HHS Public Access

Author manuscript

Dev Dyn. Author manuscript; available in PMC 2022 December 01.

Published in final edited form as:

Dev Dyn. 2021 December ; 250(12): 1796–1809. doi:10.1002/dvdy.380.

A show of Hands: Novel and conserved expression patterns of teleost *hand* paralogs during craniofacial, heart, fin, peripheral nervous system and gut development

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Abstract

Background: *Hand* genes are required for the development of the vertebrate jaw, heart, peripheral nervous system, limb, gut, placenta, and decidua. Two *Hand* paralogues, *Hand1* and *Hand2*, are present in most vertebrates, where they mediate different functions yet overlap in expression. In ray-finned fishes, *Hand* gene expression and function is only known for the zebrafish, which represents the rare condition of having a single *Hand* gene, *hand2*. Here we describe the developmental expression of *hand1* and *hand2* in the cichlid *Copadichromis azureus*.

Results: *hand1* and *hand2* are expressed in the cichlid heart, paired fins, pharyngeal arches, peripheral nervous system, gut, and lateral plate mesoderm with different degrees of overlap.

Conclusions: *Hand* gene expression in the gut, peripheral nervous system, and pharyngeal arches may have already been fixed in the lobe- and ray-finned fish common ancestor. In other embryonic regions, such as paired appendages, *hand2* expression was fixed, while *hand1* expression diverged in lobe- and ray-finned fish lineages. In the lateral plate mesoderm and arch associated catecholaminergic cells, *hand1* and *hand2* swapped expression between divergent lineages. Distinct expression of cichlid *hand1* and *hand2* in the epicardium and myocardium of the developing heart may represent the ancestral pattern for bony fishes.

Keywords

cichlid; epicardium; fin; gut; *hand1* ; *hand2* ; heart; myocardium; oral teeth; pharyngeal arches; sympathetic

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AUTHOR CONTRIBUTIONS

Samantha Reynolds: Investigation. **Christian Pierce:** Investigation. **Benjamin Powell:** Investigation. **Alexandra Kite:** Investigation. **Nicholas Hall-Ruiz:** Investigation. **Thomas Schilling:** Funding acquisition; writing-original draft. **Pierre Le Pabic:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; writing-original draft.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

1 | INTRODUCTION

Hand (Heart And Neural crest Derived expressed) genes code for basic Helix-Loop-Helix (bHLH) transcription factors of the Twist class that play roles in cell specification, differentiation and tissue patterning during vertebrate and invertebrate development.[1–4] In *Drosophila*, a single *Hand* gene is required for cardiac and vascular development.[2] As a result of the whole genome duplication events that took place in the chordate lineage preceding the evolution of vertebrates,[5] the single ancestral chordate *Hand* gene[6,7] was duplicated into 2 paralogues, *Hand1* (*eHand*) and *Hand2* (*dHand*), that are present in most extant vertebrates (Ensemble.org). *Hand* genes are required for the development of a host of vertebrate evolutionary novelties: the jaw, the left/right ventricles of the four-chambered heart, the sympathetic division of the peripheral nervous system, the tetrapod limb, as well as the placenta and decidua of placental mammals.[8–17] Consequently, *Hand* gene expression must have dramatically changed during the course of chordate evolution and understanding these changes and their timing may provide insights into the evolution of these morphological innovations.

Hand1 and *Hand2* expression patterns have been described in model species of mammalian (mouse, *Mus musculus*),[8,18] avian (chicken, *Gallus gallus*)[19] and amphibian (frog, *Xenopus laevis*)[20,21] classes. The developmental roles of tetrapod *Hand1* and *Hand2* have been best studied in the mouse embryo, where *Hand1* and *Hand2* have distinct functions in heart, pharyngeal arch (PA), limb, sympathoadrenal lineages of the peripheral nervous system (PNS) and gut development.[8–17,22] In addition, *Hand1* is required for extra-embryonic mesoderm development.[9]

In contrast, *Hand* gene expression and function outside tetrapods has only been examined in a ray-finned fish species, the zebrafish (*Danio rerio*, order Cypriniformes), the genome of which contains just a single *Hand* orthologue (*hand2*).[23–27] This condition is not conserved among most extant ray-finned fishes, which have two *Hand* genes, *hand1* and *hand2*, indicating that *hand1* was lost in the lineage leading to the Cypriniforms (Ensemble.org). *Hand1* requirements for the development of multiple structures in the mouse embryo and its loss in Cypriniforms poses multiple questions. What is the expression and function of *hand1* in non-cypriniform ray-finned fishes? Did the loss of *hand1* have any consequences for the development, anatomy, and/or morphology of the cypriniform body plan? A thorough description of *hand1* and *hand2* expression patterns in a non-cypriniform teleost is a first step towards answering these questions.

Here, we describe the expression patterns of *hand1* and *hand2* during the development of the teleost *Copadichromis azureus* (*Caz*; Cichlidae).[28] We find that both genes are expressed in the developing cichlid heart, paired fins, PAs, PNS, and gut with different degrees of overlap, while *hand1* alone is expressed in the lateral plate mesoderm (LPM), and *hand2* alone is expressed in median fins (dorsal and anal). *Caz hand1* and *hand2* expression patterns are most similar to each other in the trunk PNS. In the developing *Caz* heart, *hand1* expression is detected in the epicardium alone, while *hand2* is detected only in the myocardium. In the *Caz* gut mesenchyme, *hand1* is expressed first and more robustly than *hand2*. In the *Caz* fin mesenchyme, *hand1* expression is uniform, while *hand2* is expressed in a posterior domain resembling the tetrapod limb zone of polarizing activity (ZPA) in both paired and median fins. In the *Caz* pharyngeal arches, both *hand* genes are expressed ventrally, although *hand1* expression is restricted to a ventral subset of the *hand2* domain.

2 | RESULTS AND DISCUSSION

2.1 | *Hand1* alone is expressed in the cichlid lateral plate mesoderm

Derivatives of the vertebrate LPM include the heart and cardiovascular system, blood, smooth muscle, and limb skeleton.[29] A single *Hand* gene is expressed in the LPM of basal chordates (amphioxus, lamprey).[6,30] Mouse *Hand2*, but not *Hand1*, is expressed in the LPM.[8,18,19] Similarly *hand2* is expressed in the zebrafish LPM,[27,31] while it is not expressed in the LPM of teleosts with two *hand* genes in their genomes, such as medaka and Nile tilapia.[32] Because of the close evolutionary relationship between *Caz* and Nile tilapia, we expected Caz *hand2* expression to be absent from the LPM, while *hand1* expression in this species remained to be determined.

To address these issues we performed whole-mount in situ hybridization (ISH) for both *hand1* and *hand2* in *Caz* embryos over the first few days of development. At 36 hours postfertilization (hpf), *hand1* was robustly expressed in a region directly lateral to the pharyngeal arches (PAs), which we interpret as the anterior LPM, including the heart field (Figure 1A), and also lateral to the posterior paraxial mesoderm in a region that we interpret as the posterior LPM (Figure 1A). *hand2* expression was not detected at this stage (Figure 1B). At 54 hpf, *hand1* expression was detected in the heart tube and two bilateral LPM patches lying lateral to the embryo body (Figure 1C), while *hand2* was strongly expressed in the developing heart and neural crest (NC) of the PAs but was not detected in the LPM (Figure 1D). Bilateral domains of *hand1* expression were detected in anterior LPM at the position of the future pectoral fins (Figure 1C) and in posterior LPM connecting to two bilateral lines of expression flanking the developing hindgut (Figure 1C). This *Caz hand1* expression resembles that of *hand2* in the zebrafish posterior LPM, where *hand2* regulates the size of the intermediate mesoderm.[31]

Our results show that the expression of *hand1*, but not *hand2*, in the cichlid LPM differs from mouse or zebrafish *Hand* gene expression, in which *hand2*, but not *hand1*, is expressed in the LPM.[8,18,19,27] The lack of *hand2* expression in cichlid LPM is consistent with previous reports in teleosts with a two *hand* gene complement.[32] These opposite and mutually exclusive expression patterns suggest that a single *hand* gene is sufficient for LPM development and that LPM expression switched from one *hand* ortholog to the other in various bony fish lineages.

2.2 | *hand1* and *hand2* are expressed in the atrium, ventricle, and outflow tract of the cichlid heart

During vertebrate heart morphogenesis cardiac precursors derived from the anterior LPM form a linear, primitive heart tube that is subsequently patterned along the anterior-posterior (A-P) axis into the presumptive atrium(a), ventricle(s), and outflow tract. *Hand* genes are diversely expressed in the developing heart fields of model vertebrate embryos. In the chick, both genes are homogeneously expressed and required redundantly for heart looping.[19] In mouse, initially *Hand2* expression spans the entire primitive heart tube while *Hand1* expression is restricted to the presumptive left ventricle and outflow tract. Thereafter, *Hand2* expression becomes the highest in the presumptive right ventricle.[8,33] Mouse knockouts have shown specific requirements for *Hand1* and *Hand2* in left and right ventricle formation, respectively.[8–10] In Xenopus, *Hand1* is homogeneously expressed throughout the heart tube,[34] while *Hand2* expression is not detected.[21] In zebrafish, which lacks *hand1, hand2* is expressed throughout the heart tube and required for myocardial lineage specification and heart looping.[27]

At 54 hpf when the heart tube has formed, the expression of both Caz *hand1* and *hand2* was uniform along the entire tube, albeit in different layers (Figure 1C,D; Figure 2A,B). *hand1* was detected in the outer epicardial layer (Figure 2A), while *hand2* was detected in the inner myocardial layer (Figure 2B). During heart looping (78 hpf, Figure 1E, F), *hand1* was still primarily expressed in the epicardium of the entire outflow tract and ventricle but became restricted in the atrium to the region immediately adjacent to the ventricle (Figure 2C). *hand2* was also still primarily expressed in the myocardium of the entire outflow tract, ventricle and atrium, with the strongest expression in the thickening ventricle myocardium and very weak expression in the atrium (Figure 2D). By 7 days postfertilization (dpf) (Figure 1M,N), *hand1* expression remained restricted to more peripheral regions of the outflow tract and ventricle, while not detected in the atrium (Figure 2E). In contrast, *hand2* expression was excluded from the cardiac periphery, with robust expression in the outflow tract core and inner ventricle, and weaker expression in the atrial myocardium (Figure 2F). In addition, *hand1* was robustly expressed throughout the pericardial sac (Figure 2E), while pericardial *hand2* expression was weaker and restricted anteriorly (Figure 2F).

The uniform expression of *hand* genes along the A-P axis of the cichlid heart tube is consistent with previous reports for zebrafish *hand2*, Xenopus *Hand1* and both chicken *Hand* orthologs.[19,21,27] The restricted *Hand* gene expression observed along the A-P axis of the mouse heart tube patterns the left and right ventricles,[8] a morphological innovation that evolved in the tetrapod lineage leading to mammals. Stronger expression in the outflow tract and ventricle during heart looping was reported for zebrafish *hand2*.[26]

Layer-specific expression of *hand* orthologues in the heart was reported in a few organisms and at particular stages. Layer-specific expression of zebrafish *hand2* expression was not reported, yet it is required specifically for myocardium development, the particular layer where *hand2* is detected in our study.[27] In mouse, *Hand2* is expressed in the myocardium, but not the epicardium, similar to cichlid *hand2*.[8] In addition, mouse *Hand1* expression was primarily reported in the myocardium, yet lineage analyses showed that *Hand1*-expressing cells become restricted to the epicardium,[14] which is where cichlid *hand1* is detected in our study.

Taken together, our results suggest that the ancestral expression of *Hand* orthologs before the split between ray- and lobe-finned fish lineages was uniform along the A-P axis of the heart tube, and that *hand1* was expressed in the epicardium and *hand2* was expressed in the myocardium. The loss of *hand1* in Cypriniforms does not correlate with any known morphological alteration of the heart, suggesting that *hand1* was dispensable for heart development at the time of its loss.

2.3 | Cichlid paired fins express *hand1* and *hand2*, median fins express *hand2* alone

Hand2 is dynamically expressed in mouse and chick limb buds through multiple stages.[19,35] Its posterior expression in the early limb bud is required for *Shh* expression in the Zone of Polarizing Activity (ZPA).[11,35,36] By contrast, *Hand1* is expressed anteriorly and ventrally in chick and mouse limb buds, and no limb defects have been reported in mouse *Hand1* mutants.[22]

The teleost fore- and hindlimb homologues, the pectoral and pelvic fins, respectively, develop over distinct periods. Pectoral fin buds become visible during pharyngeal arch segmentation, similar to tetrapod forelimb buds,[37–39] while pelvic fin buds appear during metamorphosis, the timing of which is species-specific.[38,40–42] Teleost *hand2* fin expression was previously reported for the zebrafish pectoral fin alone, where it is expressed in the posterior mesenchyme and required for *shh* expression similar to tetrapods.[27] *hand1* expression in fins has remained undescribed in teleosts before this study.

hand1 and *hand2* expression were both detected, albeit differently, in the cichlid pectoral fin bud from 72 hpf to 9 dpf (Figure 1C–P). In the early bud (72-96 hpf), *hand1* expression appeared uniform across the A-P extent of the pectoral fin mesenchyme, while *hand2* expression appeared restricted to the posterior mesenchyme alone (Figure 1E–P, Figure 3A,B). As fin development proceeded, *hand1* and *hand2* expression became restricted to the fin base (Figure 3C,D), with *hand1* expression along the entire A-P extent of the fin (Figure 3C), and *hand2* expression restricted to the posterior fin (Figure 3D).

Pelvic fin expression of *hand1* and *hand2* was detected starting at 8 dpf, the beginning of cichlid metamorphosis.[38,41] At this stage, pelvic fin buds lay laterally on the yolk sac (Figure 1O,P). Similar to the pectoral fin, *hand1* expression was uniform throughout the pelvic fin bud mesenchyme (Figure 3E), while *hand2* expression was restricted posteriorly (Figure 3F).

In median fins, *hand2* expression, but not *hand1*, also became detectable at the onset of metamorphosis (6 dpf; Figure 1L), although 48 hours prior to its detection in the pelvic fin buds. Weak expression was first detected within the larval finfold at the posterior edges of the presumptive dorsal and anal fins (Figure 1L). Expression became more robust at later stages (Figure 1N,P).

The ZPA-like expression of *hand2* in cichlid fins suggests a conserved role in appendage A-P patterning between teleosts and mammals, while the uniform *hand1* expression in the cichlid fin mesenchyme suggests a different requirement than in mammals. The previously undescribed *hand2* expression in median fins, structures lost in mammals, is likely ancestral for ray- and lobe-finned fishes as the ZPA-like expression of *shh*, activated by *Hand2* in mouse limbs,[11] is regulated by a common cis-regulatory element in zebrafish paired and median fins[43] and present in shark median fins.[44] Median fin *hand2* expression was likely not previously described in zebrafish due to the late onset of metamorphosis in this species.[45]

2.4 | *hand1* and *hand2* expression in the cichlid gut mesenchyme

In mouse, *Hand1* and *Hand2* are expressed in distinct populations of the intestinal mesenchyme: *Hand1* is expressed in the enteric mesoderm, while *Hand2* is expressed in enteric NC cells.[12–14] Mouse *Hand1* is required for enteric muscle organization, not differentiation,[46] while *Hand2* is required for the differentiation of enteric neurons.[12] In zebrafish, *hand2* is first expressed in the intestinal mesoderm-derived mesenchyme at early stages of intestinal development, similar to mouse *Hand1* [12,25] and becomes also expressed in enteric NC cells at later stages. Zebrafish *hand2* is required for (a) the migration and proliferation of enteric NC cells along the intestine, (b) their differentiation, (c) intestinal smooth muscle development,[25] and (d) gut looping.[47] Thus, zebrafish *hand2* is responsible for the biological activities of both *hand1* and *hand2* shown in mouse, in addition to requirements for enteric neuron migration and gut looping.

Caz hand1 expression was detected in bilateral lines of mesenchyme flanking the gut at 54 hpf (Figure 1C) and became restricted to the gut region extending from the pectoral fin to the anus at subsequent stages (Figure 1E). Expression was first restricted to the ventral side of the gut (Figure 1E, G), became localized dorsally in the region proximal to the anus at 5 dpf (Figure 1I), and was detected dorsally and ventrally thereafter (Figure 1K,M,O). *hand2* gut expression started 2 days after *hand1* was first detected (4 dpf) and its general pattern mirrored that of *hand1*: it was first detected ventrally before extending to the dorsal aspect of the gut tube, although *hand2* salt-and-pepper expression was less robust than that of *hand1* (Figure 4A,B). Observations of *hand1* and *hand2* gut expression at higher magnification, together with sagittal histological sections from same-staged larvae of Nile tilapia (*Oreochromis niloticus*), showed that both genes are expressed in the mesenchyme lining the basal side of the intestinal epithelium, although *hand1* is expressed in more cells than *hand2* (Figure 4A–C). *hand1* and *hand2* gut expression became increasingly restricted to the distal gut/anal region over the next few days of development (Figure 1K–P).

The salt-and-pepper *hand2* expression in the *Caz* gut mesenchyme, and the more robust *hand1* expression suggest that, as in mouse, *Caz hand1*, and *hand2* may be required for smooth muscle and enteric neuron development, respectively. However, the use of molecular markers specifically expressed in these two lineages will be necessary to support this possibility.

2.5 | *hand1* and *hand2* expression in the pharyngeal arches

In mouse and chick, *Hand1* and *Hand2* expressions overlap in the ventral pharyngeal arches (PAs): *Hand1* expression is restricted to the ventral (distal)-most PA region, while *Hand2* extends further dorsally.[8,18,19,48] NC ablation of *hand1* alone does not affect pharyngeal development,[49] whereas distal mandibular structures (Meckel's symphysis, lower incisors) are affected when loss of *Hand1* is combined with a *Hand2* heterozygous loss-of-function.[50] In addition, altering the dimerization affinities of *Hand1* in phosphorylation mutants results in severe mid-facial clefting.[51] *Hand2* is required for lower jaw patterning in mice and zebrafish, where it is a downstream target of Edn1.[24,33,52] It also represses bone differentiation in the lower jaw independently from its DNA-binding role by direct binding to the transcription factor Runx2.[53]

Expression of *hand1* and *hand2* in NC was first detected in the cichlid PAs at 54 hpf (Figure 1C,D; Figure 2A,B), by which stage NC cells are post-migratory.[41,54] *hand1* expression was salt-and-pepper in the ventral domains of PA1-4 (Figure 2A), while *hand2* was robustly expressed ventrally in the rings of NC that surround the mesodermal core of each pharyngeal arch (Figure 2B).[55] In addition, *hand1* was intensely expressed in small clusters of variably positioned cells located ventrally in the zone of contact between PA1 and 2 (Figure 2A). These cells were identified as arch associated catecholaminergic cells (aac's).[55] By 78 hpf, both genes were expressed in PA1-7, although *hand1* expression was restricted to the ventral-most domain of *hand2* expression in each PA (Figure 5A,B). *hand1* expression subsequently became restricted to PA1-2 at 5 dpf and thereafter, while *hand2* remained expressed in PA1-7 (Figure 1I–P). As the musculoskeletal derivatives of the PAs started to differentiate during jaw elongation (6 pdf; Figure 1K,L; Figure 5C–F), *hand1* and *hand2* expression was not detected in cartilages, such as Meckel's cartilage in PA1 and the ceratohyal cartilage in PA2 (Figure 5G–J). Both genes were expressed in pharyngeal mesenchyme and differentiating muscles within their respective domains (Figure 5G, H). At 9 dpf, *hand1*, and *hand2* were both detected in the oral mesenchyme and developing teeth of the lower jaw (Figure 5I,J), but not of the upper jaw (Figure 5G,H). *hand1* expression was more robust anterior to Meckel's cartilage, while *hand2* expression was stronger posterior to Meckel's cartilage (Figure 5I,J). *hand1* and *hand2* were not detected in the dental mesenchyme of the upper and lower pharyngeal jaws (Figure 5M–P).

Our results show that PA expression of *hand1* is very similar in *Caz* to the mouse, and that *hand2* expression is very similar in *Caz*, zebrafish, and mice, except for aac's, which express *hand2* in zebrafish[55] and *hand1* in *Caz*. The presence of *hand1* expression in the dental mesenchyme of the *Caz* lower oral jaw, and its absence from the upper oral jaw and the upper/lower pharyngeal jaws makes it seem unlikely that the singular loss of *hand1* in the genomes of Cypriniforms underlies oral tooth loss in this lineage.

2.6 | *hand1* and *hand2* expression in the peripheral nervous system

The sympathetic branch of the peripheral nervous system (PNS) regulates homeostasis in vertebrates and is entirely derived from NC cells.[56] Sympathetic neurons and supporting cells lie either in paravertebral ganglia lying bilaterally ventral to the spinal column or in prevertebral ganglia located between paravertebral ganglia and target organs. Paravertebral ganglia include the sympathetic trunk ganglia, where both *Hand1* and *Hand2* are expressed in mice, whereas their prevertebral ganglionic expression includes the paraaortic ganglia and adrenal medulla.[15,18,19] *Hand2* is required for noradrenergic neuron differentiation, where it also activates *Hand1* expression, which in contrast is not required.[15–17] A similar requirement for *Hand2* was shown in zebrafish for noradrenergic neuron differentiation in cervical sympathetic ganglia and, unlike in mice, in the locus coeruleus of the central nervous system.[23] *hand2* expression was not reported in zebrafish sympathetic trunk ganglia and its requirement for sympathetic trunk noradrenergic neuron differentiation was not tested due to early lethality of the zebrafish *hands off* mutant.[23,27]

In the cichlid trunk, both *hand1* and *hand2* expression was detected in sympathetic ganglia starting at 6 dpf (Figure 1K–P and Figure 6A–C). Sympathetic cell clusters expressing either gene became connected along the A-P axis during the next 2 days of development, resulting in bilateral chains running on either side of the dorsal aorta along the entire trunk and tail (Figure 6L). We interpret these ganglionic chains as sympathetic trunks. In the head, *hand1* and *hand2* were also detected in the vagal (cranial nerve X) ganglion starting at 5 dpf (Figure 1I–P). Sagittal histological sections from same-staged larvae of Nile tilapia confirmed this structure as the vagal (Figure 6F,I). Ventral observations after removal of pharyngeal structures revealed a physical connection between *hand1* and *hand2* expressing components of the PNS of the head and trunk (Figure 6J–L). In teleosts, sympathetic chains extend into the head, where they are associated with cranial nerves.[57] Our results show that, as in mouse, cichlid *hand* genes are expressed in the sympathetic branch of the PNS.

3 | CONCLUSIONS

Overall, a comparison of our results in cichlids with other vertebrates (Table 1) suggests an evolutionary history of *Hand* genes in which expression in some embryonic structures such as the gut, PAs, and the sympathetic PNS were fixed in the common ancestor of lobe- and ray-finned fish. In contrast, in the appendages, *Hand2* expression appears to have been fixed, while *Hand1* expression diverged in lobe- and ray-finned fish lineages. Evolution of the four-chambered heart in mammals, and the roles for *Hand1* and *Hand2* in left-right ventricle patterning, respectively, imply a lineage-specific divergence in *Hand* gene expression between lobe- and ray-finned fishes. Our results suggest that a primitive role for *Hand1* and *Hand2* may have been in specifying the epicardium and myocardium, respectively. Lastly, LPM expression was shuffled between *Hand1*, in non-cypriniform teleosts, and *Hand2* in zebrafish and mammals,[8,27] A similar expression swap took place in aac’s between non-cypriniforms (*hand1* expression) and cypriniforms (*hand2* expression).

In the cypriniform lineage, our results suggest that the loss of *hand1* could have impacted fin and/or mandibular development. The function of *hand1* in teleost fin development is unknown, and the cypriniform fin is representative of the ancestral teleost configuration,[40] suggesting that loss of *hand1* may not have had an effect on fin development in this lineage. In contrast, the absence of oral teeth is a defining feature of Cypriniforms and correlates with the loss of *hand1* in this lineage. However, our results do not strongly support a causal role for *hand1* loss in the loss of oral teeth in Cypriniform evolution as it is expressed in dental mesenchyme of the cichlid lower, but not upper jaw. Thus, the morphological effects of the loss of *hand1* on developing fins, jaws, and teeth as well as other cell types at later stages in the Cypriniform lineage remain a mystery. Experimental tests of *hand1* function in non-Cypriniform teleosts will be required to address these hypotheses.

4 | EXPERIMENTAL PROCEDURES

4.1 | Animal care

All animals were reared and euthanized following protocols approved by the IACUC at the University of North Carolina, Wilmington. *C. azureus* brood stocks were purchased from a pet store and maintained at 28°C in 55-gal tanks. Adults were fed Northfin Food Cichlid Formula 3 mm sinking pellets. Fertilized eggs were collected from mouth-brooding females a few hours after fertilization. Embryos were reared in cichlid egg tumblers and staged under a microscope following Fujimura and Okada (2007).[38]

4.2 | Image capture

Observations and images were either made using a dissecting microscope Leica M165FC equipped with Planapo 1.6X M-series objective and a Leica DFC7000T camera controlled by the LAS X software, or using a Zeiss Axioskop 2 FS Plus compound microscope equipped with a Qimaging Micropublisher 6 camera controlled by Ocular software. Image processing was performed in Adobe Photoshop. Figures were prepared in Adobe Illustrator.

4.3 | In situ hybridization

Embryos and larvae were fixed and processed for whole-mount in situ hybridization as described in Le Pabic et al., (2007)[58] with the following modifications: for the proteinase K treatment step, specimens 5 dpf and younger were incubated for 10 minutes and specimens 6 dpf and older were incubated for 20 minutes in a 60 μg/mL solution. Prehybridization was overnight and probe incubation was 48 hours. Post-hybridization washes were 2 x 15 minutes in 2XSSCT and 3 x 2 hours 0.2XSSCT at 65° C. Block incubation was overnight and antibody incubation was 48 hours. In situ hybridization probes were generated from RT-PCR amplified regions of *C. azureus hand1* and *hand2* cDNAs using the following primers: CA_Hand1_F: TTACCAGCATCACCACCAC, CA_Hand1_R: TTCTTCTCGGCTCCTAAGGA, CA_Hand2_F: GTCATGCACCATCACGACA, CA_Hand2_R: CAGTTCCAGAGCCCAGACAT. The amplified *C. azureus hand1* fragment was 501 bp long and over 99% identical (500/501 identities) to its *M. zebra* ortholog (Malawi cichlid reference genome, sequence ID XM_004551130.2). The amplified *C. azureus hand2* fragment was 696 bp long and 100% identical to its *M. zebra* ortholog (Malawi cichlid reference genome, sequence ID XM_004562238.3). mRNA was extracted from deyolked 54hpf *C. azureus* embryos. In situ hybridization was conducted using antisense probes prepared from pGem-t-easy vectors (Promega) containing cDNA fragments corresponding to *C. azureus hand1* and *hand2*.

Funding information

National Institute of Dental and Craniofacial Research, Grant/Award Number: R01DE13828

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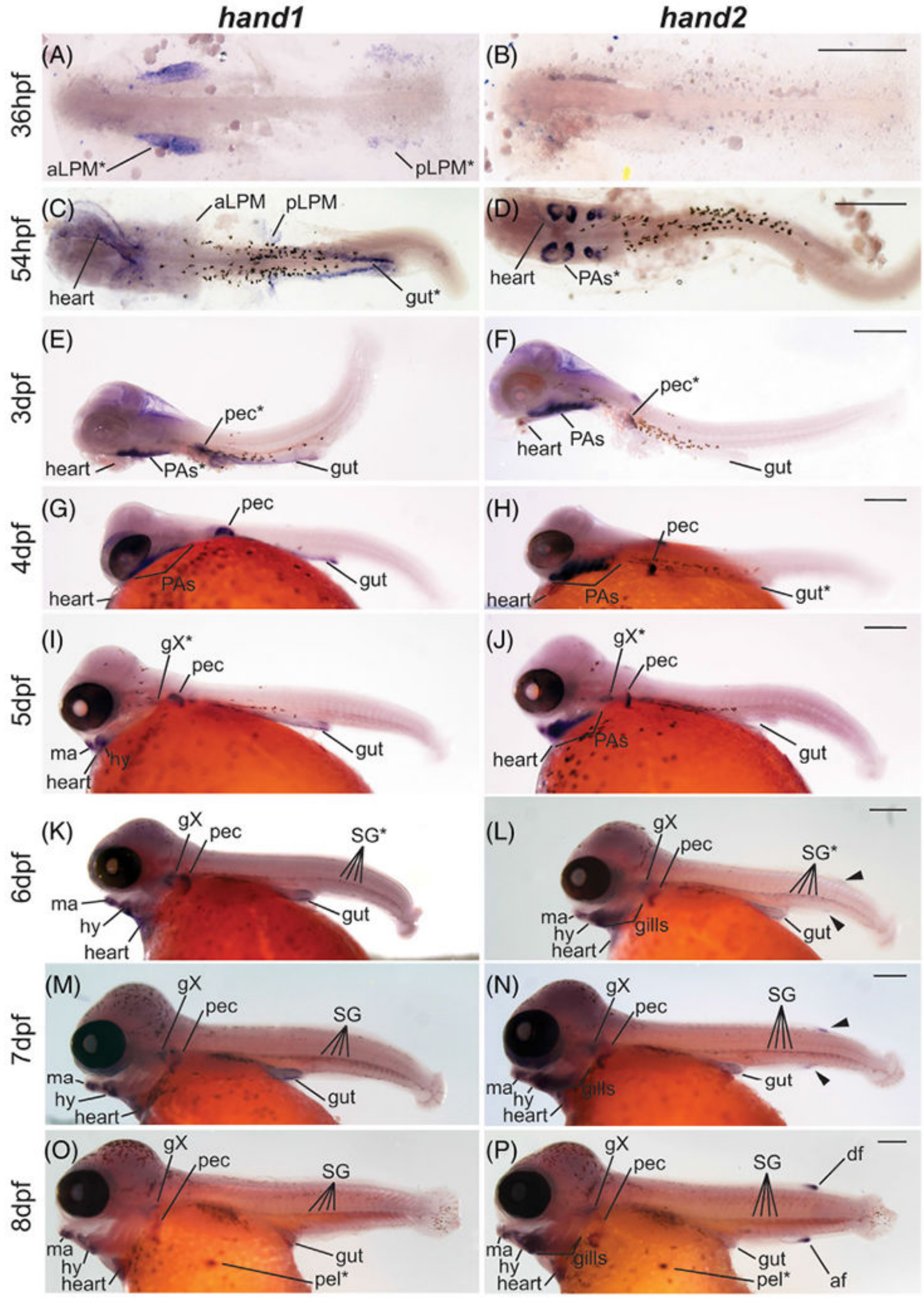

FIGURE 1.

hand1 and *hand2* expression in 2 to 8dpf *C. azureus*. A-B: *hand1*, A, and *hand2*, B, expression at 36 hpf in de-yolked embryos (dorsal view). *hand1* expression is detected in anterior and posterior lateral plate mesoderm, while *hand2* is not. C-D, *hand1*, C, and *hand2*, D, expression in 54 hpf de-yolked embryos (ventral view). *hand1* is detected in the heart, lateral plate mesoderm and gut, C, and *hand2* expression is detected in the heart and pharyngeal arches, D. E and F, *hand1*, E, and *hand2*, F, expression in 3 dpf de-yolked embryos (lateral view). *hand1* and *hand2* expression is detected in the heart, pharyngeal arches, and pectoral fins. *hand1* alone is expressed in the gut, E. G and H, *hand1*, G, and *hand2*, H, expression at 4 dpf. *hand1* and *hand2* are still detected in the heart, pharyngeal arches, and pectoral fins. *hand2* is first detected in the gut, H. I and J, *hand1*, I, and *hand2*, J, expression at 5 dpf. *hand1* and *hand2* are still detected in the heart, pharyngeal arches, pectoral fins, and gut. Their domains of expression now include vagal ganglia (gX). *hand1* pharyngeal arch expression has become restricted to the mandibular and hyoid arches, I. K and L, *hand1*, K, and *hand2*, L, expression at 6 dpf. *hand1* and *hand2* are still detected in the heart, pharyngeal arches, pectoral fins, gut, and vagal ganglia. *hand1* and *hand2* are first detected in trunk sympathetic ganglia. *hand2* is first detected in the presumptive dorsal and anal fins (arrowheads), L. M and N, *hand1*, M, and *hand2*, N, expression at 7 dpf. *hand1* and *hand2* are still detected in the heart, pharyngeal arches, pectoral fins, gut, vagal, and trunk sympathetic ganglia, and in the dorsal and anal fins. O and P, *hand1*, O, and *hand2*, P, expression at 8 dpf. *hand1* and *hand2* are still detected in the heart, pharyngeal arches, pectoral fins, gut, vagal and trunk sympathetic ganglia, and in the dorsal and anal fins. Their domains of expression include pelvic fins starting at this stage. Anterior to the left. * indicates first detection stage in labeled structure. aLPM, anterior lateral plate mesoderm; pLPM, posterior lateral plate mesoderm; PA, pharyngeal arches; pec, pectoral fin; SG, sympathetic ganglia; ma, mandibular arch; hy, hyoid arch; gX, vagus ganglion; df, dorsal fin; af, anal fin; pel, pelvic fin. Scale: 100 µm

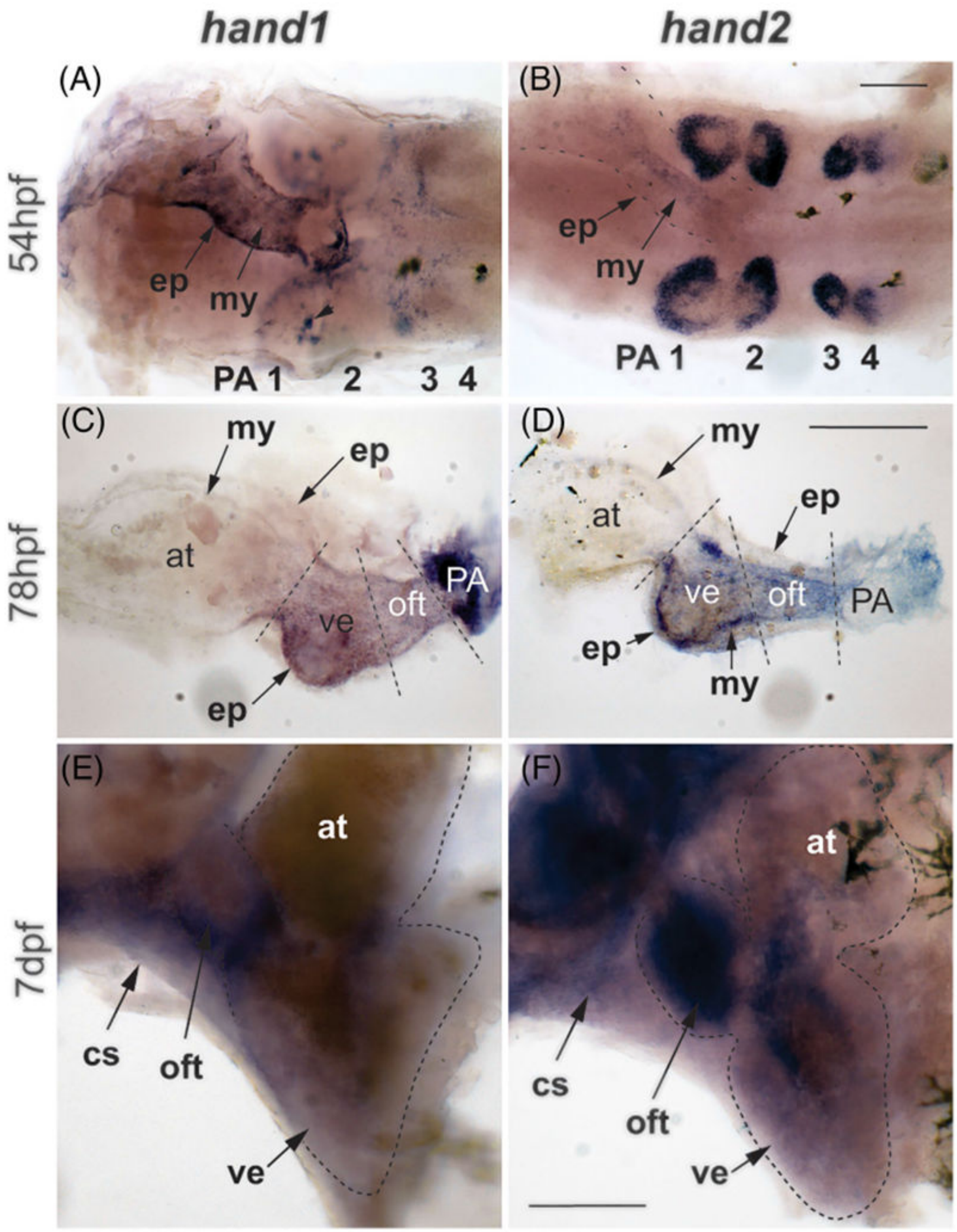

FIGURE 2.

hand1 and *hand2* expression in the developing cichlid heart. A and B, *hand1*, A, and *hand2*, B, expression in the 54 hpf heart (ventral view). *hand1* expression is detected in the outer heart layer, which we interpret as epicardium (ep), while *hand2* expression is detected in the inner heart layer, which we interpret as presumptive myocardium (my). *hand1* is also expressed in arch associated catecholaminergic cells[55] (arrowhead). C and D, *hand1* C, and *hand2*, D, expression in the 78 hpf dissected heart (ventral view). C, *hand1* expression is detected in the epicardium of ventricle, outflow tract, and proximal atrium. D, *hand2* expression is robust in the myocardium of the ventricle and outflow tract, and weak in the atrium. E and F, *hand1* (E) and *hand2* (F) expression in the 7 dpf heart (lateral view). E, *hand1* is expressed in the outer layers of the ventricle and outflow tract and not detected in the atrium. F, *hand2* is expressed in the inner layers of the atrium, ventricle, and outflow tract. Both genes are also expressed in the cardiac sac. at, atrium; cs, cardiac sac; oft, outflow tract; ve, ventricle. Scale: 25 μm

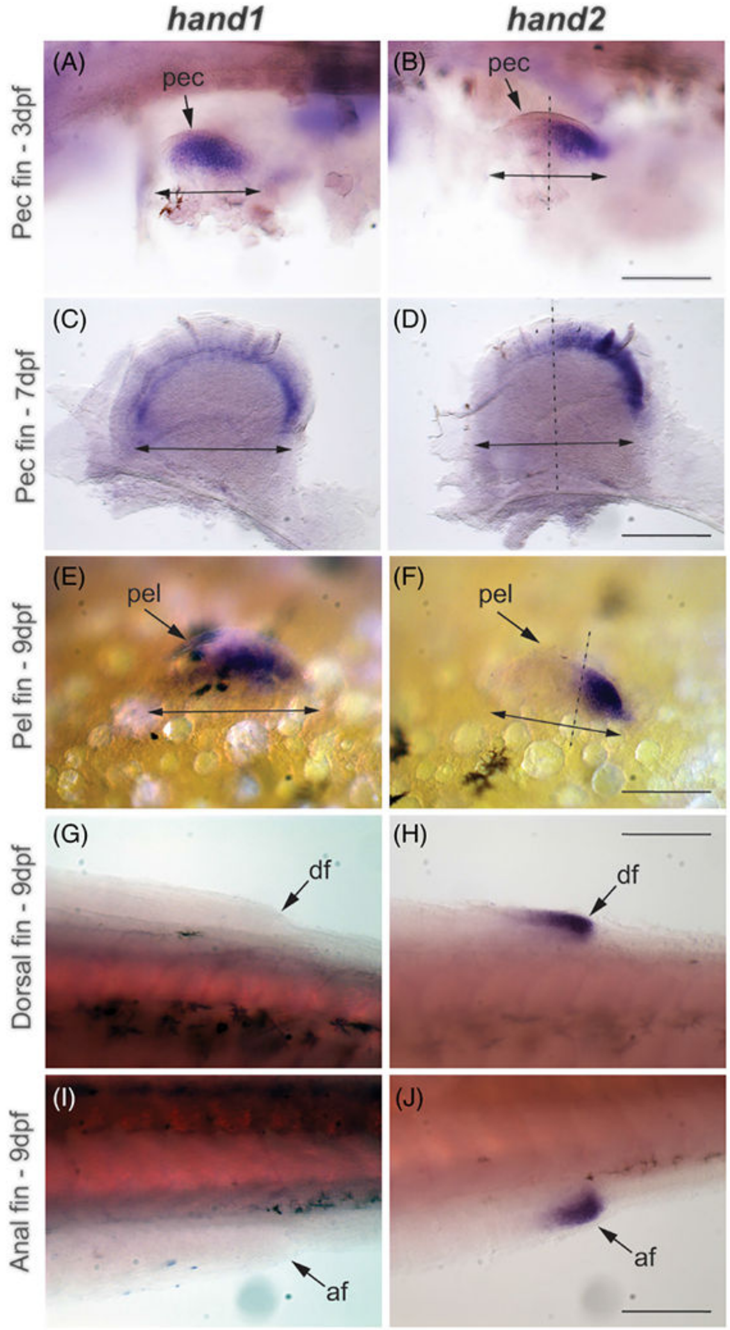

FIGURE 3.

hand1 and *hand2* expression in the developing cichlid paired and median fins. A and B, *hand1*, A, and *hand2*, B, expression in the 3 dpf pectoral fin. *hand1*, A, is expressed in the entire fin mesenchyme, while *hand2*, B, is expressed in the posterior mesenchyme. C and D, *hand1*, C, and *hand2*, D, expression in the 7 dpf pectoral fin (anterior to the left). *hand1*, C, fin base expression is continuous along the A-P axis, while *hand2*, D, expression is restricted to the posterior half of the fin base. E and F, *hand1*, E, and *hand2*, F, expression in the 9 dpf pelvic fin. *hand1*, E, is expressed in the entire fin mesenchyme, while *hand2*, F, is expressed in the posterior mesenchyme. G and H, *hand2*, H, but not *hand1*, G, is expressed in the posterior mesenchyme of the 9 dpf dorsal fin. I and J: *hand2*, I, but not *hand1*, J, is expressed in the posterior mesenchyme of the 9 dpf anal fin. Scale: 25 μm. pec, pectoral fin; pel, pelvic fin; df, dorsal fin; af, anal fin

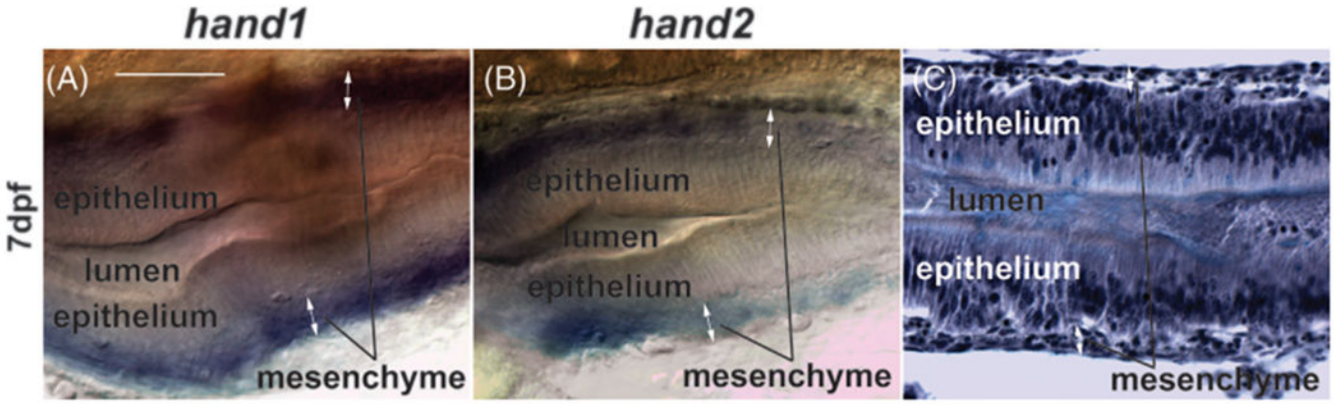

FIGURE 4.

hand1 and *hand2* expression in the developing cichlid gut. A, *hand1* expression throughout the 7 dpf intestinal mesenchyme. B, *hand2* salt and pepper expression in the 7 dpf intestinal mesenchyme. C, sagittal section showing gut histology at 7dpf. Scale: 50 μm

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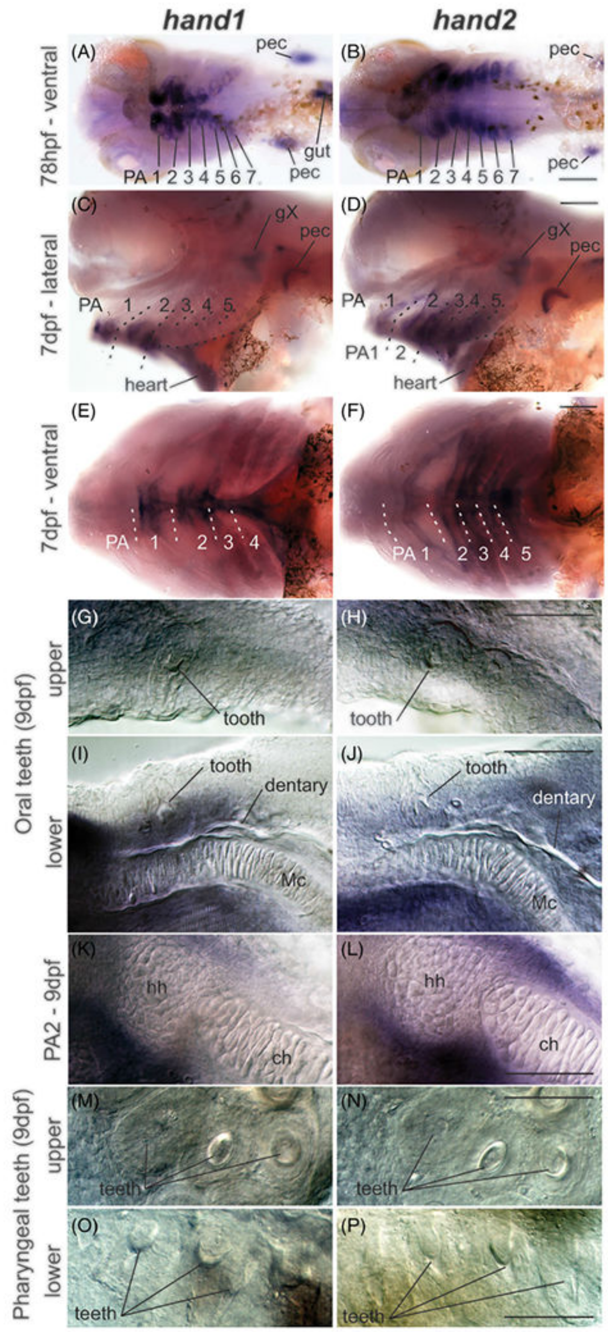

FIGURE 5.

hand1 and *hand2* expression in the developing cichlid pharyngeal arches. A and B, *hand1*, A, and *hand2*, B, overlapping expression in the 3 dpf pharyngeal arches. *hand1*, A, expression is more restricted than *hand1* to the ventral-most domain. C to F: *hand1* (C,E) and *hand2* (D,F) expression in the 7 dpf pharyngeal arches. *hand1* (C, lateral; E, ventral) expression is restricted to pharyngeal arches 1 to 2, while *hand2* (D, lateral; F, ventral) is expressed in all arches. G and H: *hand1*, G, and *hand2*, H, expression is not detected in the 9 dpf upper jaw dental mesenchyme. Dorsal to the top. I and J, *hand1*, I, and *hand2*, J, expression in the 9 dpf lower jaw. *hand1*, I, expression is strongest at the midline (left in image), and in the dental mesenchyme. *hand2* expression is detected in the mesenchyme posterior Meckel's cartilage. Neither gene is detected in Meckel's cartilage. Anterior to the top. K and L, *hand1*, K, and *hand2*, L, expression in the 9 dpf hyoid arch. *hand1* and *hand2* are both expressed in midline mesenchyme in the region connecting to the cardiac outflow tract. *hand2* is also expressed in mesenchyme around the hypo- and cerato-hyal cartilages, L, Anterior to the top. M to P, *hand1* (M,O) and *hand2* (N,P) expression is not detected in the 9 dpf upper (M,N) and lower (O,P) pharyngeal jaw dental mesenchyme. Anterior to the top. ch, ceratohyal; gX, vagal ganglion; hh, hypohyal; Mc, Meckel's cartilage; PA, pharyngeal arch; pec, pectoral fin. Scale: 50 μm

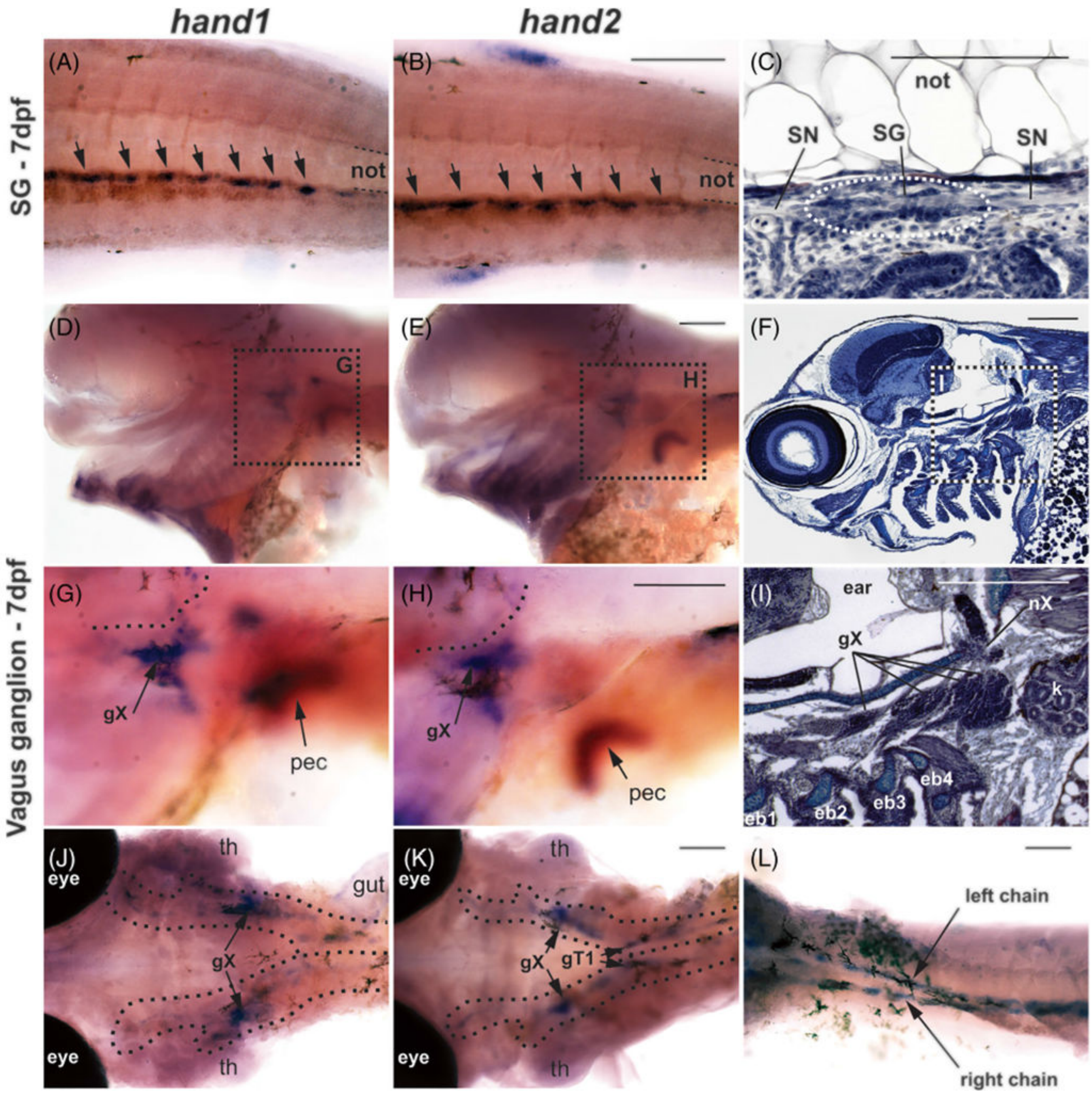

FIGURE 6.

hand1 and *hand2* expression in the developing cichlid PNS. A to C, *hand1*, A, and *hand2*, B, expression in sympathetic ganglia at 7 dpf. C, sagittal section showing sympathetic ganglia at 7 dpf (arrows). D to I, *hand1* and *hand2* are expressed in the vagal ganglion. D and G, *hand1* expression in the vagal ganglion in lateral view. E and H, *hand2* expression in the vagal ganglion in lateral view. F and I, sagittal section showing vagal ganglion histology. J to L, *hand1*, J, and *hand2*, K, expression in the peripheral nervous system extends into the head region and labels the left and right sympathetic ganglion chains, L. Scale: 50 μm. eb1-4, epibranchial 1-4; gT1, first trunk sympathetic ganglion; gX, vagal ganglion; k, kidney; my, myotome; not, notochord; pec, pectoral fin; SG, sympathetic ganglion; SN, sympathetic nerve; th, thymus

TABLE 1

Summary of *Hand* gene expression in chordate embryos

taxon region	Cephalochordates amphioxus	Agnathans lamprey	Gnathostomes: Ray-Finned fishes: zebrafish	Gnathostomes: Ray-Finned fishes: cichlid		Gnathostomes: Tetrapods: frog		Tetrapods: chick		Tetrapods: mouse	
	AmphiHand	***LjHandA***	***hand2***	***hand1***	***hand2***	***Hand1***	***Hand2***	***Hand1***	***Hand2***	***Hand1***	***Hand2***
LPM	yes[6,31]	yes[6]	yes[27,32]	yes	no[33]	yes[35]	no[21]	CC[19]	yes[19]	no[18]	yes[8]
Heart	subintestinal vessel anlag*[31]	yes[6]	unif.[27]	AP unif. epicard.	AP unif. myocard.	unif. [35]	no[21]	unif. [19]	unif. [19]	LV + OFT myocard. [19]	AP unif. then RV myocard.[19] see[14]
Append.	NA	NA	post. (pc) [27]	unif. (pc,pl,dr,an)	post. (pc,pl,dr,an)	?	?	ventral [22]	post. [36]	no bud expr.[18,22]	post. [11,36]
Gut	post. hindgut[31]	ventral to gut[6]	mderm + enteric NC[12]	mchyme	mchyme	?	punctate[21]	?	?	mderm 14	enteric NC[12]
PA's	mderm[6]	yes[6]	NC ventral + aac's[24]	NC ventral most + aac's	NC ventral	yes[35] ventral most	NC ventral[21]	yes[19]	yes[19]	NC ventral most[18,19,49]	NC ventral[8,19]
PNS	NA	NA	cerv. g. [23]	ST	ST	?	?	ST[19]	ST[19]	ST[15,17,18]	ST[15,16]

Abbreviations: aac, arch associated catecholaminergic cells; an, anal fin; AP, anterior-posterior; Append., appendages; CC, cardiac crescent; cer. g., cervical ganglion; epicard., epicardium; expr, expression; LPM, lateral plate mesoderm; LV, left ventricle; mchyme, mesenchyme; mderm, mesoderm; NC, neural crest; OFT, outflow tract; PA, pharyngeal arches; pc, pectoral fin; pi, pelvic fin. dr, dorsal fin; PNS, peripheral nervous system; post., posterior; RV, right ventricle; ST, sympathetic trunks; unif., uniform.