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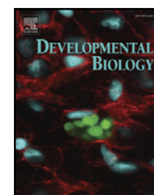

Endoderm-derived Sonic hedgehog and mesoderm Hand2 expression are required for enteric nervous system development in zebrafish

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ABSTRACT

The zebrafish enteric nervous system (ENS), like those of all other vertebrate species, is principally derived from the vagal neural crest cells (NCC). The developmental controls that govern the migration, proliferation and patterning of the ENS precursors are not well understood. We have investigated the roles of endoderm and Sonic hedgehog (SHH) in the development of the ENS. We show that endoderm is required for the migration of ENS NCC from the vagal region to the anterior end of the intestine. We show that the expression of *shh* and its receptor *ptc-1* correlate with the development of the ENS and demonstrate that hedgehog (HH) signaling is required in two phases, a pre-enteric and an enteric phase, for normal ENS development. We show that HH signaling regulates the proliferation of vagal NCC and ENS precursors *in vivo*. We also show the zebrafish *hand2* is required for the normal development of the intestinal smooth muscle and the ENS. Furthermore we show that endoderm and HH signaling, but not *hand2*, regulate *gdnf* expression in the intestine, highlighting a central role of endoderm and SHH in patterning the intestine and the ENS.
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Introduction

The enteric nervous system (ENS) is the largest subdivision of the peripheral nervous system and is derived from the neural crest (Furness, 2006; Le Douarin and Kalcheim, 1999). In all vertebrate species studied the majority of the ENS is derived from vagal neural crest cells (NCC) (Burns, 2005; Heanue and Pachnis, 2007; Newgreen and Young, 2002b). ENS NCC migrate from the vagal region and then associate with the rostral end of the intestine. Subsequently these ENS precursors migrate rostro-caudally along the length of the intestine, continuing to proliferate while they migrate, then differentiate to form a wide variety of neuronal subtypes (Furness, 2006). The migration, proliferation and differentiation of the ENS precursors in the developing gut is dependent on reciprocal signaling interactions between the precursors, the gut mesenchyme, and the gut endoderm. Molecules in the environment as well as lineage restrictions within the ENS precursors determine the number and specific cell fates acquired by these precursors. Failure of ENS precursors to colonize the complete length of the gut results in the absence of enteric ganglia along varying lengths of the colon (colonic aganglionosis) (Heanue and Pachnis, 2007; Newgreen and Young, 2002a,b). This is the most common cause of congenital intestinal obstruction in humans and is clinically referred to as Hirschsprung's disease (HSCR) (Brooks et al., 2005; Gershon and Ratcliffe, 2004).

The molecular mechanisms that control the specification, proliferation and differentiation of the enteric neural crest have been studied extensively *in vivo* and *in vitro*. A number of transcription factors have been implicated in ENS development, including Mash1 (Guillemot et al., 1993), Phox2b (Pattyn et al., 1999), SOX10 (Herbarth et al., 1998; Kapur, 1999; Pattyn et al., 1999; Southard Smith et al., 1998), Hox11L1 (Tlx2) (Hatano et al., 1997; Shirasawa et al., 1997), Hoxb5 (Kuratani and Wall, 1992; Pitera et al., 1999), HAND2 (Cserjesi et al., 1995; D'Autreaux et al., 2007; Hendershot et al., 2007; Howard et al., 1999; Srivastava et al., 1995; Wu and Howard, 2002) and AP-2alpha (Barrallo-Gimeno et al., 2004; Knight et al., 2003; O'Brien et al., 2004). Several secreted signaling molecules and their associated receptors have also been identified that control directly and indirectly the morphogenesis of the ENS. These include GDNF (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Schuchardt et al., 1994), Neurturin (Heuckeroth et al., 1999, 1998; Rossi et al., 1999), Endothelin-3 (Baynash et al., 1994; Hosoda et al., 1994; Yanagisawa et al., 1998), BMP 2/4 (Chalazonitis et al., 2004; Goldstein et al., 2005; Sukegawa et al., 2000; Wu and Howard, 2002), NT-3 (Chalazonitis et al., 2001, 1994), CNTF (Chalazonitis et al., 1998), and Indian hedgehog (Ramalho-Santos et al., 2000). Perturbation of the function of most of these transcription factors and signaling molecules/receptors leads to defects in the ENS. Furthermore, mutations in

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some of these genes have been identified in patients affected with HSCR (Amiel and Lyonnet, 2001; Brooks et al., 2005; Puri et al., 1998).

Recently we have shown that endoderm-derived cues are essential for normal ENS development in zebrafish (Pietsch et al., 2006). To further investigate the role of endoderm in zebrafish ENS development and identify specific endoderm dependent signaling pathways involved in this developmental process, we have focused on the *in vivo* function of the hedgehog (HH) signaling pathway, and specifically Sonic hedgehog (SHH), in zebrafish ENS development.

SHH is a member of the Hedgehog family of signaling molecules that was identified by its homology to the Drosophila segment polarity gene *hedgehog*. SHH controls the differentiation and proliferation of numerous cell types in a variety of tissues (Ingham and McMahon, 2001; McMahon et al., 2003). In all vertebrates examined SHH is expressed along the rostro-caudal extent of the gut endoderm (Bitgood and McMahon, 1995; Echelard et al., 1993; Krauss et al., 1993; Marigo et al., 1995; Roberts et al., 1995; Stolow and Shi, 1995). Subsequent studies have demonstrated that SHH signaling is required for normal patterning of the gut mesenchyme and the ENS plexuses in mouse and avian systems (Fu et al., 2004; Ramalho-Santos et al., 2000; Sukegawa et al., 2000). More recently it has been shown *in vitro* that SHH regulates ENS NCC proliferation (Fu et al., 2004). This is significant as proliferation has been recently shown to be a major mechanism driving the invasion of ENS NCC along the gut (Simpson et al., 2007). Furthermore, Fu and colleagues' study suggested that SHH modulates ENS NCC's chemotactic responsiveness to GDNF. GDNF has been previously shown to act as a chemoattractant for ENS precursors (Natarajan et al., 2002; Young et al., 2001).

In this work we have further investigated the role of endoderm in zebrafish ENS development. We show that endoderm-derived factors are required not only for the migration of ENS precursors along the intestine, but also for the initial migration of NCC from the vagal premigratory crest to the anterior end of the intestine. We have also investigated the function of the *hand2* transcription factor that is expressed in the intestinal mesoderm-derived mesenchyme in zebrafish at early stages of intestinal development. We find that *hand2* function is not required for the initial migration of vagal NNCs to the anterior end of the intestine but it is necessary for the migration/proliferation of ENS precursors along the intestine and their subsequent differentiation. We show that SHH secreted from the intestinal endoderm is necessary and sufficient to enable NCC to migrate from the premigratory vagal region to the anterior end of the intestine. We also show that SHH is subsequently required for the migration of ENS NCC along the intestine. We show that SHH acts as a mitogen and is required for the normal proliferation of vagal NCC that gives rise to the ENS precursors. Finally we present data showing that endoderm and HH signaling are required for the normal expression of *gdnf* in the zebrafish intestine and that the absence of *gdnf* expression, when HH signaling is perturbed, correlates with the lack of migration of vagal neural crest cells to and along the intestine.

Materials and methods

Zebrafish maintenance and breeding

Fish were raised and kept under standard laboratory conditions at 28.5 °C (Westerfield, 1993). Embryos were staged and fixed at specific hours or days post fertilization (hpf or dpf) as described Kimmel et al. (1995). To better visualize internal structures in some experiments embryos were incubated with 0.2 mM 1-phenyl-2-thiourea (Sigma) to inhibit pigment formation (Westerfield, 1993). *handsoff* (Yelon et al., 2000) fish line was obtained from Deborah Yellon and the *syu* (Brand et al., 1996; Schauerte et al., 1998) and *smu* fish lines were obtained from Philip DiIorio.

Immunohistochemistry

Embryos were processed for immunohistochemistry as previously described (Raible and Kruse, 2000). Differentiated enteric neurons were revealed with the anti-Hu mAb 16A11 (Molecular Probes) that labels differentiated neurons (Marusich et al., 1994) or by immunohistochemically staining embryos obtained from the *HuC-GFP* transgenic line (Park et al., 2000) that expresses GFP in differentiated neurons with a rabbit polyclonal anti-GFP antibody (Molecular Probes). Vagal neural crest cells and migrating ENS precursors were identified in *FoxD3-GFP* transgenic fish line (Gilmour et al., 2002; Lister et al., 2006) immunocytochemically using a mouse monoclonal anti-GFP antibody and the rabbit polyclonal anti-GFP antibody (Molecular Probes). Proliferating cells were identified using a monoclonal anti-phosphohistone H3 antibody (Upstate) (Ajiro et al., 1996). Cells undergoing apoptosis were identified using an anti-activated Caspase-3 rabbit polyclonal antibody (BD Biosciences). The anti-Hu mAb was visualized using an Alexa Fluor 568 anti-mouse IgG antibody (Molecular Probes), the rabbit polyclonal antibodies were visualized using either an Alexa Fluor 568 or an Alexa Fluor 488 anti-rabbit IgG antibody (Molecular Probes) and the mouse anti GFP antibody was visualized using an Alexa Flour 488 anti-mouse IgG antibody (Molecular Probes).

Whole-mount in situ hybridization

Embryos were collected and processed for whole-mount *in situ* hybridization as previously described (Thisse et al., 1993). Digoxigenin-labeled riboprobes used in this study were synthesized from templates linearized and transcribed as follows: *crestin* (Rubinstein et al., 2000), *Sac*I and T7; *phox2b* (Shepherd et al., 2004), *Not*I and T7; *hand2* (Angelo et al., 2000) *Bam*H1 and T7; *myh11* (Wallace et al., 2005b) *Bam*H1 T7; alpha-*smooth muscle actin* (α *SMA*) (Georgijevic et al., 2007) *Bam*H1 T7; *gdnf* (Shepherd et al., 2001) *Not*1 T3; *ptc-1* (Concordet et al., 1996), *Bam*HI and T3; *sHH* (Ekker et al., 1995), *Hin*dII and T7; *ret* (Bisgrove et al., 1997), NotI and T7; and *foxA3* (Odenthal and Nusslein-Volhard, 1998), *Apa*I and T3. Digoxigenin-labeled probes were visualized with NBT/BCIP coloration reactions. Double-label wholemount fluorescent *in situ* hybridization (FISH) was carried out as described (Filippi et al., 2007). Confocal z-stacks of whole-mount FISH embryos were recorded using a Zeiss LSM 510 laser scanning confocal microscope. Cross-sections of *in situ* hybridized embryos were made by embedding embryos in 1 ml gelatin-albumin (25 mg gelatin, 1.3 g BSA, 0.9 g saccharose, in 4.5 ml PBS) for 1 h at room temperature. The gelatin–albumin was then replaced with 1 ml of gelatin–albumin +35 μl of 50% glutaraldehyde. To harden the embedding mixture embryos were incubated at 4 °C overnight. Sections were cut with a Leica vibratome at 10–20 μm.

Embryonic microinjections

shh mRNA was synthesized using the mMessage mMachine kit (Ambion) and injected at a concentration of 150 ng/μl. Approximately 4 nl of diluted mRNA was injected into 1- to 2-cell embryos using a gas-driven microinjection apparatus (PV820 WPI) through a micropipette.

sox32 (casanova) morpholino antisense oligonucleotide (Gene Tools) was designed to the previously described morpholino translation blocking sequence (Dickmeis et al., 2001). *hand2* morpholino was designed to target the translation start site of the gene to the following sequence:

5′ CCTCCAACTAAACTCATGGCGACAG 3′

The morpholino phenocopied the *handsoff* zebrafish deletion mutant (Yelon et al., 2000). A 5-base pair mismatch *hand2* control morpholino failed to illicit any phenotype. The oligos were resuspended in sterile filtered water and diluted to working concentrations in a range between 1 and 5 μg/μl. Approximately 1 nl of diluted morpholino was injected into 1- to 2-cell embryos using a gas-driven microinjection apparatus.

Cyclopamine treatment

Cyclopamine (Toronoto Research Company) was dissolved in 100% ethanol to generate a 10 mM stock solution. Subsequently this stock solution was further diluted in embryo media to give a working concentration of 10 μM. Wild type embryos were soaked in the cyclopamine containing embryo medium at 28 °C for 24–36 hpf or 36–60 hpf after which the embryo media was replaced with fresh embryo media that did not contain cyclopamine. Embryos were then incubated until they were fixed and processed for immunohistochemistry or *in situ* hybridization.

Transplantation experiments

Wild-type donor embryos used in transplant experiments were from an AB/TL strain. Host embryos were generated from an in cross of *syu* or *smu* heterozygotes. Donor embryos at the 1- to 2-cell stage were injected with 1.2 ng of *sox32* mRNA combined with 2% of tetramethylrhodamine lysine fixable biotin dextran (10 K MW_r, Molecular Probes) to convert most of the donor cells to an endodermal cell fate (Holzschuh et al., 2005; Stafford et al., 2006). *sox32* mRNA was synthesized using the mMessage mMachine kit (Ambion) as described above. At the sphere stage, 20–40 donor cells were transplanted into unlabelled sibling host embryos. Embryos were then cultured in embryo medium with 10 U/ml penicillin and 10 U/ml streptomycin. Host embryo ENS development was determined in 60 hpf embryos by *phox2b in situ* hybridization. Donor cells from the transplant were detected in hosts using an avidin-biotinylated complex (ABC kit, Vectastain) and a DAB substrate. Embryos were embedded and sectioned as described in the wholemount *in situ* hybridization section. Genotypes of the host embryos were determined by phenotypic appearance typical for *shh*−/−, *smu*−/− or wild type embryos.

Results

Requirements for endoderm and mesodermal hand2 expression in zebrafish ENS development

In zebrafish ENS precursors first migrate from the post-otic (vagal) region to the anterior end of the intestine beginning around 30 hpf and continue to do so until 40 hpf. At this stage the intestine is little more than a rod of endoderm that has not yet formed a lumen (Ng et al., 2005; Wallace et al., 2005a; Wallace and Pack, 2003). A thin layer of loosely associated mesodermal-derived mesenchyme surrounds the endoderm that will form the smooth muscle of the intestine beginning around 60 hpf (Georgijevic et al., 2007; Wallace et al., 2005a). Between 34 and 36 hpf *phox2b* expressing ENS precursors can be first observed closely associated with the anterior end of the intestinal endoderm (Elworthy et al., 2005; Shepherd et al., 2004). Subsequently ENS precursors migrate as two symmetric streams either side of the rod of endoderm reaching the distal end of the

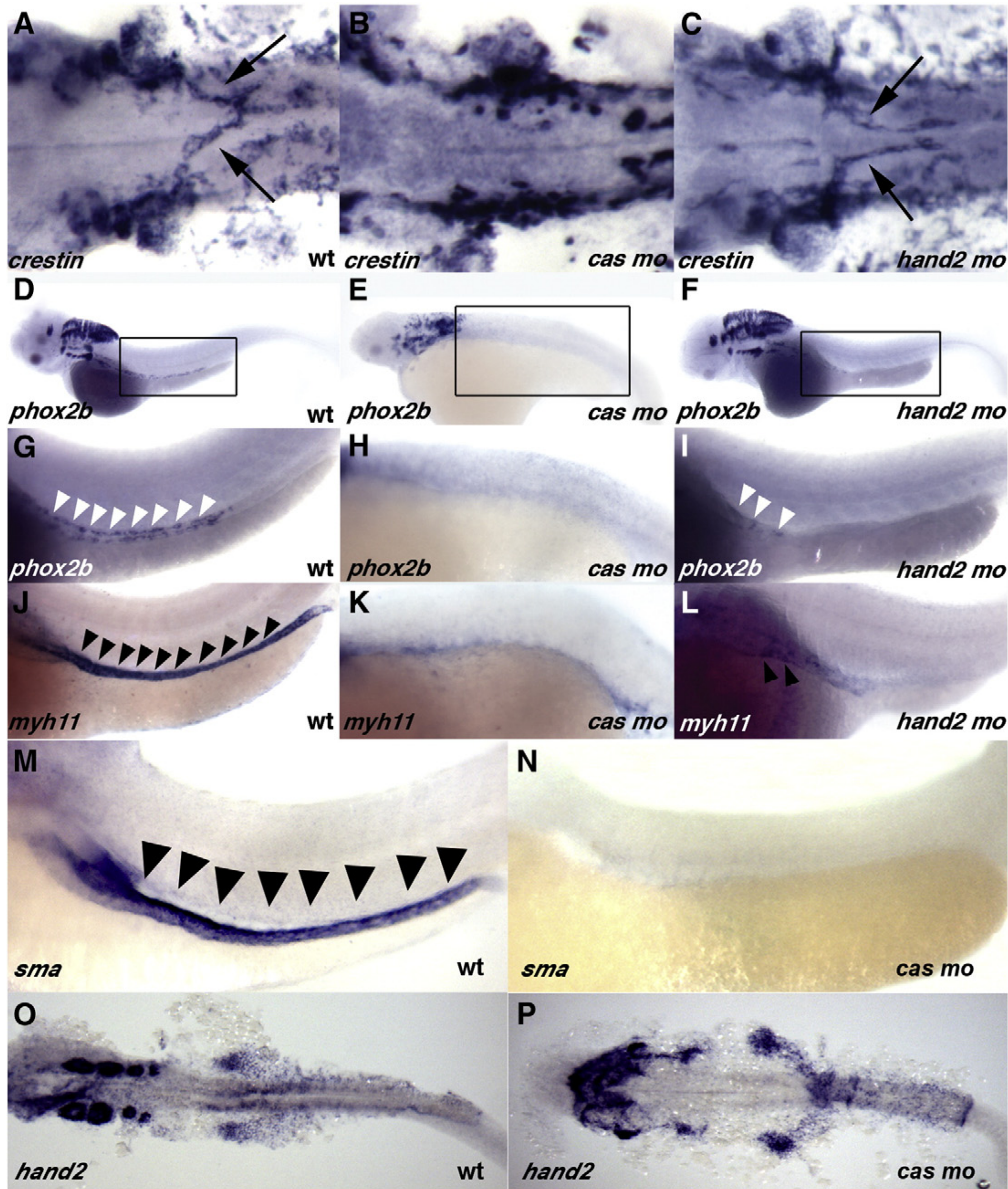

Fig. 1. Endoderm and mesodermal *hand2* function are required for normal ENS and intestinal smooth muscle development but *hand2* is not required for the initial migration of vagal neural crest to the anterior end of the intestine and endoderm is not required for mesodermal *hand 2* expression. (A, D, G, J, M, O) wild-type embryos, (B, E, H, K, N, P) *cas/sox32* morphant embryos and (C, F, I, L) *hand2* morphants. (A–C) Ventral view of the vagal region of 36 hpf embryos that have been hybridized with riboprobes for *crestin* showing a failure of vagal NCC migration to the anterior end of the intestine in *sox32* morphants but no affect on these NCC in *hand2* morphants. (D–F) Lateral view of 60 hpf embryos that have been hybridized with riboprobes for *phox2b*. (G–I) Close up lateral view of the intestine of 60 hpf embryos that have been hybridized with riboprobes for *phox2b* showing a failure of *phox2b* expressing cells to populate the entire length of the intestine in *cas/sox32* and *hand2* morphants. (J–L) Close up lateral view of the intestine of 60 hpf embryos that have been hybridized with riboprobes for *myh11* showing a reduction/loss of *myh11* expressing intestinal smooth muscle cells in the intestine of *cas/sox32* and *hand2* morphants. (M, N) Lateral view of the intestine of 72 hpf embryos wholemount *in situ* hybridized embryos that have been hybridized with riboprobes for α *SMA*. Black boxes in panels D–F are the regions that are shown in close up in panels G–I. Arrowheads (M) indicate the α*SMA* expressing cells in the intestine. (O, P) Ventral view of 30 hpf wholemount *in situ* hybridized embryos that have been hybridized with an antisense probe for *hand2*. Arrows (A, C) indicate the migrating enteric precursors. White arrowheads (G, I) indicate *phox2b* expressing cells in the intestine. Black arrowheads (J, L) indicate *myh11* expressing cells in the intestine. Black arrowheads (M) indicate the α*SMA* expressing cells in the intestine Anterior is to the left.

intestine by 60 hpf (Elworthy et al., 2005; Shepherd et al., 2004). Previously we have shown that endoderm is required for the normal migration of the ENS precursors along the intestine (Pietsch et al., 2006) (Fig. 1). To determine whether endoderm is required earlier in ENS development for the migration of ENS precursors from the premigratory vagal crest region to the ventral midline, we hybridized *sox32 (casanova)* morphants at 36 hpf with probes for *crestin* or *ret*. *sox32* morphants exhibit cardiabifida and completely lacked intestinal endoderm (Dickmeis et al., 2001; Kikuchi et al., 2001). No distinct chevron of migrating ENS NCC precursors was observed in the morphant embryos (Figs. 1A, B). To further determine if other aspects of intestinal development are perturbed in the *sox32* morphants we examined whether the intestinal mesoderm-derived mesenchymal cells that gives rise to the intestinal muscle layers continue to develop normally in these morphants. Expression of the smooth muscle markers *myh11* and *alpha smooth muscle actin (αSMA)* are severely perturbed or absent in *sox32* morphant embryos (Figs. 1J, K, M, N). By contrast expression of the lateral plate mesoderm marker *hand2* is not perturbed in *sox32* morphants (Figs. 1O, P).

We next investigated the function of *hand2* in the development of zebrafish ENS. In the zebrafish intestine *hand2* is expressed in the lateral plate mesoderm-derived mesenchyme at stages prior to and during the period when ENS NCC's are migrating along the intestine (Figs. 1O and 2B). We hypothesized that zebrafish *hand2* has functions in intestinal development that include those that are normally carried out by *hand1* in mouse. Mouse *hand1* is expressed in intestinal mesenchyme whereas mouse *hand2* is not (D'Autreaux et al., 2007). We therefore determined the effect of morpholino knockdown of *hand2* on intestinal smooth muscle development and ENS NCC migration. We also determined whether the *hand2* morphant intestinal smooth muscle and ENS phenotypes were the same as that in *handsoff* mutant fish that have a deletion of the *hand2* gene (Yelon et al., 2000). Consistent with our hypothesis, *handsoff* mutant embryos and *hand2* morphant embryos fail to express the intestinal smooth muscle markers *myh11* at 60 hpf (Fig. 1L). In addition ENS precursors fail to migrate along the intestine at 60 hpf in *hand2* morphants as determined by *phox2b in situ* (Figs. 1F, I). Subsequently there is a significant decrease in the number of differentiated ENS neurons at 96 hpf in morphants as determined by Hu immunoreactivity (Fig. 2E). To determine if the initial migration of ENS precursors from the premigratory vagal neural crest to the anterior end of the intestine is also perturbed in *hand2* morphants we fixed morphants at 36 hpf and examined *crestin* expression. In contrast to the *sox32* morphants, migration of the ENS precursors to the anterior end of the intestine is not perturbed in the *hand2* morphants (Figs. 1B, C). Taken together these experiments suggest that both endoderm and *hand2* are required for normal ENS and intestinal smooth muscle development in zebrafish. However only endoderm is required for the migration of ENS precursors from the premigratory vagal neural crest region to the anterior end of the intestine.

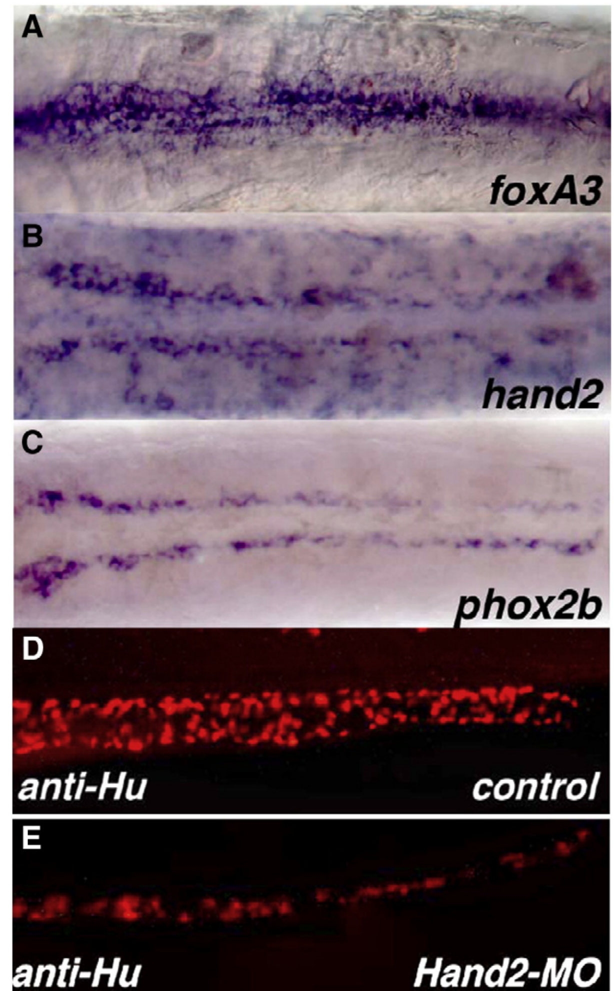

Fig. 2. Comparison of the expression of *hand2* to endodermal marker *foxA3* and ENS precursors marker *phox2b* and the reduction of ENS neurons in *hand2* morphants. (A–C) Ventral views of the intestine of 60 hpf wholemount *in situ* hybridized embryos that have been *in situ* hybridized with riboprobes for *foxA3* (A), *hand2* (B) and *phox2b* (C). (D, E) Lateral views of the intestine of 96 hpf wild type (D) and *hand2* morphant (E) embryos that have been stained with an anti-Hu antibody showing that there is a reduction in the overall neuron number in *hand2* morphants as compared to wild type. (A–C) Yolk has been removed from the embryos. Anterior is to the left.

Expression of Sonic hedgehog and hedgehog signaling pathway components during ENS development

Previous studies have shown that SHH acts a mitogen *in vitro* for purified enteric neural crest cells and in turn modulates the ENS NCC migratory stimulating activity of GDNF (Fu et al., 2004). Furthermore, previous studies in mouse and avian have shown expression of *shh* correlates with ENS development and genetic or pharmacological perturbation of HH activity in the intestine causes ENS patterning defects (Ramalho-Santos et al., 2000; Sukegawa et al., 2000).

To determine if *shh* and a component of its receptor, *patched-1 (ptc1)*, have expression patterns that correlate with the migration of ENS NCC precursors from the premigratory vagal crest region to the anterior end of the intestine and along it, we examined the expression patterns of these genes. The location and distribution of ENS NCC was determined in age-matched embryos/sections using *crestin* or *phox2b*. *shh* expression is first detected at the anterior end of the intestine around 30 hpf (Figs. 3A, B). Expression is restricted to this anterior region of the intestine just prior to the stage when ENS NCC first migrate from the vagal premigratory neural crest to this part of the gut. This pattern of expression continues through 36 hpf, the stage at which the chain of ENS precursors migrating to the anterior end of the intestine can be clearly seen (Fig. 1A). *ptc1* is widely expressed throughout the mesenchyme adjacent to the endoderm and is expressed by ENS NCC (Figs. 3G–L). This pattern of *shh* and *ptc1* expression is consistent with SHH having a direct role in zebrafish ENS development.

ENS development is perturbed in hedgehog signaling pathway mutants and in cyclopamine treated embryos

To determine the functional role of SHH in zebrafish ENS development, we examined the expression of ENS NCC markers *phox2b*, *ret* and *crestin* in the hedgehog pathway homozygous mutants *sonic you (syu)*, that has a deletion in the *shh* gene (Brand et al., 1996; Schauerte et al., 1998), and *smooth muscle-omitted (smu)*, that has a

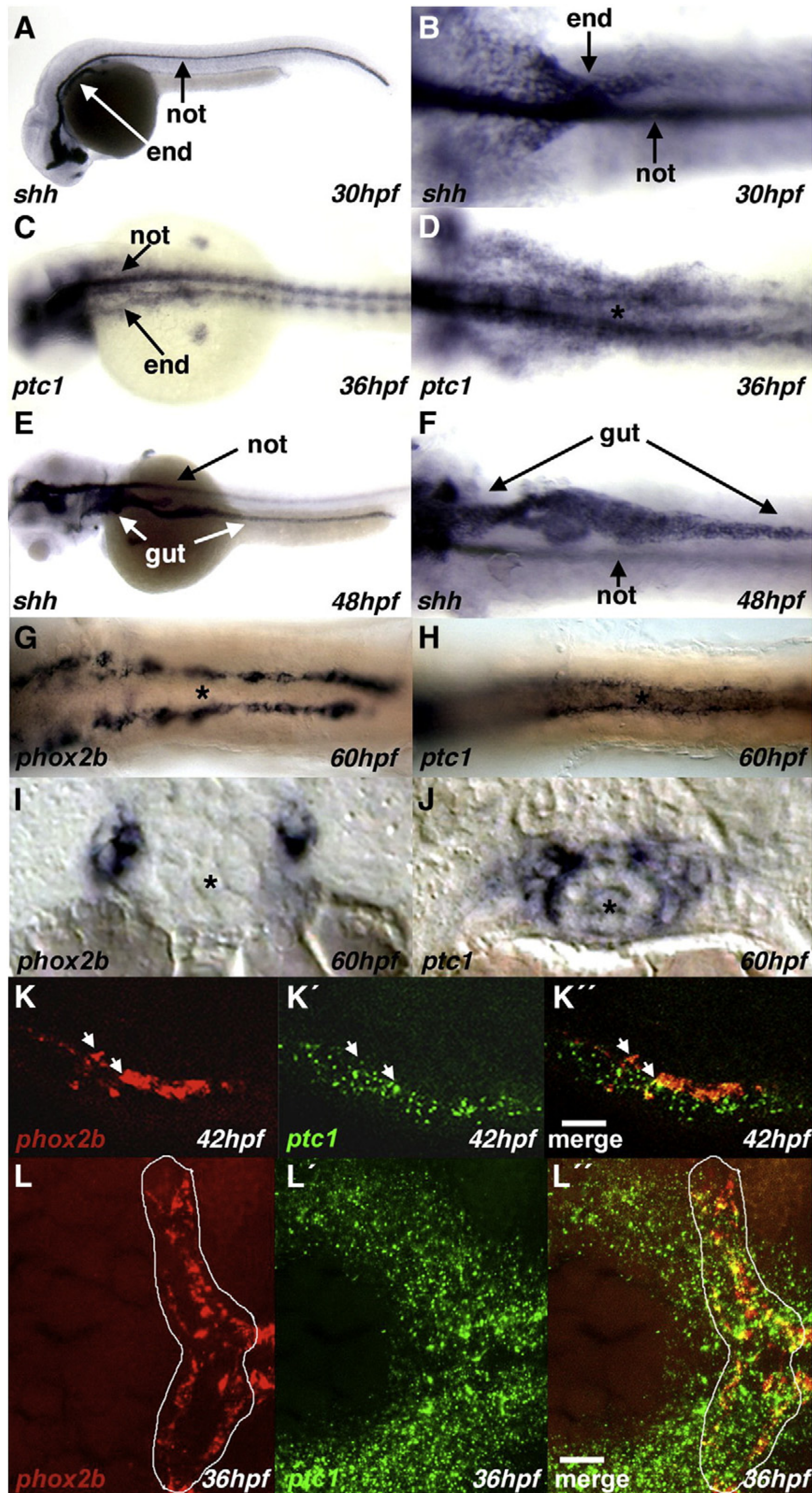

Fig. 3. The expression pattern of *shh* and *ptc-1* correlate with the development of ENS. (A, B, E, F) Wholemount embryos hybridized *in situ* with a *shh* antisense probe at the indicated developmental stages. (C, D, H) Wholemount embryos hybridized *in situ* with a *ptc-1* antisense probe at the indicated developmental stages. (G) 60 hpf wholemount *in situ* hybridized embryos hybridized with a *phox2b* antisense probe to reveal the distribution of the ENS NCC in the intestine. (I) Cross-section taken through a 60 hpf embryo hybridized with a *phox2b* antisense probe to reveal the distribution of ENS NCC in the intestine. (J) Cross-section taken through a 60 hpf embryo hybridized with a *ptc-1* antisense probe. At all stages examined, *shh* and *ptc-1* are expressed in regions that correlate with the development of the ENS. Furthermore, comparison of the pattern of *ptc-1* expression in the intestine at 60 hpf shows that *phox2b* expressing ENS NCC are located in the *ptc-1* expression domain. (K–K″) Comparison of the pattern of *ptc-1* expression (K′, green channel) to that of *phox2b* (K, red channel) in the intestine at 42 hpf by double fluorescent *in situ* hybridization shows that *phox2b* expressing ENS NCC co-express ptc-1 (K″, merge channels). Expression was documented by confocal stacks of images (L–L″). Ventral view of the vagal region of a 36 hpf wildtype embryo showing a comparison of the pattern of *ptc-1* expression (L′, green channel) to that of *phox2b* (K, red channel) by double fluorescent *in situ* hybridization showing pre-enteric ENS NCC's express *ptc-1* (K″, merge channels). (A) Lateral view of embryo. (B, D, F, G, H, K–K″, L–L″) Ventral views of embryos with the yolk removed. (C, E) Dorsal views of embryo. not (A, B, C, E, F) indicates notochord. end (A–C) indicate endoderm. * (D, G, H, I, J) indicates the gut lumen. Arrows (K–K″) indicate *ptc-1 phox2b* coexpressing cells. The cells delineated by the white line in (L, L″) are the migrating pre-enteric ENS NCC. Scale bar (K″, L″) is 20 μm. In all wholemounts (A–H) anterior is to the left.

mutation in zebrafish *smoothened (smo)* which completely abolishes all HH signaling (Barresi et al., 2000; Chen et al., 2001; Varga et al., 2001). At 60 hpf both *syu* and *smu* mutants completely lack any ENS precursors along the length of the intestine as compared to wild type controls (Figs. 4A–C). To determine if the initial migration of ENS precursors to the anterior end of the intestine is similarly perturbed in these mutants, the pattern of *ret* and *crestin* expression was examined in 36 hpf *syu* and *smu* mutants as compared to wildtype embryos. No migrating ENS NCC's were observed in either mutant using either marker (Figs. 4D–I).

To further define the precise temporal requirements for HH signaling in normal ENS development we treated wildtype zebrafish embryos with cyclopamine at specific time points during embryogenesis. Cyclopamine is a steroidal alkaloid that inhibits activation of the HH signaling pathway by directly binding to Smo (Chen et al., 2002; Cooper et al., 1998; Incardona et al., 1998). In addition, this experiment also determined if the absence of ENS observed in the *syu* and *smu* mutants is a secondary phenotype, due to the loss of HH signaling at earlier stages of embryogenesis that perturbed vagal crest specification and patterning. Previously it has been shown that HH signaling is required for survival, migration and patterning of cranial neural crest populations in xenopus, avian and zebrafish (Ahlgren and Bronner-Fraser, 1999; Barresi et al., 2000; Brand et al., 1996; Brito et al., 2006; Charrier et al., 2001; Dunn et al., 1995; Kimmel et al., 2001; Varga et al., 2001; Wada et al., 2005). However HH signaling is not directly required for neural crest formation in zebrafish (Ungos et al., 2003). To determine the precise temporal requirement for HH signaling in ENS development, embryos were treated with cyclopamine either from 24–36 hpf, during the pre-enteric phase of ENS development, or from 36–60 hpf, referred to as the enteric phase of ENS development in this paper. The embryos were then fixed at 60 hpf and process for *in situ* hybridization with a *phox2b* probe to determine if the pattern of ENS precursor migration along the intestine was perturbed. The pattern of intestinal smooth muscle development was also assessed at 60 hpf, based on *myh11* expression and at 72 hpf based on α *SMA* expression. Other cyclopamine treated embryos were fixed at 96 hpf to determine the state of differentiation of ENS neurons by anti Hu immunohistochemistry.

Cyclopamine treatment caused an almost complete loss of ENS precursors along the intestine in 24–36 hpf treated embryos as compared to controls (Figs. 5A, B). A similar though slightly less severe reduction in the number of ENS precursors was seen in 36–60 hpf treated embryos (Fig. 5C). Under both experimental conditions the small number of *phox2b* expressing precursors remaining were restricted to the anterior end of the intestine up to somite 7. Similarly at 96 hpf no differentiated enteric neurons could be detected along the length of the gut in 24–36 hpf cyclopamine treated embryos, while only a very few neurons could be seen in 36–60 hpf cyclopamine treated embryos as compared to control embryos (Figs. 5G–I).

Consistent with previous experiments examining the affect of temporal treatment of cyclopamine on DRG development we observed varying affects on DRG number and size depending on the period of cyclopamine treatment (Ungos et al., 2003). Surprisingly expression of the intestinal smooth muscle markers *myh11* at 60 hpf and α *SMA* at 72 hpf were equally perturbed in both 24–36 hpf and 36–60 hpf treated embryos (Figs. 5D–I). However expression of *myh11* was less affected in the pharyngeal arches in 24–36 hpf embryos than the 36–60 hpf treated embryos.

To determine if the initial migration of ENS precursors from the vagal premigratory crest to the anterior end of the intestine is perturbed in the 24–36 hpf treated embryos we fixed treated embryos at 36 hpf and stained with *crestin*. At 36 hpf no chevron of ventromedial migrating vagal ENS NCC could be detected in the 24–36 hpf cyclopamine treated embryos (Fig. 6). Taken together these data suggest that there are two phases of HH signaling required for normal ENS development in zebrafish. HH signaling is required in a pre-enteric phase when the ENS NCC precursors migrate to the anterior end of the intestine between 24 and 36 hpf. Subsequently HH signaling is required from 36–60 hpf when the ENS NCC precursors migrate along the intestine.

Proliferation of vagal NCC and ENS precursors is decreased in cyclopamine treated embryos

To determine the mechanism that causes the failure of the ENS NCC to populate the intestine in cyclopamine treated embryos we examined whether there is an increase in apoptosis or a decrease in proliferation in treated embryos.

To assess if there is an increase in apoptosis in cyclopamine treated embryos we determined the pattern of activated Caspase 3 immunoreactivity in vagal NCC and ENS precursors at 30 hpf and 48 hpf in control and experimental embryos. We used *foxD3::gfp* embryos for these studies as they express green fluorescent protein (GFP) in vagal NCC and ENS precursors (Gilmour et al., 2002; Lister et al., 2006). No increase in apoptosis was observed in these cells in cyclopamine

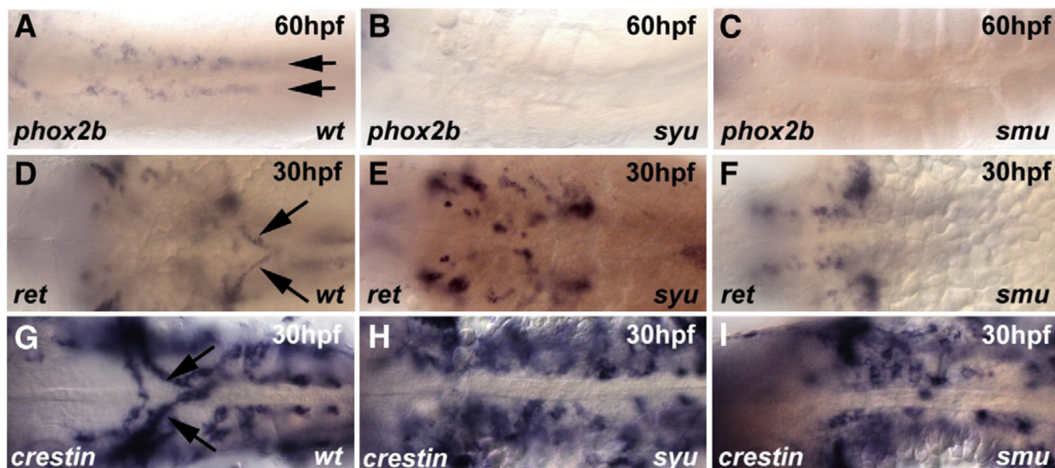

Fig. 4. Hedgehog pathway mutants lack enteric ganglia. (A–I) Dorsal views, anterior to the left. Enteric ganglia colonizing the gut express *phox2b* in wild type (A, black arrows) while $syu^{-/-}$ (B) and $smu^{-/-}$ (C) embryos show no *phox2b* expression around the gut tube. (D) Expression of *ret* in 36 hpf wild type embryos in migrating enteric precursors (black arrows). In $syu^{-/-}$ (E) and $smu^{-/-}$ (F) no *ret* expressing migrating cells can be detected. Wild type embryos at 36 hpf show *crestin* expression in vagal neural crest cells migrating to the anterior gut (G, black arrows). $syu^{-/-}$ (H) and $smu^{-/-}$ (I) embryos lack *crestin* expressing vagal neural crest cells.

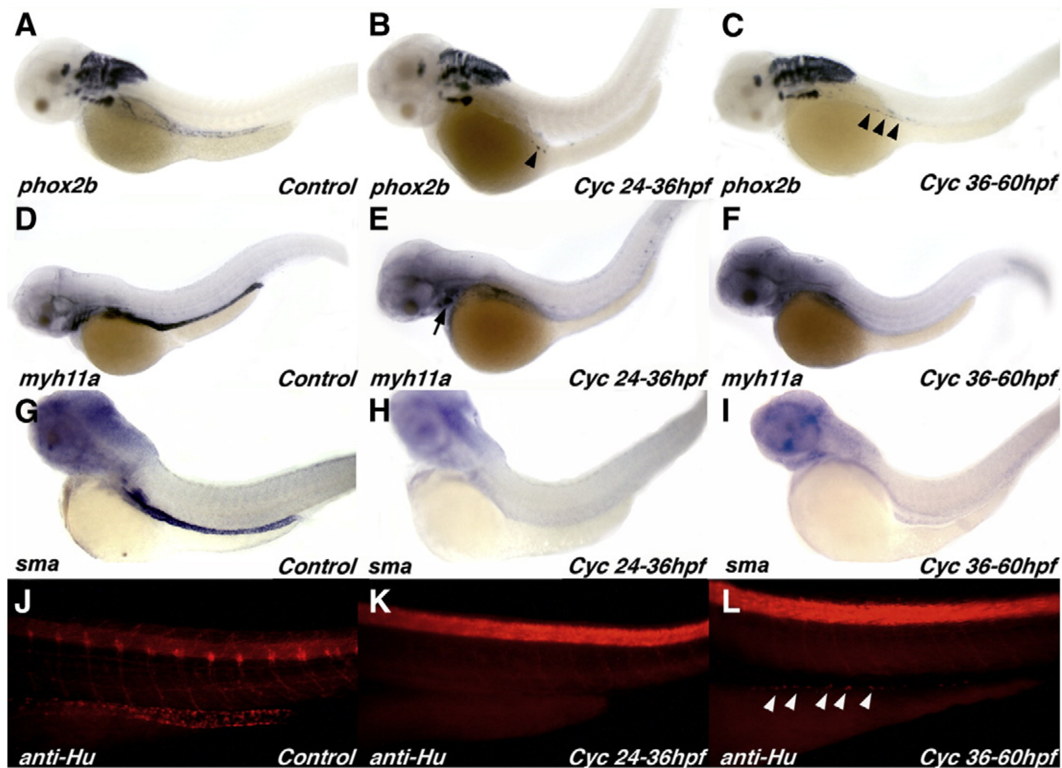

Fig. 5. Cyclopamine treatment causes a failure of enteric precursors to populate the entire length of the intestine and to differentiate into enteric neurons and also results in a failure of intestinal smooth muscle differentiation. (A, D, G, J) Wildtype control embryos, (B, E, H, K) 24–26 hpf cyclopamine treated embryos (C, F, I, L) 36–60 hpf cyclopamine treated embryos. (A–C) Lateral view of 60 hpf wholemount embryos hybridized *in situ* with a *phox2b* antisense probe to reveal the distribution of ENS NNC precursors in the intestine. Treatment with cyclopamine results in a significant reduction/absence of ENS precursors along the length of intestine. (D–E) Lateral view of 60 hpf wholemount *in situ* hybridized embryos hybridized with a *myh11* antisense probe. Treatment with cyclopamine results in a loss of this early smooth muscle marker expression in the intestine. (G–I) Lateral view of 72 hpf wholemount *in situ* hybridized embryos hybridized with a α *SMA* antisense probe. Treatment with cyclopamine results in a loss of this late smooth muscle marker expression in the intestine. (J–L) Lateral views of 96 hpf *HuC-gfp* embryos stained with anti-GFP antibody to shows a significant reduction in the number of enteric neurons in the intestine of cyclopamine treated embryos. Treatment conditions are indicated in lower right corner of each panel. Black arrowheads (B, C) indicate the few *phox2b* expressing cells in the intestine of cyclopamine treated embryos. Arrow (E) indicates pharyngeal arch *myh11* expression. White arrowheads (L) indicate differentiated neurons in the intestine. Anterior is to the left.

treated embryos (Figs. 7A, C data not shown). This suggests that apoptosis is not the mechanism that causes the loss of ENS precursors in cyclopamine treated embryos.

We next investigated whether there is a decrease in proliferation in the vagal NCC and ENS precursors due to the cyclopamine treatment. 30 hpf cyclopamine treated and control *foxD3::gfp* transgenic embryos were immunocytochemically stained with an anti-phosphohistone H3 antibody to identify proliferating cells (Ajiro et al., 1996; Pietsch et al., 2006). A statistically significant decrease was observed in the number of proliferating vagal NCC's and ENS precursors in cyclopamine treated embryos as compared to controls (Figs. 7B, D). Only 6.8±4.3% of vagal NCC and ENS precursors were seen to be proliferating in cyclopamine treated embryos vs. 21.2±5.1% in control embryos (n=5). This statistically significant result (P=0.002 using Student's t-test) suggests that reduced proliferation of the vagal NCC causes the loss of ENS precursors in the intestine and is consistent with recent data from chick that suggests cell proliferation drives neural crest cell invasion of the intestine (Simpson et al., 2007).

The number of ENS precursors in the intestine is increased by over expression of Sonic hedgehog

Given that a loss of HH signaling caused a reduction in ENS precursors, we wanted to determine if overexpression of SHH early in

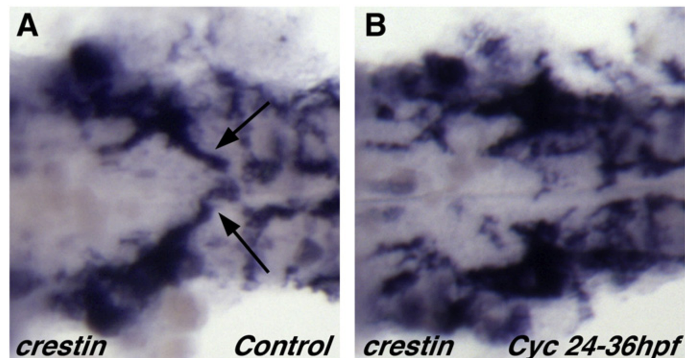

Fig. 6. ENS NCC fail to migrate from the vagal region to the anterior end of the intestine in 24–36 hpf cyclopamine treated embryos. (A, B) Ventral view of the anterior end of the intestine in 36 hpf wildtype (A) and 24–36 hpf cyclopamine treated (B) embryos that have been hybridized with an antisense probe to *crestin*. Arrows indicate migrating ENS NCC. Anterior is to the left.

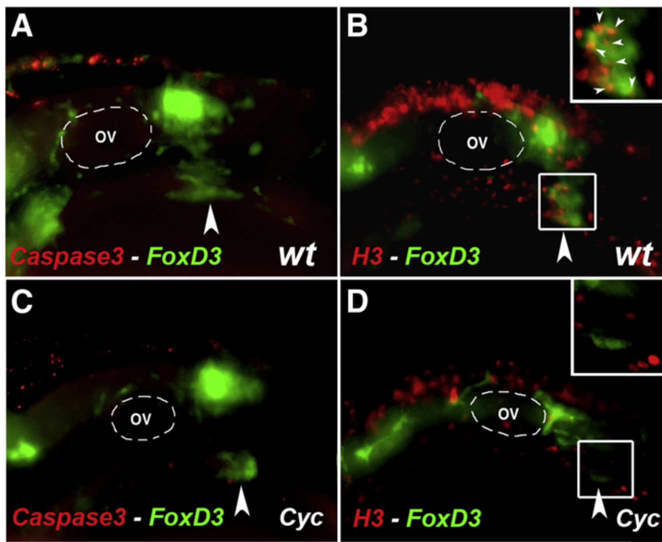

Fig. 7. Cyclopamine treatment causes no change in apoptosis but a decrease proliferation in the vagal NCC and ENS NCC. (A–D) Lateral views of the vagal region of 30 hpf wildtype (A, B) and 24–30 hpf cyclopamine treated (C, D) Foxd3:GFP transgenic embryos. Embryos were immunocytochemically double stained with anti gfp antibody (green) (A–D) to reveal the distribution of vagal NCC and ENS NCC's and anti activated caspase 3 antibody (red) (A, C) to reveal apoptotic cells and anti-phosphohistone H3 (red) (B, D) to reveal proliferating cells. OV (A–D) indicates otic vesicle. Large arrowheads (A–D) indicate the stream of vagal NCC that gives rise to ENS. White boxes (B, D) indicate the region that is shown in close up in the insert. Small white arrowheads (B) indicate proliferating cells. Anterior is to the left.

embryogenesis would cause an increase in the number of ENS precursors. Previous *in vitro* studies have shown that SHH acts as a mitogen for purified ENS NNCs (Fu et al., 2004). To determine if SHH has a mitogenic affect *in vivo* on the ENS precursors we injected embryos with *shh* mRNA at the one cell stage. The number of ENS precursors was then determined in these injected embryos by *phox2b* expression at 72 hpf. Overexpression of SHH consistently led to an increase in the number of *phox2b* expressing precursors in the intestine, with 33±2.5 *phox2b* expressing ENS precursors being present in the intestine of *shh* injected embryos vs. 24±2 in controls (Fig. 8).

Transplantation of wild type endoderm into sonic you mutants rescues ENS development

To further demonstrate that endoderm-derived SHH is necessary and sufficient for normal ENS development, we took a genetic chimeric approach to introduce wildtype cells that express SHH into the endoderm of *syu* and *smu* mutants to determine if these wildtype cells could rescue ENS development. Wildtype cells were transplanted into the *syu* and *smu* mutants at the early blastula stage. The wild type cells were targeted to the endoderm as the donor embryos were injected at the one cell stage with *sox32* mRNA along with a rhodamine biotinylated dextran lineage tracer (Holzschuh et al., 2005; Stafford et al., 2006). Experimental embryos were allowed to develop to 60 hpf at which time the development of the ENS was determined by *phox2b* expression. In contrast to *syu*−/− embryos that have no *phox2b* precursors in the intestine at 60 hpf (Fig. 4B) in 100% of the experimental embryos where wild type cells were transplanted into *syu* hosts, *phox2b* expressing ENS could be detected adjacent to the intestine (Figs. 9A–E) (2 independent experiments with 7/7 and 22/22 syu−/− embryos with transplanted wt endoderm). By contrast, no *phox2b* precursors could be detected in the intestines of *smu* mutants that received wildtype endodermal cell transplants (Fig. 9F) (8/8 smu−/− embryos with wildtype endoderm no phox2b expression could be detected in the intestine.

gdnf expression is perturbed in sox32 morphant embryos and cyclopamine treated embryos

Recent *in vitro* studies have shown that SHH acts to promote proliferation of ENS precursors while modulating responsiveness of ENS NCC's to the migratory stimulating activity of GDNF (Fu et al., 2004). Previously, GDNF has been shown to be a chemoattractant for ENS NCC's *in vitro* and regulates ENS NCC proliferation (Gianino et al., 2003; Natarajan et al., 2002; Young et al., 2001). Furthermore, knockdown of *gdnf* and its receptor complex causes an ENS NCC migration defect in zebrafish (Shepherd et al., 2001, 2004). To determine if the defects in ENS precursor migration observed in *sox32* morphants, *hand2* morphants and cyclopamine treated embryos were associated with changes in *gdnf* expression, we assessed *gdnf*'s expression at 36 and 60 hpf in these experimental embryos.

In *sox32* morphant embryos and 24–36 hpf cyclopamine treated embryos there is no expression of *gdnf* at the anterior end of the intestine at 36 hpf (Figs. 10C, D). By contrast *hand2* morphant embryos have a comparatively normal expression of *gdnf* at the anterior end of the intestine at this age (Fig. 10B). This pattern of *gdnf* expression in the *hand2* morphants is consistent with the normal pattern of ENS NCC migration from the vagal region to the anterior end of the intestine that we see in these morphants (Fig. 1C). At 60 hpf, when

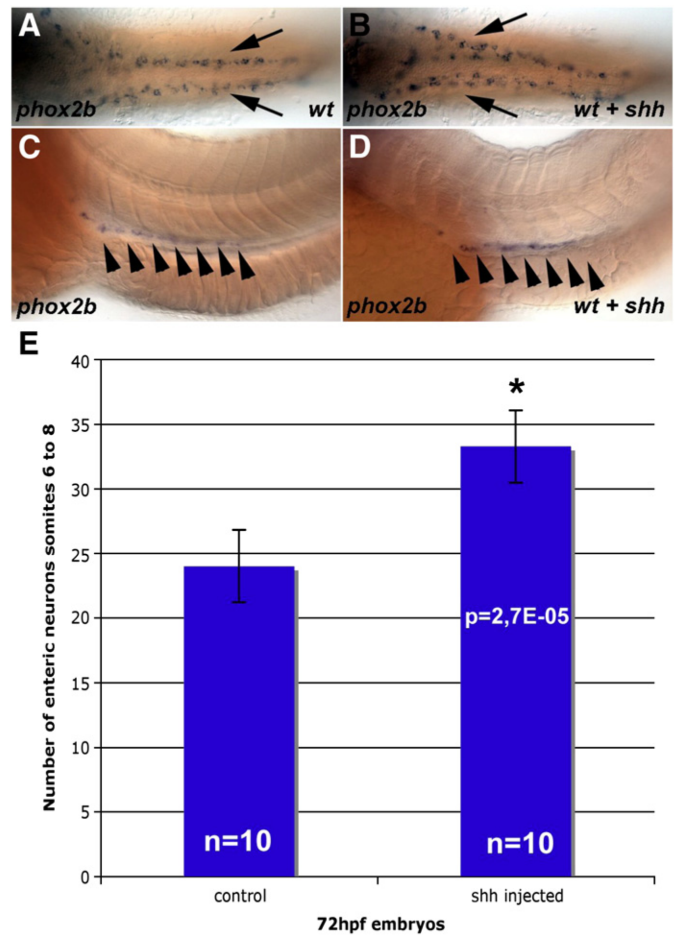

Fig. 8. *shh* over-expression leads to an increase in enteric neurons. Dorsal (A, B) and lateral (C, D) views of *phox2b* in enteric ganglia of 72 hpf control (A, C) and *shh* injected embryos (B, D). (E) Bar graph summarizes the results from one of three independent *shh* injection experiments. Enteric ganglia from *shh* injected embryos contained more *phox2b* expressing cells around the gut (P=2.7e−05; Student's t-test). The error bars represent the standard deviation. Arrows and arrowheads (A–D) indicate *phox2b* expressing ENS precursors.

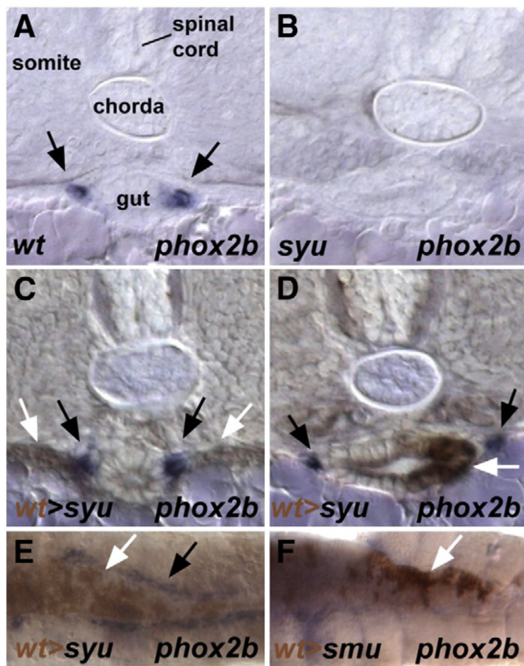

Fig. 9. Transplanted wild type endoderm rescues the enteric ganglia in *syu* mutants. (A–D) Cross-sections taken through 72 hpf embryos. (E, F) ventral views with anterior to the left of 72 hpf embryos. In wild type embryos *phox2b* expressing enteric neurons are located on the lateral edge of the gut tube (A). No *phox2b* expressing cells are found around the gut of syu mutant embryos (B). Transplanted *sox32* injected cells are recruited to the endoderm of *syu* mutants and rescue the enteric ganglia formation (C–E). Endodermal transplants from *sox32* injected wild type embryos are not able to rescue enteric ganglia formation in *smu* mutants (F). Black arrows indicate to *phox2b* expressing enteric neurons (A, C, D, E); white arrows point to transplanted endoderm (C–F).

gdnf is normally expressed along the complete length of the intestine, *sox32* morphant embryos and cyclopamine treated embryos have no *gdnf* expression along the length of the gut (Figs. 10K, M, N, O). As at 36 hpf, the pattern of *gdnf* expression at 60 hpf is comparatively normal in *hand2* morphants (Fig. 10L). Taken together these data suggest that endoderm and HH signaling are required for *gdnf* expression in the zebrafish intestine while *hand2* is not. Moreover the ENS defect observed in *hand2* morphants at 60 hpf and 96 hpf is not due to defects in *gdnf* expression.

Discussion

Our results provide evidence that endoderm-derived SHH/ hedgehog signaling is specifically required in two distinct phases of ENS development: i) a pre-enteric phase that is necessary for the normal migration of zebrafish ENS NCC precursors from the vagal crest to the anterior end of the intestine ii) an enteric phase that is required for the ENS NCC migration along the intestine. We show that proliferation of vagal NCC and ENS NCC is reduced when HH signaling is perturbed and that SHH acts as a potent mitogen for ENS NCC *in vivo*. We show that zebrafish *hand2* function is not required in the pre-enteric phase of ENS development but is required for enteric stages of ENS development. Furthermore we show that *hand2* function is required for the initial stages of development for intestinal muscle suggesting that zebrafish *hand2* has functions in ENS/intestinal development that normally require *hand1* in other species. Finally, we show that *in vivo* perturbation of either endodermal development or HH signaling results in loss of intestinal *gdnf* expression. These studies add to the previously described roles of HH signaling in neural crest and ENS development and provide evidence for an early requirement for HH signaling in ENS development in a pre-enteric phase. We also provide *in vivo* evidence that endoderm-derived HH signaling modulates *gdnf* expression in the intestine while *hand2* does not.

Role of endoderm in ENS development

In zebrafish, vagal neural crest-derived ENS precursors first migrate towards the anterior end of the intestine between 30 and 36 hpf forming a distinct chain of migrating precursors. Previously we have demonstrated a requirement for endoderm for the anterior–posterior migration of the ENS precursors along the length of the intestine (Pietsch et al., 2006). While the central role of endoderm in ENS development is not surprising, the requirement for endoderm in the ventromedial migration of the vagal crest-derived ENS NCC, as

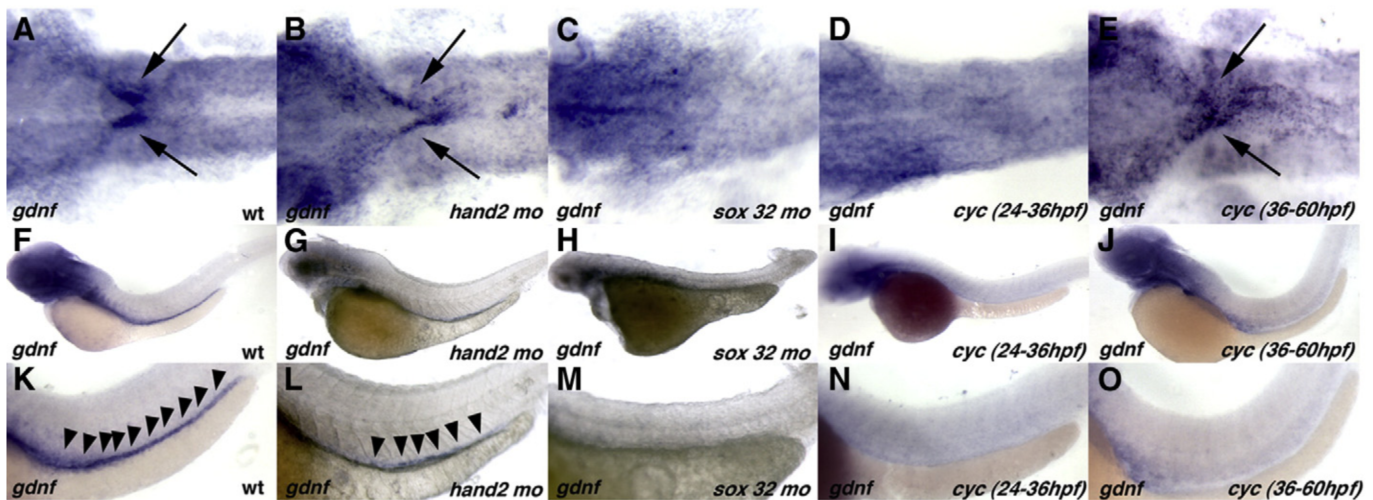

Fig. 10. Expression of *gdnf* in the intestinal mesenchyme requires endoderm and HH signaling but not *hand2* function. (A, F, K) wildtype control embryos, (B, G, L) *hand2* morphants (C, H, M) *cas/sox32* morphant embryos, (D, I, N) 24–26 hpf cyclopamine treated embryos and (E, J, O) 36–60 hpf cyclopamine treated embryos. (A–E) Ventral views of 36 hpf wholemount embryos that have been hybridized with a *gdnf* antisense probe and the yolk removed. In *sox32* morphant (C) and 24–36 hpf cyclopamine treated embryos (D) there is a loss of *gdnf* expression at the anterior end of the intestine. (F–J) Lateral view of 60 hpf wholemount embryos, that have been hybridized with a *gdnf* antisense probe, showing the loss of intestinal *gdnf* expression in *sox32* morphants and cyclopamine treated embryos. (K–O) Lateral view of the intestine of 60 hpf wholemount embryos that have been hybridized with a *gdnf* antisense probe. Arrows (A, B, E) indicate *gdnf* expression at the anterior end of the intestine. Arrow heads (K, L) indicate the *gdnf* expression along the length of the intestine. Anterior is to the left.

revealed by the loss of the chain of these cells in *sox32* morphants, is novel. To date the tissues, mechanisms and molecules involved in patterning the migration of the vagal ENS NCCs to the anterior end of the intestine have not been directly addressed. By determining that the endoderm is the key signaling center for this initial migration to occur, we have been able to focus on secreted molecules produced by this tissue that are responsible for this activity. This has led us to determine that SHH is an essential molecule for this migration to occur.

Evolutionary differences in hand2 function in zebrafish as compared to mouse

In most vertebrate species there are two *hand* genes. In mice and avian *hand2* is expressed in the developing ENS while *hand1* is expressed in the mouse intestinal mesenchyme (Cserjesi et al., 1995; D'Autreaux et al., 2007; Hendershot et al., 2007; Wu and Howard, 2002). By contrast in zebrafish there is only a single *hand* gene. At the sequence level the zebrafish *hand* gene is more orthologous to *hand2* than *hand1* (Angelo et al., 2000). However the expression of zebrafish *hand2* in the intestine is more like that of *hand1* in mouse at early stages of intestinal development as it is expressed in the intestinal mesenchyme (Angelo et al., 2000; D'Autreaux et al., 2007) (Fig. 1). At later stages of intestinal development zebrafish *hand2* appears to be expressed in both the intestinal mesenchyme and ENS precursors (Fig. 2). In mouse, *hand1* is expressed in the intestinal muscle cells and is required for vascular smooth muscle development while *hand 2* is exclusively expressed in enteric neurons and ENS NCC (D'Autreaux et al., 2007; Morikawa and Cserjesi, 2004). The function of Hand1 has not been addressed in mouse intestinal muscle development due to the early embryonic lethality of the Hand1 null mouse at E9–9.5 (Morikawa and Cserjesi, 2004). However, murine Hand2 has been shown to be required for normal differentiation of enteric neurons but not for the migration of ENS precursors along the intestine (D'Autreaux et al., 2007; Hendershot et al., 2007). Our data raises the possibility that in zebrafish *hand2* is responsible for the biological activities of both *hand1* and *hand2* found in other species. Future studies will determine whether this is the case.

Function of Sonic hedgehog signaling on ENS development

Our results demonstrating the loss of ENS precursors in HH pathway mutants and cyclopamine treated embryos, as well as the increase in number of ENS NCC in SHH overexpressing embryos, is consistent with previous studies that have examined the effect of SHH on ENS NCC (Fu et al., 2004). Our results also support the findings from other studies that have demonstrated that different axial populations of NCC respond differently to SHH.

SHH has been shown to play an important role in survival of both neural tube and cranial neural crest. Injections of function blocking anti-SHH antibody into chick cranial mesenchyme results in a loss of branchial arch structures and is associated with significant cell death in both the neural tube and the neural crest (Ahlgren and Bronner-Fraser, 1999). More recent chick studies have demonstrated that ventral foregut endoderm-derived SHH is specifically required at early stages of jaw development, between somite stages 5 and 7, for the survival of branchial arch 1 NCC (Brito et al., 2006). Similarly, cyclopamine treatment of *Xenopus* embryos results in a reduction of craniofacial cartilages and promotes cell death in explants of cranial neural crest (Dunn et al., 1995). More recent mouse studies, in which *smo* was genetically removed from migratory neural crest, have also demonstrated a requirement for hedgehog signaling in craniofacial and cardiac NCC survival (Goddeeris et al., 2007; Jeong et al., 2004). In contrast to cranial NCC, when HH signaling is perturbed during dorsal root ganglion (DRG) development in avian, xenopus or zebrafish, there is no obvious increase in cell death within trunk crest (Ahlgren and Bronner-Fraser, 1999; Dunn et al., 1995; Ungos et al., 2003). This has led to the suggestion that the anti-apoptotic effect of SHH is restricted to cranial neural crest populations. However we do not see any obvious increase in cell death in the premigratory vagal neural crest that gives rise to the ENS NCC when HH signaling is perturbed (Figs. 7A, C), though we cannot definitively rule out that there is no cell death in these cells as we have no specific markers for the ENS precursors in the premigratory NCC that could reveal this. The apparent lack of cell death in the ENS NCC precursors though is consistent with data from mouse *in vitro* studies (Fu et al., 2004). In these studies no difference was observed in the number of apoptotic cells in ENS NCC-derived neuropsheres cultured in the presence or in the absence of SHH (Fu et al., 2004).

Our finding that SHH is necessary for ENS NCC migration to the anterior end of the intestine from the vagal region in the pre-enteric phase of ENS development is novel and significant. This result is somewhat consistent with other studies that have shown SHH has a role in determining the pattern of migration of a number of other neural crest populations. In mice SHH signaling is required for the normal pattern of migration of cardiac and trunk neural crest (Washington Smoak et al., 2005). Similarly HH signaling is required for the normal pattern of migration of trunk NCC DRG precursors in zebrafish, as revealed by HH pathway mutants (Ungos et al., 2003). In addition SHH determines the pattern of migration of cranial NCC that form the anterior neurocranium in zebrafish (Wada et al., 2005). While it is possible that SHH could act as a chemoattractive cue for ENS precursors from the vagal premigratory NCC to the anterior end of the intestine our results instead show that HH regulates the proliferation of the vagal NCC. As a result the ENS defect in cyclopamine treated embryos appears to arise due to there being insufficient ENS precursors generated within the premigratory vagal NCC when HH signaling is perturbed. This finding is consistent with recent studies that show NCC proliferation drives the invasion of ENS precursors along the length of the chick intestine (Simpson et al., 2007). We now extended this model and suggest that proliferation drives the pre-enteric migration of ENS NCC precursors form the vagal premigratory NCC region to the anterior end of the intestine.

During the enteric phase we propose that SHH continues to act as a mitogen for ENS precursors and drive their migration along the intestine, as well as acting as a morphogen patterning the intestinal mesenchyme. This *in vivo* mitogenic activity of SHH for ENS NCC is a comparatively novel finding. Previously SHH has been shown to be a mitogen for ENS neurospheres *in vitro* (Fu et al., 2004). We now show by *shh* RNA over-expression a similar mitogenic activity *in vivo*, however we cannot rule out the possibility that this activity is an indirect effect and is due to the increased secretion of other mitogens from the intestinal mesenchyme rather than SHH acting directly as an ENS NCC mitogen. The putative increased secretion of other mitogens could result from the morphogenetic actions of SHH on the intestinal mesenchyme. Previous studies in mouse and chick have demonstrated that perturbation of HH signaling results in intestinal and ENS patterning defects (Ramalho-Santos et al., 2000; Sukegawa et al., 2000). These studies demonstrated that SHH is the key factor that patterns the radial axis of the intestine and led to the proposal that SHH inhibits smooth muscle differentiation in the intestine (Ramalho-Santos et al., 2000; Sukegawa et al., 2000). A number of previous studies have shown that SHH secreted from the endodermal epithelium induces expression of BMP4 in the adjacent non-smooth muscle mesenchyme (Narita et al., 1998; Roberts et al., 1995, 1998; Sukegawa et al., 2000). More recent studies have suggested that down regulation of BMP signaling in the intestinal mesenchyme is also required for intestinal smooth muscle differentiation (De Santa Barbara et al., 2005). Our data shows that blocking SHH signaling with cyclopamine results in a failure of intestinal smooth muscle differentiation in the intestinal mesenchyme of zeb-

rafish, counter to what would have been predicted from the previous models. This suggests that other factors are involved in the intestinal smooth muscle differentiation, rather than just a simple down regulation/inhibition/lack of BMP signaling, and a SHH dependent myogenic factor is required to induce *myh11* and α *SMA* expression in zebrafish.

As SHH patterns the radial axis of the intestine it will also indirectly act as an ENS NCC guidance cue for the migrating precursors within the intestine. As previously mentioned, SHH induces BMP expression in the intestinal mesenchyme. Recently BMP signaling has been shown to regulate PSA-NCAM levels on NCC (Fu et al., 2006). Increased BMP signaling leads to the increased addition of PSA to NCAM. The increased amount of PSA-NCAM on the cell-surface of the ENS NCC in turn inhibits their migratory ability. As cyclopamine treatment should cause a decrease in BMP expression in the intestinal mesenchyme, we would predict that if this model also applies to zebrafish, there would be an increase in the migratory behavior of the ENS precursors due to reduced PSA-NCAM. However this does not appear to be the case based on our *in situ* analysis. We propose that the key activity of SHH on ENS precursors, during the enteric phase of ENS development, is as a mitogen rather than as an indirect regulator of ENS precursors migratory ability. We believe that when HH signaling is perturbed during this enteric phase the reduced/lack of proliferation of the ENS NCC precursors in the intestine will mask any changes in their migratory behavior.

In addition to BMP's role in regulating the migratory behavior of ENS NCC along the intestine, GDNF has been shown in avian, mouse and zebrafish to be critical for stimulating the migratory behavior of these precursors along the intestine (Natarajan et al., 2002; Shepherd et al., 2001, 2004; Young et al., 2001). Furthermore, SHH has been shown to regulate GDNF's migratory stimulatory behavior for ENS NCC's *in vitro* while GDNF acts to inhibit SHH's mitogenic activity (Fu et al., 2004). Our data is consistent with SHH regulating GDNF's activities in ENS development, as perturbation of endoderm development or perturbation of SHH signaling results in a loss of *gdnf* expression in the intestinal mesenchyme. Our results are also consistent with our previous studies that show knockdown of *gdnf* or its receptor complex perturbs the migration of ENS precursors along the intestine (Shepherd et al., 2001, 2004). Strikingly though, when we knockdown expression of *gdnf* or its receptor, we do not see any perturbation in the pre-enteric migration of ENS NCC from the vagal crest to the intestine (Shepherd et al., 2001, 2004). As a result SHH must be acting either directly or indirectly, via the production of some unknown chemoattractant, to direct the vagal crest-derived ENS NCC to the anterior end of the intestine. Future studies will attempt to determine what the molecular basis of this activity is. These studies will also address whether SHH acts directly or indirectly on the ENS precursors in the enteric phase of ENS development. Current genetic chimera techniques do not effectively target ENS precursors due to the small number of ENS NCC in the premigratory vagal neural crest. We are currently developing techniques that will more effectively target these cells. We will also determine if SHH directly regulates GDNF expression. What is clear is that there is a careful balance between the GDNF, HH and BMP signaling pathways in the development of the intestine and the ENS and if this balance is perturbed it results in intestinal patterning defects as well as intestinal aganglonosis.

In summary, our analysis of the role of endoderm-derived SHH in the development of the ENS has demonstrated that HH signaling is required in two phases, a pre enteric and an enteric phase for normal ENS development (Fig. 11). We show that HH signaling regulates migratory behavior and proliferation of the ENS precursors *in vivo*. We have also clarified the role of *hand2* in zebrafish intestinal development by demonstrating that the single zebrafish *hand* gene has functions correlated with both *hand* genes found in other vertebrates. Finally, we show that endoderm and HH signaling regulates *gdnf* expression in the intestine, highlighting a central role of endoderm and SHH in patterning the intestine and the ENS.

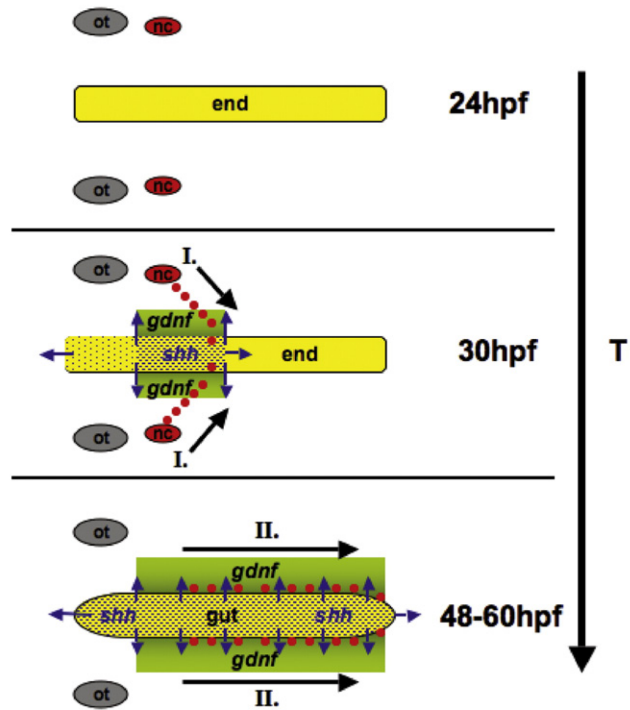

Fig. 11. Model for the two phases of SHH signaling required for intestinal *gdnf* expression and normal ENS development. Diagrams illustrate the relationship between ENS development, endodermal expression of SHH and the expression of *gdnf* in the intestinal mesenchyme. At 24 hpf prior to migration of ENS NCC from the vagal region there is no expression of *shh* in the endoderm or expression of *gdnf* in the intestinal mesenchyme. Around 30 hpf *shh* begins to be expressed in the anterior region of the intestinal endoderm. This expression coincides with the expression of *gdnf* in the adjacent intestinal mesenchyme and the beginning of the migration of the ENS NCC from the vagal region to the anterior end of the intestine. We refer to this as phase I or the pre-enteric phase of ENS development. By 48 hpf *shh* is expressed along the whole length of the intestine and *gdnf* is now expressed in the adjacent mesenchyme along the whole length of the intestine. In this phase II of ENS development, the enteric phase, ENS NCC migrate along the whole length of the intestine reaching the distal end by 60 hpf.

Acknowledgments

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