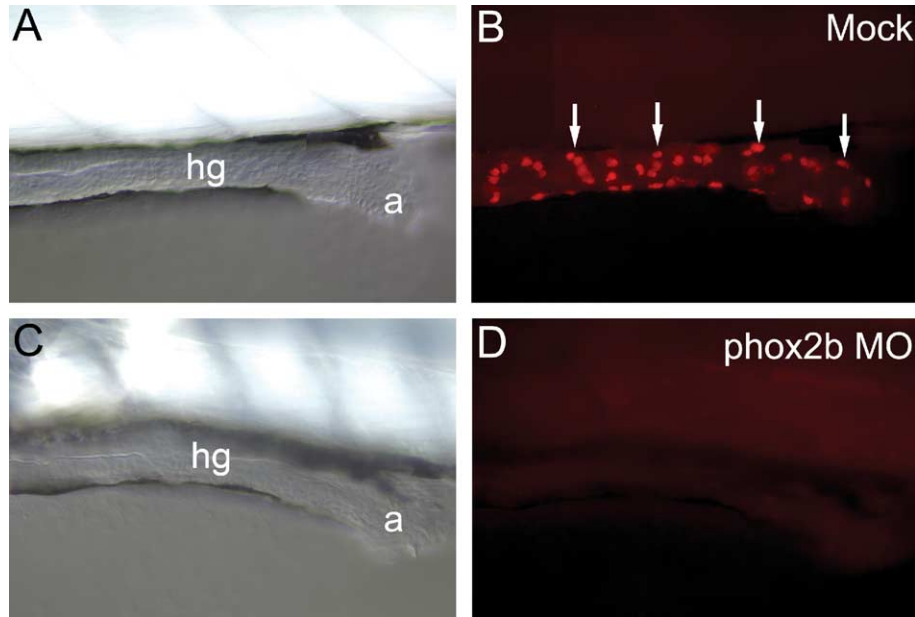
Fig. 5. Injection of *phox2b* morpholinos generates partial phenocopies of the *sox10* enteric neuron phenotype. Lateral views of posterior hindgut of 5 dpf wild-type embryos injected with 2 ng dose of *phox2b.a* (C, D) morpholino to show severe reduction in numbers of Hu-positive enteric neurons (arrows) compared with uninjected controls (A, B). DIC images of same embryos confirm their normal gross morphology (A, C). hg, hindgut; a, anus.

been described in the hindgut using Hu as a marker of differentiated neurons at 3–5 dpf and *sox10* and *ret* as markers of ENS precursors at 60 hpf (Dutton et al., 2001; Kelsh and Eisen, 2000). We used in situ hybridization with *sox10*, *ret* and *phox2b* probes, examined both as whole-mounts and in transverse section, to define the position of ENS progenitors during wild-type development, focusing on development in the anterior gut, in the region of the pectoral fin bud (Fig. 7). Between 35 and 60 hpf, cells expressing all of these marker genes were seen in close proximity to the developing anterior gut. In whole-mounts, a progressive rostro-caudal migration of ENS progenitors was seen as an increasingly more posterior extent of marker expression (e.g. for *phox2b*, Fig. 7C,O,AA). Although at 60 hpf in transverse sections of the hindgut all ENS progenitor markers were expressed in two chains of cells along the sides of the gut, in anterior regions at this stage such a pattern was only seen for *phox2b* and *ret* (Fig. 7AB,AD). In contrast, *sox10* was expressed in cells that completely encircle the gut (Fig. 7Z). At 45 and 35 hpf, *sox10*, *phox2b* and *ret* were all expressed in two lines of cells either side of the developing foregut (Fig. 7N,P,R), consistent with our previous observations (Shepherd et al., 2004). We conclude that ENS progenitors expressing all three-marker genes initially migrate in an antero-posterior direction along the developing gut as two chains of cells. Later migrating progenitor cells encircle the gut.

When we examined *sox10* mutant embryos we saw dramatic defects in ENS development at these early stages. Neither *phox2b* nor *ret* expression were detected in any cells associated with gut at any of these 3 stages, although expression in the hindbrain (*phox2b*) and pronephros (*ret*)

were unaffected (Fig. 7I–L,U–X,AG–AJ). Interestingly, *sox10* expression was seen in cells in the characteristic position of ENS progenitors, but in very reduced numbers; consequently, in most sections these cells were absent (Fig. 7G,H,S,T,AE,AF).

As noted before (Shepherd et al., 2004), unambiguous ENS progenitors could not be identified by mRNA in situ hybridization at earlier stages. However, we did note at 24 hpf an aberrant accumulation of *sox10* labeled neural crest cells retarded on proximal regions of the medial migratory pathway (data not shown). Expression of *crestin* has been described as a general marker of premigratory neural crest and of early stages of differentiation of neural crest derivatives (Luo et al., 2001). Using *crestin* as a further general marker of neural crest at this stage revealed a dramatic reduction of *crestin* expression in all domains except the cartilage precursors in the branchial arches in *sox10* mutants (Fig. 8). Strikingly, in *sox10* mutants premigratory neural crest cells showed weaker expression and most medial pathway cells lacked expression, as did cells associated with the cranial ganglia. Hence, although *crestin* does not constitute an informative marker for ENS precursors in *sox10* mutant embryos, this probe does reveal defects in premigratory as well as migrating cells in *sox10* mutants.

## 3. Discussion

The *sox10*, *phox2b* and *ret* expression patterns in zebrafish confirm the evolutionary conservation of many aspects of ENS development. ENS progenitor migration
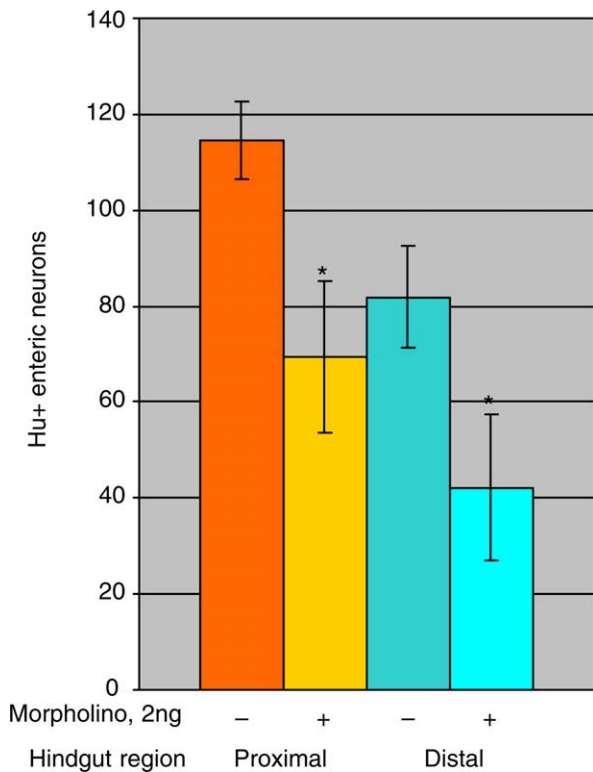
Fig. 6. Quantitation of *phox2b* morpholino injections shows significant reduction in hindgut enteric neurons. Numbers of Hu-positive enteric neurons in proximal (orange; corresponding to the region of somites 4–9) or distal (blue; below somites 10–14) hindgut of 5 dpf wild-type embryos are compared with those of siblings injected with 2 ng of *phox2b.a* morpholino. Embryos show a graded distribution of enteric neurons in the hindgut, with higher numbers in proximal regions, but numbers are much reduced in morpholino-injected embryos. Bars represent means ± SEM. Difference between uninjected and injected embryos was statistically significant (*Student's *t*-test, $P < 0.001$).

occurs initially as two chains along the side of the developing gut, with cells only subsequently migrating circumferentially around the gut primordium. This situation is reminiscent of, but perhaps rather simpler than, that recently demonstrated in mouse (Young et al., 1999, 2004). In mice most migrating cells do not express neuronal markers, consistent with our observations that Hu is expressed after a long delay compared with *sox10* (Young et al., 1999, 2004). Furthermore, *sox10* is expressed significantly before *phox2b* or *ret*. Finally, our data suggest that early differentiating neurons maintain *phox2b* expression, consistent with studies in mice showing that all differentiated neurons maintain Phox2b (Young et al., 2003).In the mouse, *Phox2b* is required for all autonomic neuron development (Pattyn et al., 1999). Recently the human *Phox2b* orthologue, *PMX2B*, has been implicated in a case of short-segment Hirschsprung disease (Benailly et al., 2003). Our zebrafish data provide evidence that a partial loss of function phenotype might include HSCR. We were unable to address definitively whether in zebrafish Phox2b is required for all enteric

neurons, due to pleiotrophic effects of Phox2b knockdown. However, some morphants had no neurons in the hindgut, suggesting that at least in this terminal region all enteric neurons are Phox2b-dependent in zebrafish, as they are in mouse.

Surprisingly, we did not observe *phox2b* expression maintained in sympathetic progenitors. In occasional sections, but only at 48 hpf, we observed *phox2b*-expressing cells in a position adjacent to the dorsal aorta, and clearly separated from *phox2b*-expressing ENS progenitors (data not shown). This may indicate transient *phox2b* expression in at least a subset of sympathetic progenitors. Zebrafish *phox2a* is expressed in sympathetic ganglia (Guo et al., 1999b). Cloned PCR products from two distinct pairs of degenerate primers all corresponded to the same *phox2b* gene and genomic and EST database searches gave no indication of a second zebrafish *phox2b* gene (data not shown). It is possible that in zebrafish *phox2a* may act without *phox2b* to support sympathetic neuron development. Consistent with this, our *phox2b* morphants showed numerous sympathetic neurons even when the enteric neurons were severely depleted.

Failure of non-ectomesenchymal derivative fate specification is a common theme in zebrafish and mouse *sox10* mutants (Dutton et al., 2001; Elworthy et al., 2003; Kelsh and Eisen, 2000; Kelsh and Raible, 2002; Kim et al., 2003). We show here that the ENS requires Sox10 for fate specification. In *sox10* mutants a few cells expressing *sox10* transcripts do reach the gut primordium and remain there until *phox2b* and *ret* are expressed in ENS progenitors of wild-types. Nevertheless, these cells fail to express *phox2b* or *ret*. Although the potency of *phox2b*-expressing ENS progenitors has not been fully explored in zebrafish, our observations of co-expression of a neuronal marker Hu in a subset of *phox2b*-expressing cells is consistent with studies in mouse showing that both undifferentiated precursors and differentiated neurons express Phox2b (Young et al., 1999). In mouse overlapping Sox10 and Phox2b expression is seen in multipotent ENS progenitors that will generate both enteric neurons and glia (Young et al., 2003). Thus, it seems likely that in zebrafish specification of ENS progenitors themselves fails in the absence of Sox10 function.

Failure of peripheral glial specification has been demonstrated in mouse *Sox10* mutants, however in glial development *Sox10* also has a second function as an activator of terminal differentiation genes (Britsch et al., 2001). This accounts for why in glia, in contrast to neuronal and pigment lineages, *Sox10* expression is maintained throughout embryonic development in all vertebrates examined (Cheng et al., 2000; Dutton et al., 2001; Kuhlbrodt et al., 1998a; Paratore et al., 2001; Peirano et al., 2000). Our data for the ENS suggest another function for *sox10*. The number of neural crest cells reaching the gut primordium, even at very early stages, is highly reduced in *sox10* mutants, indicating that *sox10* is required for ENS progenitor
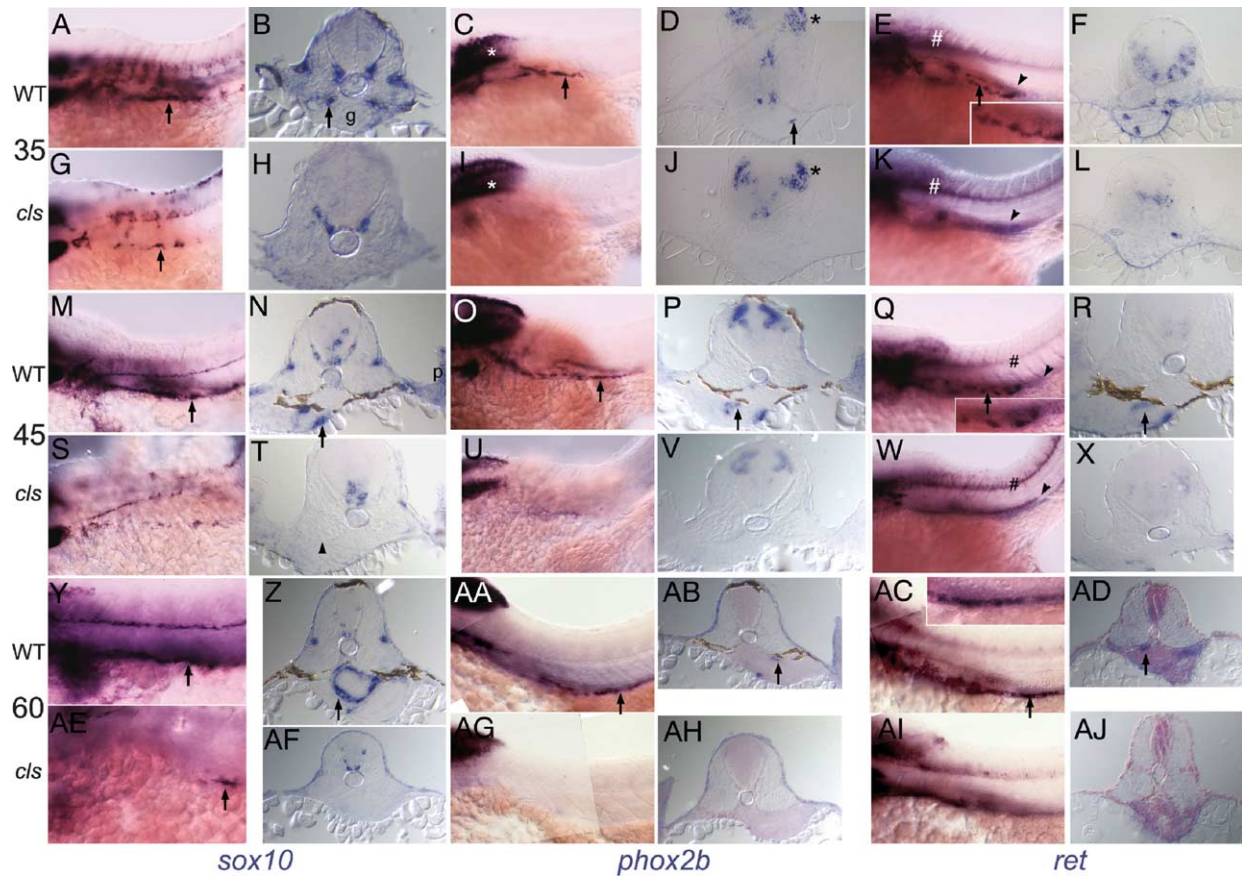
Fig. 7. *sox10* mutants show severe and early defects in enteric nervous system development. Lateral views (A, C, E, G, I, K, M, O, Q, S, U, W, Y, AA, AC, AE, AG, AI) or transverse sections (B, D, F, H, J, L, N, P, R, T, V, X, Z, AB, AD, AF, AH, AJ) of whole-mount in situ hybridizations of anterior trunk at 35 (A–L), 45 (M–X) and 60 hpf (Y–AJ) for enteric nervous system markers *sox10* (A, B, G, H, M, N, S, T, Y, Z, AE, AF), *phox2b* (C, D, I, J, O, P, U, V, AA, AB, AG, AH) or *ret* (E, F, K, L, Q, R, W, X, AC, AD, AI, AJ) in wild-type (A–F, M–R, Y–AD) and *sox10 (cls)* mutant (G–L, S–X, AE–AJ) embryos. At all stages, wild-type ENS progenitors express all three genes (arrows), whereas *sox10* mutants show occasional *sox10*-positive cells in this location, but do not express *phox2b* nor *ret*. As an internal positive control, note that expression of *phox2b* in the hindbrain (*, C, D, I, J) and *ret* in the ventral neural tube (#, E, K) is not affected. Note too that in wild-type embryos *ret* is expressed in both a chain of ENS progenitor cells (arrow and inset in E, Q and AC) and more medially in the tightly packed cells of the forming pronephros (partially out of focus in this focal plane; arrowhead, E, Q); the former is absent in *sox10* mutants, whereas the latter is unaffected (K, W, AI). ENS progenitor marker expression extends more posteriorly along the gut with increasing age (e.g. compare C, O, AA). *sox10* expression consistently extends more posteriorly than the other two markers at equivalent stages (e.g. compare A, C, E). Transverse sections reveal changes in progenitor distribution around gut primordium with age. ENS progenitors at 35 hpf initially lie in contact with gut primordium (arrow, B), forming two longitudinal columns migrating along the gut, but have spread around the developing gut by 60 hpf (Z). Note the identical positioning of *sox10*, *phox2b* and *ret* positive progenitors with respect to the developing gut (e.g. N, P, R). ENS progenitors are not usually seen in sections of *cls* mutant embryos (H, J, L, T, V, X, AF, AH, AJ), although expression in CNS remains. Interestingly, *sox10* and *phox2b* expression is prominent and overlapping in the pectoral fin mesenchyme (p) of wild-types (N, P), whereas *phox2b* appears to be strongly reduced in *cls* mutants (T, V). NB All whole-mount embryos treated with PTU to inhibit melanin synthesis. g, gut primordium.

migration to the gut. This result contrasts with the role of *Phox2b* in mice, since ENS progenitors reach the foregut in *Phox2b* knockouts, but then fail to migrate or differentiate (Pattyn et al., 1999). Our data support Kapur's suggestion that ENS development in mouse *Sox10* mutants fails prior to arrival of ENS progenitors at the gut (Kapur, 1999). We have shown definitively that non-ectomesenchymal neural crest cells in cranial and trunk regions die in zebrafish *sox10* mutants (Dutton et al., 2001), but initial studies using TUNEL reveal no concentration of cell death in the region of the migrating enteric precursors nor on the forming gut (Dutton and Kelsh, unpublished observation). Thus, definitive analysis of the fate of ENS progenitors will

require the identification of fate-specific markers expressed in the pre-enteric phase of their development.

In mice *Sox10* acts to delay differentiation of sympathetic and enteric neurons allowing the progenitors to migrate to the correct embryonic locations (Kim et al., 2003; Paratore et al., 2002; Zhu et al., 2004). Although we see defects in ENS progenitor migration to the gut primordium in zebrafish *sox10* mutants, we do not see precocious expression of the neuronal markers examined. *crestin* is the first general marker of premigratory NC to show decreased expression in *sox10* mutants (*sox10*, *foxD3* and *sna-2* expression in premigratory NC are unaffected; Dutton et al., 2001; Kelsh and Eisen, 2000).
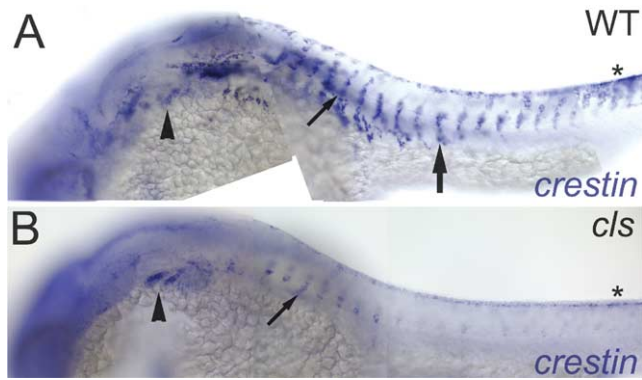
Fig. 8. Widespread defects in *crestin* expression in *sox10* mutants extend to premigratory stages of neural crest development. Lateral views of head and trunk of 24 hpf wild-type (A) and *sox10* mutant (B) zebrafish to show expression of *crestin*. Whilst in wild-types (A) *crestin* is strongly expressed in premigratory neural crest cells (asterisk) and in neural crest cells migrating on the medial pathway (large arrow), expression in these sites is dramatically reduced in *sox10* mutants (B). In contrast, expression in neural crest cells in the branchial arch streams (arrowhead) is unaffected (A, compare B). A few cells on the medial pathway with relatively normal *crestin* expression levels in *sox10* mutants (small arrow, A) may represent sensory neuron precursors (see Kelsh and Eisen (2000)).

*crestin* does not appear to encode a protein and may be functionless (Rubinstein et al., 2000), nevertheless its expression is associated with all trunk premigratory NC and all differentiating lineages. It is lost from all lineages as overt differentiation becomes apparent (Luo et al., 2001).

In summary, we have shown that *phox2b* function in enteric neuron development is conserved in zebrafish. *sox10* is required in specification of ENS progenitors during the enteric phase of ENS development and at an earlier stage for the migration of pre-enteric ENS progenitors to the gut primordium. Reduced *crestin* expression in zebrafish *sox10* mutants adds to the body of evidence showing defects in non-ectomesenchymal progenitors in premigratory stages of NC development (Dutton et al., 2001).

# 4. Experimental procedures

## 4.1. Isolation of zebrafish phox2b ortholog

Degenerate primers for *phox2b* were designed from sequences of the human and mouse Phox2b proteins using the CODEHOP program with zebrafish codon usage. Primer pairs gagtcagggtcggtggacttngcytcytt + gccttacaagctgttcaccgaycayggngg and ttctcgttcagtcctccgtgrtcngtraa + ggcctccggattccagtayaayccnat were chosen to avoid similarity with Phox2a. These primer pairs each gave RT-PCR products of the expected sizes from 38 or 63 hpf embryos. Sequences from these products were used to design primers for overlapping 5′ and 3′ Smart RACE (Clontech). Using 38 hpf embryos; 5′ RACE with primer

ctcgcgagtcagatttcttcccagtgc and 3′ RACE with primer agtacaacccaatcaggacgacgtttgg gave products with identical sequence in the overlapping region. The RACE product sequences were used to design primer pair ccatcgatgtgcagctttccttctccac + ggctctctctttctttttggc to amplify the coding region from 63 hpf embryo cDNA for insertion in pCSHSP as a Cla1 to Xba1 fragment. The zebrafish *phox2b* cDNA sequence has GenBank accession number AY846871. Zebrafish *phox2b* was mapped on the radiation hybrid panel LN54 by PCR with primers gtgcagctttccttctccactga + aacgtcgtcctgatgggattgta.

## 4.2. Fish husbandry and mutant strains

Mutant strains used consisted of $cls^{m618}$ and $cls^{tw11}$; their phenotypes are indistinguishable (Dutton et al., 2001; data not shown). For morpholino injections, embryos from F1 hybrid wild-types derived from an AB × WIK cross were used.

## 4.3. Whole-mount mRNA in situ hybridization and immunofluorescence

Embryos were processed for whole mount mRNA in situ hybridization or immunofluorescence by standard methods. Hu antibody, *sox10*, and *ret* riboprobes were obtained as previously described (Dutton et al., 2001; Kelsh and Eisen, 2000). *phox2b* riboprobe was synthesized from 3′ RACE product in pGEMT (Promega) with T7 RNA polymerase after Not1 cleavage.

## 4.4. Morpholino injections

AB wild-type embryos ranging from one to eight cell stages were injected with up to 4.6 ng of either of two non-overlapping *phox2b* morpholinos, *phox2b.a* (5′-CATTGAAAAGGCTCAGTGGAGAAGG-3′) or *phox2b.b* (5′-CCAAGGAAAAATACACACAAACGTC-3′), diluted with 1 × Danieau's solution with Phenol Red. Embryos mock-injected with 1 × Danieau's solution with Phenol Red acted as a negative control.

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