Hedgehog signaling is directly required for the development of zebrafish dorsal root ganglia neurons

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Summary

Hedgehog (Hh) signal transduction is directly required in zebrafish DRG precursors for proper development of DRG neurons. Zebrafish mutations in the Hh signaling pathway result in the absence of DRG neurons and the loss of expression of *neurogenin1* (*ngn1*), a gene required for determination of DRG precursors. Cell transplantation experiments demonstrate that Hh acts directly on DRG neuron precursors. Blocking Hh pathway activation at later stages of embryogenesis with the steroidal alkaloid, cyclopamine, further reveals that the requirement for a Hh signal response in DRG precursors correlates with the onset of *ngn1* expression. These results suggest that Hh signaling may normally promote DRG development by regulating expression of *ngn1* in DRG precursors.

Key words: Dorsal root ganglia, Hedgehog, Cyclopamine, Neurogenin, Neural crest, Zebrafish

Introduction

The dorsal root ganglia (DRG) are the metamerically organized peripheral sensory ganglia important for transmitting somatosensory information from the body to the brain. The neural crest cells that give rise to the neurons and glia of the DRG originate from the dorsal aspect of the neural tube and migrate ventrally to populate the ganglion at the ventrolateral edge of the spinal cord. The establishment of the DRG involves precise coordination of migration patterns, regulation of cell number, and the complex problem of generating multiple cell types within the same local environment. Little is known about the signals that specify DRG precursors from the neural crest.

Neurogenin (Ngn) transcription factors are key regulators of DRG development. *ngn1* and *ngn2* are both expressed in the dorsal root ganglia (Ma et al., 1998; Ma et al., 1999). *ngn2* is expressed in a subset of migrating neural crest precursors, whereas *ngn1* expression appears later in the nascent DRG (Ma et al., 1999; Perez et al., 1999). Inactivation of *ngn1* and *ngn2* completely blocks development of DRG neurons (Ma et al., 1999), and overexpression of *ngn1* in neural crest cells biases them to contribute to the DRG (Perez et al., 1999). In zebrafish, a single *ngn* gene, *ngn1*, performs many of the functions controlled by the two separate mammalian neurogenins (Andermann et al., 2002; Cornell and Eisen, 2002). Blocking Ngn1 function by injection of antisense morpholino oligonucleotides disrupts development of zebrafish DRG neurons. Understanding the signals that regulate neurogenin gene expression in neural crest cells should shed light on the early steps of DRG development.

In zebrafish, neural crest cells that give rise to the DRG are amongst the earliest cells to migrate from the dorsal neural tube on a ventral pathway between neural tube and somite (Raible et al., 1992). Observation of labeled neural crest cells in living zebrafish embryos revealed that DRG precursors subsequently undergo a characteristic dorsal migration from a position lateral to the notochord to their final position lateral to the neural tube (Raible and Eisen, 1994). *ngn1* is first expressed in DRG precursors once they reach this final position (Andermann et al., 2002; Cornell and Eisen, 2002). Together, these results suggest that DRG precursors may be specified by local signals from notochord, somite or neural tube.

Several studies in avian embryos support the idea that signals released from nearby tissues are involved in DRG specification. A role for somitic mesenchyme in producing the segmental arrangement of the DRG is well-established (Kalcheim and Teillet, 1989). Manipulation of rostrocaudal polarity of somites alters DRG spacing and size, and can influence proliferation of DRG precursors (Goldstein et al., 1990; Goldstein and Kalcheim, 1991). Axial structures have also been implicated in the development of the DRG. Removal of the neural tube results in loss of DRG and produces unsegmented sympathetic ganglia (Teillet and Le Douarin, 1983). Similarly, placement of an impermeable membrane between the neural tube and migrating neural crest eliminates the development of the DRG (Kalcheim and Le Douarin, 1986). Consistent with these results, loss of either axial signals in zebrafish *floating head* (*flh*) mutant embryos (Halpern et al., 1995; Talbot et al., 1995) or loss of paraxial tissues in the zebrafish *spadetail* (*spt*) mutant (Kimmel et al., 1989; Griffin et al., 1998) severely disrupts DRG development (J.U. and D.W.R., unpublished). All of these results indicate a role for diffusible signals, either from axial or paraxial tissues, in the development and patterning of the DRG.

Sonic hedgehog (Shh) is a secreted protein that signals from the notochord to locally pattern adjacent tissues (Krauss et al., 1993; Roelink et al., 1994). Shh specifies the differentiation of both sclerotome and epaxial muscle from paraxial mesoderm (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994). In zebrafish, Shh signaling is required for the development of slow muscle fibers and muscle pioneers (Barresi et al., 2000). Shh also induces differentiation of several cell types in the ventral neural tube through graded Shh signaling (Ericson et al., 1997). Similarly, midline Shh signaling is required for the induction of both primary and secondary motor neurons in zebrafish (Beattie et al., 1997; Lewis and Eisen, 2001).

Given its clear roles in patterning both the neural tube and somites, and its expression adjacent to the site where DRG develop, Shh is a candidate for mediating local signaling required for specification of DRG precursors. To examine the role of Shh signaling in the development of the zebrafish DRG, we have taken advantage of the availability of several zebrafish mutations that disrupt different components of the Hh signaling pathway: *sonic you* (*syu*), which encodes sonic hedgehog; *smooth muscle omitted* (*smu*), which encodes the smoothened (*smo*) receptor; *detour* (*dtr*), which encodes the transcriptional effector *gli1*, and *you-too* (*yot*), which encodes *gli2* (Brand et al., 1996; Schauerte et al., 1998; Karlstrom et al., 1999; Chen et al., 2001; Varga et al., 2001; Karlstrom et al., 2003). These mutants generally share several morphological defects, including: ventral cyclopia, curved-down tails, U-shaped somites lacking the horizontal myoseptum and circulation defects associated with disrupted dorsal aorta formation. Furthermore, we use the steroidal alkaloid, cyclopamine, to vary the timing of loss of Hh signaling during neural crest development. It has been previously demonstrated that cyclopamine inhibits activation of the Hh pathway through direct binding to Smo (Cooper et al., 1998; Incardona et al., 1998; Chen et al., 2002), and has been used previously to disrupt Hh signaling in zebrafish (Neumann et al., 1999; Chen et al., 2001; Sbrogna et al., 2003; Stenkamp and Frey, 2003). Our analysis of the timing and tissue requirements for Shh signaling reveals a cell-autonomous requirement for Hh signal transduction in DRG precursors and shows that Shh signaling functions upstream of Ngn1 to promote sensory neurogenesis.

Materials and Methods

Fish husbandry

Embryos were obtained through natural spawnings of AB* wild-type fish or heterozygous carriers of specific mutations and staged according to Kimmel et al. (Kimmel et al., 1995). The following mutant lines were used: *yot*^{ty17} encodes a C-terminally truncated Gli2 protein that blocks gli-mediated Hh signaling (Karlstrom et al., 1999); *syu*^{t4} is a deletion mutation that encompasses the *sonic-you/shh* gene (Schauerte et al., 1999); *helix*^{b392} is a strong allele of *chameleon* that was identified in a mutagenesis screen at the University of Oregon (Henion, 1996; Brand et al., 1996; Karlstrom et al., 1996; van Eeden et al., 1996); *dtr*^{ts269} is a strong loss-of-function allele of *detour/gli1* (Brand et al., 1996; Karlstrom et al., 1996; Karlstrom et al., 2003); *smu*^{b641} is a *slow muscle omitted/smoothened* loss-of-function allele that changes a glycine to an arginine in the second transmembrane domain of the Smoothened protein (Varga et al., 2001). The pHuC::GFP line is described by Park et al. (Park et al., 2000).

Cyclopamine treatment of embryos

Cyclopamine (gift from Henk Roelink) was diluted in embryo medium to 0.5 μg/ml-18 μg/ml from a stock of 4 mg/ml dissolved in 45% (w/v) 2-hydroxypropyl-β-cyclodextrin (Sigma) in phosphate buffered saline (PBS). Wild-type embryos were soaked in embryo medium containing cyclopamine and allowed to develop at 28°C. Unless otherwise specified, embryos were left in cyclopamine-containing media until they were processed for immunohistochemistry or whole-mount in situ hybridization. Embryos treated in embryo medium containing 2-hydroxypropyl-β-cyclodextrin alone were indistinguishable from untreated wild-type embryos.

Immunohistochemistry

Embryos were anesthetized in tricaine (10 mg/ml; Sigma) in embryo medium, fixed in 4% formalin in fix buffer for 2 hours at room temperature (RT) (Westerfield, 1994). Seventy-two hours post-fertilization (hpf) and older embryos were permeabilized by washing 3x30 minutes in distilled water. Embryos were incubated for 1 hour in blocking solution (2% goat serum, 1% bovine serum albumin, 1% dimethylsulfoxide, 0.1% Triton X-100 in PBS), then overnight at RT in primary antibody diluted in blocking solution. Primary antibodies used were anti-Hu (1:700; mAB 16A11) (Marusich et al., 1994), anti-zn-5 (1:400) (Fashena and Westerfield, 1999), anti-acetylated tubulin (1:1000; Sigma), anti-Islet 1 4D5 [1:200; gift from H. Roelink and Developmental Studies Hybridoma Bank (DSHB), www.uiowa.edu/~dshbwww/], anti-Lim-1/2 4F2 (1:500; gift from H. Roelink and DSHB) and anti-GFP (1:200; Molecular Probes, Eugene, OR, USA). Embryos were rinsed extensively in PBS with Triton X-100 (PBTx) and incubated overnight at RT in Alexa488- or Alexa568-conjugated secondary antibodies diluted in blocking solution (1:750; Molecular Probes). After rinsing in PBTx, embryos were transferred to 50% glycerol in PBS and mounted on bridged coverslips.

Whole-mount in situ hybridization and RNA probe synthesis

Embryos were collected from timed matings, raised at 28.5°C and carefully staged before fixing overnight at 4°C in 4% paraformaldehyde in PBS. RNA in situ hybridization was performed following Thisse et al. (Thisse et al., 1993), except embryos were hybridized at 65°C. Digoxigenin or fluorescein-labeled antisense RNA probes were generated for *ngn1* and *neurod* (Blader et al., 1997), *patched1* (*ptc1*) (Concordet et al., 1996) and *crestin* (Rubinstein et al., 2000; Luo et al., 2001) by digesting DNA with restriction enzyme and synthesizing with RNA polymerase as follows: *ngn1*, XhoI/T7; *neurod*, NotI/T3; *ptc1*, BamHI/T3; *crestin*, SacI/T7. Probes were detected using anti-digoxigenin or anti-fluorescein antibodies conjugated to alkaline phosphatase (Roche), followed by incubation with 5-bromo 4-chloro 3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT). For cryostat sectioning, embryos were cryopreserved in 30% sucrose and mounted in OCT. For plastic sectioning, embryos were processed for in situ hybridization, dehydrated in a graded ethanol series and embedded in Araldite resin (Polysciences, Warrington, PA, USA). All images were captured on a Nikon Microphot-SA microscope (Nikon, Melville, NJ, USA) using a Spot Digital Camera and software (Diagnostic Instruments, Sterling Heights, MI, USA). Images were processed using Photoshop 6.0 (Adobe, San Jose, CA, USA).

DRG counts

Embryos were processed for anti-Hu immunoreactivity at 60 hpf, mounted in 50% glycerol/PBS between bridged coverslips and oriented on their side to view DRG neurons. Each embryo was scored on one side for the presence or absence of DRG neurons and the presence of large neuronal clusters. Analysis of both sides of many embryos revealed no obligate bilateral symmetry in appearance of large clusters of neuronal cells (data not shown). Ganglia were scored

as 'normal' if the number of neurons was in the appropriate range given the age of the embryo and rostrocaudal level of the ganglion. However, given that the abnormal clusters can vary significantly in cell number, some of the ganglia that were scored as 'normal DRG' may have been abnormal clusters in the early stages of development.

Mosaic analysis

Donors were labeled by injecting 1 nl of a 3% solution of rhodamine-dextran (10,000 MW dextran, tetramethylrhodamine lysine fixable, D-3312; Molecular Probes) in 0.2 M KCl into the yolk cytoplasm of 1- to 8-cell stage embryos using an ASI pressure injection apparatus (ASI, Eugene, OR, USA). Mosaic embryos were generated by transplanting cells from blastula-stage donors into shield-stage hosts. Embryos were mounted in 3% methylcellulose (Sigma) in embryo medium containing 1% penicillin-streptomycin (Sigma). To confirm contribution of donor cells to host neural crest, in some experiments, cells from embryos of mutant heterozygote crosses were transplanted into *nacre* homozygotes that lack melanophores (Lister et al., 1999). *nacre* hosts could then be scored for the presence of neural crest-derived melanophores. All embryos were fixed at 48-72 hpf and stained with anti-Hu and anti-mouse Alexa488 to visualize DRG neurons. Stained embryos were examined on a Zeiss LSM Pascal confocal microscope.

Results

Genetic and pharmacological inhibition of Hh signaling leads to loss of DRG neurons

Hh signaling has been shown to be involved in the survival and migration of cranial neural crest populations (Ahlgren and Bronner-Fraser, 1999; Testaz et al., 2001). However, the role for Hh signaling in trunk neural crest development has not been determined. By examining expression of the pan-neuronal anti-Hu antibody in zebrafish Hh pathway mutants, we have revealed a role for Hh in development of neural crest-derived DRG neurons. In wild-type embryos at 60 hpf, most segments contain only a single Hu-positive DRG neuron (Fig. 1A). In *syu* embryos, in which the *shh* gene is disrupted, the majority of segments are completely devoid of DRG neurons (Fig. 1B). Similar DRG defects were observed in *con*, *dtr*, *yot* and *smu* mutant embryos (not shown; see Fig. 3 for numerical quantification) and after treatment of gastrula-stage embryos with cyclopamine (Fig. 1C), a steroidal alkaloid shown to inhibit Hh pathway activation (Cooper et al., 1998; Incardona et al., 1998; Chen et al., 2002). This loss of DRG neurons persists through later stages of embryonic development. Embryos treated with cyclopamine during somitogenesis still display a complete loss of DRG neurons at 7 days postfertilization (data not shown), the latest time that treated embryos survive.

Hh signaling is required for integrity of the spinal cord

In the Hh pathway mutants and in cyclopamine-treated embryos we also observe large clusters of Hu-positive cells at the ventrolateral edge of the spinal cord (Fig. 1B,C, Fig. 2K,L). These clusters of neuronal cells generally appear only in a small subset of segments and range considerably in cell number from a few neuronal cells (e.g. Fig. 1B) to more than 40 cells in a single cluster (e.g. Fig. 1C). These clusters are distributed in no obvious pattern: the size, rostrocaudal location and numbers of clusters can vary significantly from individual to individual, and they do not necessarily form bilaterally. To determine the identity of these neurons, we examined expression of specific neuronal markers. In wild-type embryos, DRG neurons are Islet- and zn-5-positive, but Lim- and znp-1-negative (Fig. 2A,D; not shown). In contrast, the abnormal ventrolateral neuronal clusters variably express all of these markers. In *dtr* mutants, many neurons in abnormal clusters express Islet-1 and zn-5 which both label DRG sensory neurons as well as spinal cord motor neurons (Fig. 2C,F) (Ericson et al., 1992; Korzh et al., 1993), leaving open the possibility that some of these cells are either motor or sensory neurons. In contrast, in *smu* mutant embryos, the majority of neurons in the large clusters express Lim-1/2, a more dorsal marker of spinal cord neurons (Fig. 2B) (Tsuchida et al., 1994), and do not label with zn-5 or Islet (Fig. 2B,E). However, the neurons in the large clusters develop disorganized projections as evidenced by expression of acetylated tubulin (Fig. 2H) and znp-1 (not shown). In tail sections of embryos treated with cyclopamine at 24 hpf, large clusters of Hu-and Islet-expressing cells (Fig. 2K,L) appear at the ventrolateral edge of the spinal cord. Ventrolateral neuronal clusters are also present at similar frequencies in *colourless*/*sox10* mutant (Dutton et al., 2001) and *mindbomb* (*mib*) morpholino (MO)-injected embryos (Itoh et al., 2003) treated with cyclopamine (not shown), suggesting that the formation of large clusters is not affected by the neural crest defects present in these embryos. Taken together, these results suggest that the large ventrolateral clusters may be spinal cord-derived neurons rather than neural crest-derived DRG sensory neurons.

It has previously been shown that *ngn1* is expressed in zebrafish DRG precursors and is required for development of

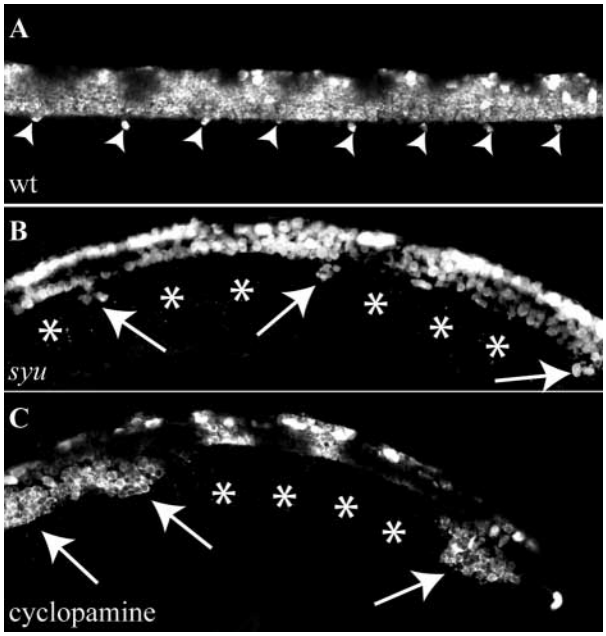

Fig. 1. Loss of Hh signaling disrupts DRG development. Confocal stacks of lateral views of embryos processed for anti-Hu at 60 hpf. (A) Normal pattern of DRG neurons (arrowheads) in wild-type (WT) embryos. (B) *syu* mutants lack DRG in many segments (asterisks), but abnormal clusters of neuronal cells (arrows) also appear. (C) WT embryos treated with 6.0 μg/ml of cyclopamine during gastrulation show similar phenotypes.

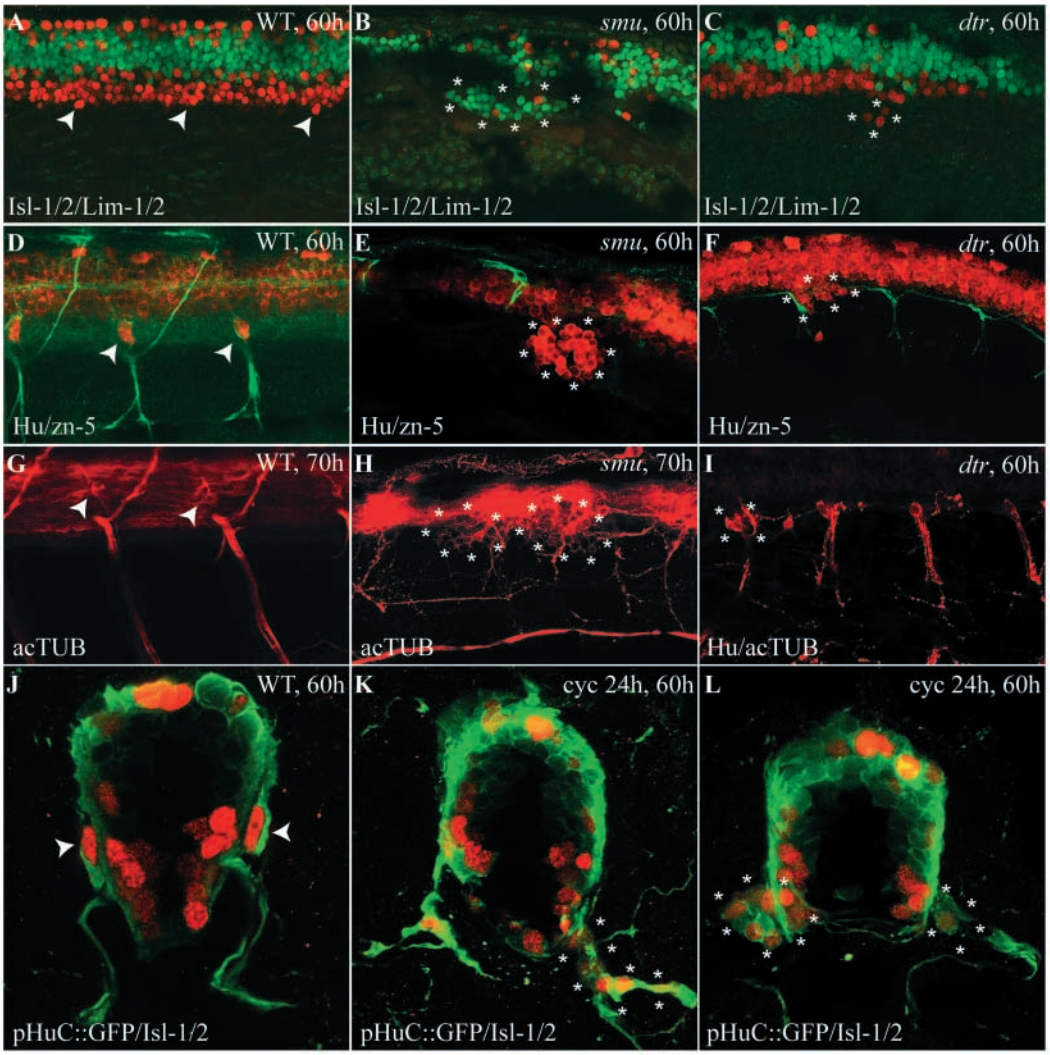

Fig. 2. Loss of Hh signaling appears to disrupt integrity of the spinal cord. (A-C) Isl-1/2 is shown in red; Lim1/2 is shown in green. (A) Wild-type (WT) expression of Isl-1/2 in DRG neurons (arrowheads) just lateral to ventral motor neuron expression and ventral to Lim-1/2 neuron expression. (B) Large clusters (asterisks) predominantly express Lim in *smu* mutant embryos. (C) Some neurons in large clusters (asterisks) in *dtr* mutants express Islet. (D-F) Hu staining is shown in red; zn-5 is shown in green. (D) WT embryos show expression of both Hu (cell bodies) and zn-5 (axons) in motor neurons as well as DRG neurons (arrowheads). (E) In *smu* mutant embryos, large clusters (asterisks) express Hu but not zn-5. (F) Some neurons in large clusters (asterisks) in *dtr* mutant embryos express both Hu and zn-5. (G,H) Acetylated tubulin is shown as red. (G) Acetylated tubulin is normally expressed in motor neuron axons as well as DRG axons (arrowheads). (H,I) Large clusters (asterisks) in *smu* and *dtr* mutant embryos display acetylated tubulin-positive projections. Both Hu and acetylated tubulin are red in I. (J-L) Confocal images of transverse sections showing pHuC::GFP in green and Isl-1/2 in red. (J) WT expression of HuC and Isl-1/2 in DRG neurons (arrowheads) located lateral to the spinal cord. (K,L) Clusters of Isl-1/2+ and HuC+ cells at the ventrolateral edge of the spinal cord (asterisks) in embryos treated with cyclopamine at 24 hpf.

zebrafish DRG neurons (Cornell and Eisen, 2002; Andermann et al., 2002). In our analysis of *ngn1* expression in the different Hh signaling mutants (see Fig. 5), we never observed expression of *ngn1* in the large clusters of cells at the ventrolateral edge of the neural tube as might be expected if these cells formed from DRG precursors. Furthermore, we found that formation of the large clusters is *ngn1*-independent. Embryos were injected at the one-cell stage with *ngn1* morpholino oligonucleotides, as described previously (Andermann et al., 2002; Cornell and Eisen, 2002), and then treated at dome stage with cyclopamine. The embryos were allowed to develop to 72 hpf and were then fixed and processed for anti-Hu immunoreactivity. DRG neurons, as well as Rohon-Beard sensory neurons, are eliminated in embryos injected with the *ngn1* MO (Fig. 3B). In contrast, large neuronal clusters still appear in the absence of Ngn1 function following treatment of the embryos with cyclopamine (Fig. 3C). These results further support the idea that the large clusters are not derived from DRG precursors.

Decreasing levels of Hh signaling leads to increasing loss of DRG neurons

The different Hh signaling mutants can be organized into a phenotypic series according to the severity of morphological defects in somite patterning, and we find that the severity of DRG phenotypes follows this series (Fig. 4). The DRG

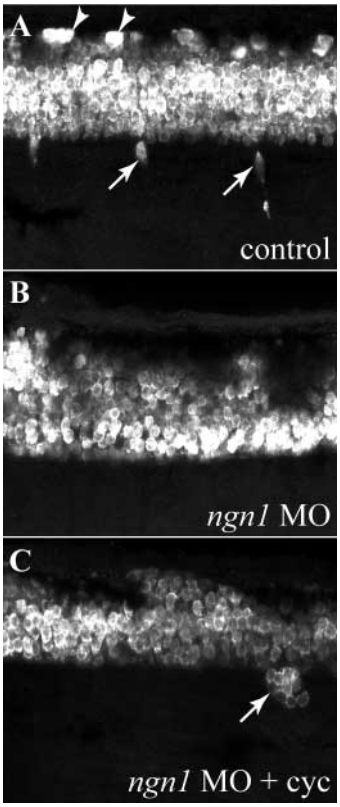

Fig. 3. Large neuronal cluster formation in the absence of Hh signaling does not require Ngn1 function. Wild-type embryos were injected with *ngn1* morpholino (*ngn1* MO) at the 1-cell stage. Injected embryos were treated with 6 μg/ml cyclopamine beginning at dome stage. Embryos were fixed at 72 hpf and processed for anti-Hu immunoreactivity. Images are confocal stacks of lateral views of whole-mount embryos. (A) Control embryo showing normal DRG (arrow) and Rohon-Beard (RB) neurons (arrowhead). (B) *ngn1* MO-injected embryo showing absence of DRG and RB neurons. The remaining Hu+ cells are spinal cord neurons. (C) *ngn1* MO-injected embryo that was also treated with cyclopamine (cyc) showing large neuronal cluster (arrow).

phenotype is much less severe in *dtr* mutant embryos, which have many segments with wild-type DRG neurons. *con* and *syu* mutant embryos display phenotypes of intermediate severity. Although normal DRG still appear in these mutants, more segments show loss of DRG neurons. *smu* mutants show more severe phenotypes with less phenotypic variability and complete penetrance (Chen et al., 2001; Varga et al., 2001). Similarly, DRG neurons rarely appear in *smu* mutant embryos (Fig. 4).

Differences in DRG phenotype appear to reflect variations in the level of Hh signaling activity in the different Hh pathway mutants. To test this idea, we analyzed the effects of treating embryos with different doses of cyclopamine (Fig. 4). Cyclopamine linearly reduces the Hh response by direct binding to Smoothened (Chen et al., 2002), and has been shown to block accumulation of zebrafish *patched1* (*ptc1*) transcripts in a dose-dependent manner (Wolff et al., 2003). Treatment of dome-stage embryos with 6.0 μg/ml of cyclopamine mimics the midline defects seen in *smu* mutant embryos, whereas treatment with lower doses (0.5 μg/ml) produces less severe effects on somite formation and more mild posterior cyclopia (not shown). Treatment of gastrula-stage embryos with a low dose of cyclopamine produces DRG phenotypes comparable to the *dtr* mutant DRG phenotype. In contrast, higher doses phenocopy the *smu* DRG defects. It should be noted that as Hh signaling activity decreases and more DRG neurons begin to disappear, the ventrolateral large clusters arise more frequently. The appearance of the large neuronal clusters in a given segment obscures our ability to score the loss of DRG neuron phenotype in that segment. Therefore, the percentage of segments with 'zero' DRG neurons shown in Fig. 4 may be underestimated.

Hh signaling mutants lose expression of *ngn1* and *neurod* in DRG precursors

The *ngn1* gene is critical for zebrafish DRG development and is the earliest known marker for sensory neuroblasts (Cornell and Eisen, 2002; Andermann et al., 2002). To determine when the development of DRG precursors is first affected by loss of Hh signaling, we examined expression of *ngn1* in Shh/midline class mutants (Fig. 5). *yot* embryos and wild-type siblings were fixed at 36 hpf and processed for in situ hybridization. Although spinal cord expression of *ngn1* appears normal in mutant embryos, expression in DRG precursors is completely eliminated in *yot* homozygotes (Fig. 5C). Similarly, expression of *ngn1* is absent in DRG precursors in *smu* mutant embryos (data not shown). Expression of the bHLH gene *neurod* normally follows expression of *ngn1* in DRG precursors. This expression is also abolished in *smu* mutants (Fig. 5). Together, these results demonstrate that Hh signaling is required prior to neurogenic bHLH expression in DRG precursors.

Neural crest develops normally in the absence of Hh signaling

In other organisms, Shh signaling influences the dorsoventral pattern of the neural tube, suggesting that the observed DRG phenotypes might reflect a disruption of neural crest induction or may result from defects in neural crest migration. Consistent with these possibilities, in addition to the DRG defects, many of the Hh pathway mutants also have defects in neural crest-derived cartilage formation in the developing jaw (Brand et al., 1996; Barresi et al., 2000; Varga et al., 2001; Kimmel et al., 2001). However, the other trunk neural crest derivatives, such as pigment cells and fin ectomesenchyme, develop normally following attenuation of Hh signaling (data not shown). To further explore the possibility that Hh signaling is required for early events in neural crest development, we examined expression of the neural crest marker *crestin* (Rubenstein et al., 2000; Luo et al., 2001) in *smu* mutant embryos (Fig. 6). The level of *crestin* expression appears comparable between wild-type and *smu* mutant embryos (Fig. 6A,B), suggesting that blocking Hh signaling does not significantly alter neural crest formation. Although *crestin* expression reveals that migration of neural crest is abnormal in *smu* mutant embryos, neural crest cells do still migrate ventrally (Fig. 6D). Moreover, although treatment of embryos with high doses of cyclopamine during somitogenesis stages has severe effects on DRG development (Fig. 6G), there is no obvious effect on the pattern of neural crest migration (Fig. 6F). Taken together, these observations suggest that Hh signaling is required in the trunk specifically for the formation of DRG neurons, and the observed effects on

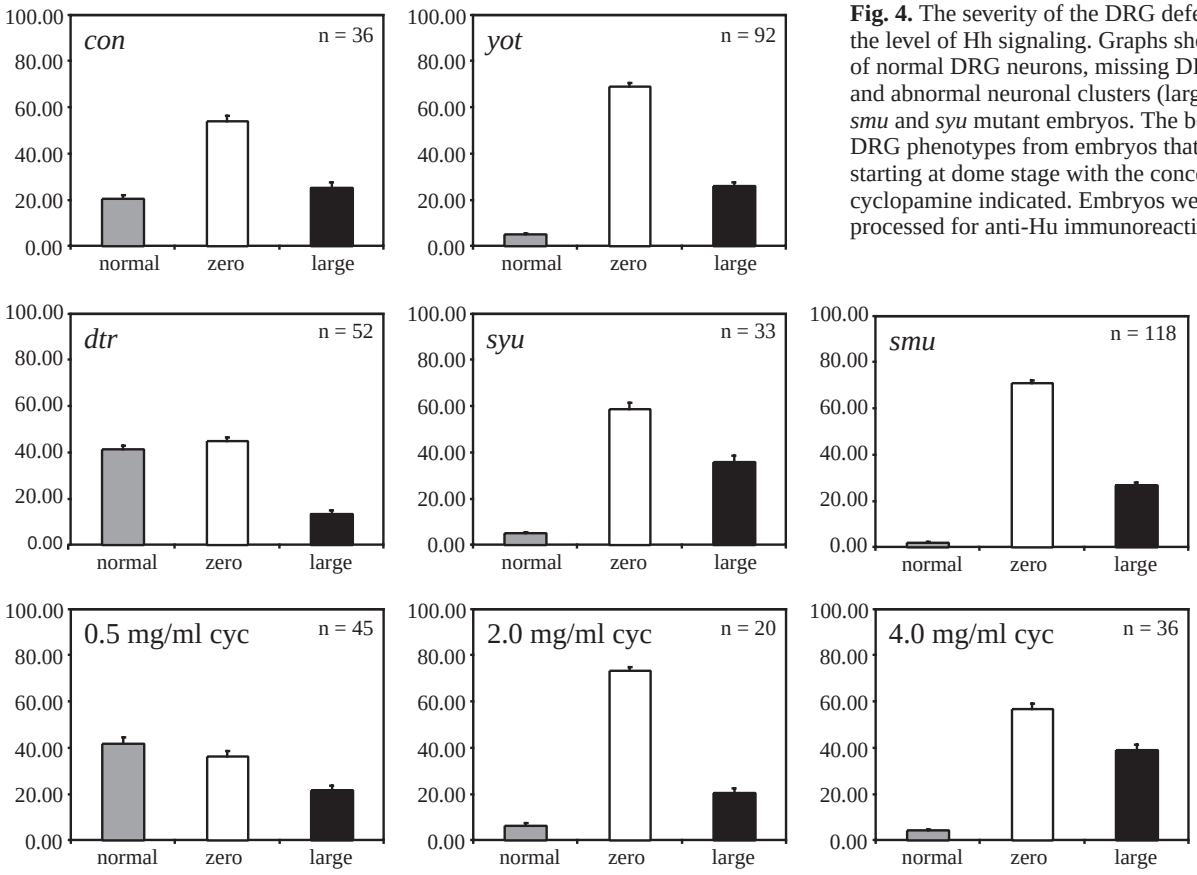

Fig. 4. The severity of the DRG defects corresponds to the level of Hh signaling. Graphs show the percentage of normal DRG neurons, missing DRG neurons (zero) and abnormal neuronal clusters (large) for *yot*, *con*, *dtr*, *smu* and *syu* mutant embryos. The bottom graphs show DRG phenotypes from embryos that were treated starting at dome stage with the concentration of cyclopamine indicated. Embryos were fixed and processed for anti-Hu immunoreactivity at 60 hpf.

DRG development are probably not the result of affecting earlier stages of trunk neural crest development.

DRG precursors require direct Hh signaling for normal DRG development

DRG precursors develop in close apposition to both the somites and neural tube. Therefore, it is possible that disruption of DRG development following loss of Hh signaling is simply a secondary effect because of the loss of patterning of these tissues. Consistent with this hypothesis, loss of either axial signals in *floating head* mutants (Halpern et al., 1995; Talbot, 1995) or paraxial tissues in *spadetail* mutants (Kimmel et al., 1989; Griffin et al., 1998) severely disrupts DRG development (data not shown). Alternatively, given that DRG precursors differentiate adjacent to strong sources of Hh signaling, neural crest cells may normally receive direct signals that instruct them to develop as DRG neurons. It is well-established that *ptc* transcription is upregulated in cells responding to Hh signals (Hidalgo and Ingham, 1990; Concordet et al., 1996). Consistent with a direct role for Hh signaling, we observe expression of *ptc1* (Fig. 7C,D) in cells in a position consistent with the location of DRG precursors (Fig. 7A,B). To confirm that *ptc* and *ngn1* were expressed in the same position, we serially sectioned embryos and

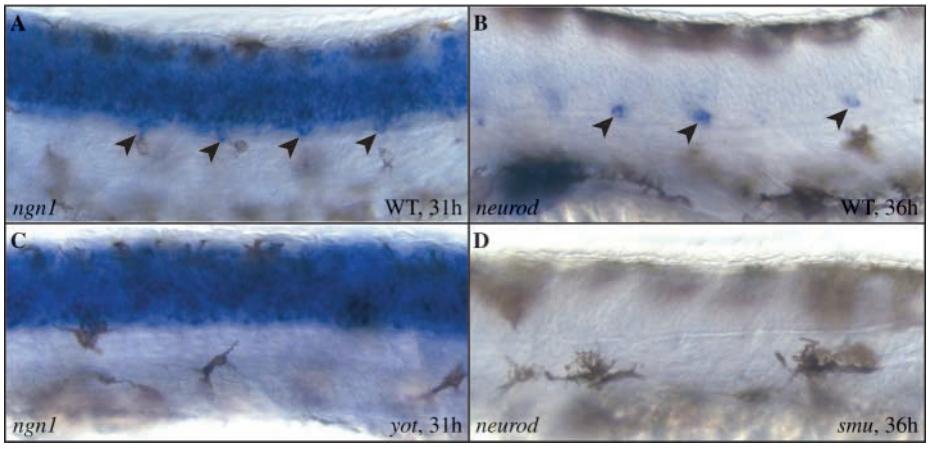

Fig. 5. Hh signaling is required for expression of *ngn1* and *neurod* in DRG precursors. *yot*, *smu* and wild-type embryos were processed for in situ hybridization at 31-36 hpf to reveal *ngn1* or *neurod* mRNA expression. (A) *ngn1* shows broad expression in the spinal cord in addition to its DRG precursor expression (arrowheads), whereas (B) *neurod* expression in the trunk is restricted to DRG precursors (arrowheads). (C,D) In *yot* and *smu* embryos, spinal cord staining is maintained, but *ngn1* and *neurod* expression in DRG precursors is eliminated.

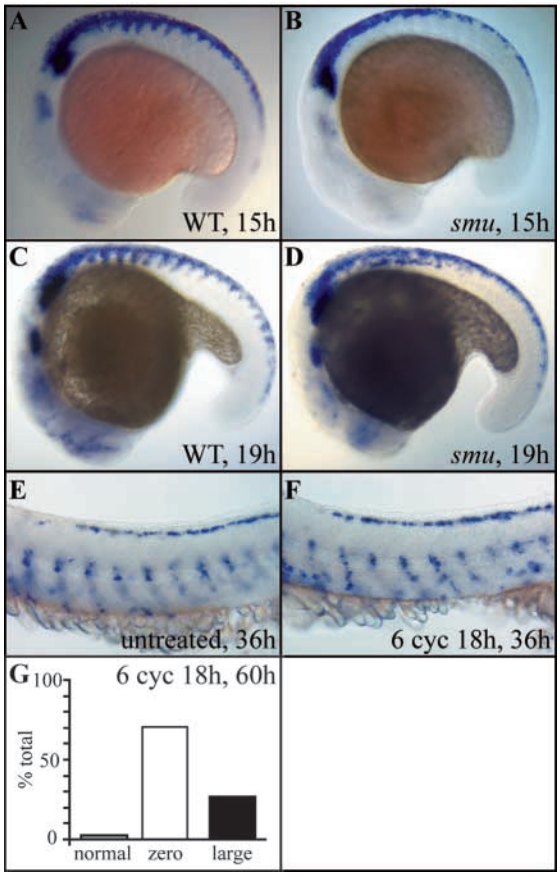

Fig. 6. Loss of Hh signaling does not result in a general disruption of neural crest development. *smu* embryos (B,D) and wild-type (WT) siblings (A,C) were probed with *crestin* to examine migratory neural crest. In *smu* embryos (B,D), levels of *crestin* expression are comparable to WT and neural crest do migrate ventrally. However, neural crest cells in *smu* embryos do not appear to be restricted to the mid-point of the somite (D). In contrast, WT embryos treated with 6 μg/ml cyclopamine beginning at 18 hpf and fixed at 36 hpf show no obvious defects in migration (F) compared with untreated siblings (E). Despite normal patterning and migration, DRG phenotypes in embryos cyclopamine-treated at 18 hpf (G) are similar to the severe disruption of Hh signaling in mutants (Fig. 4).

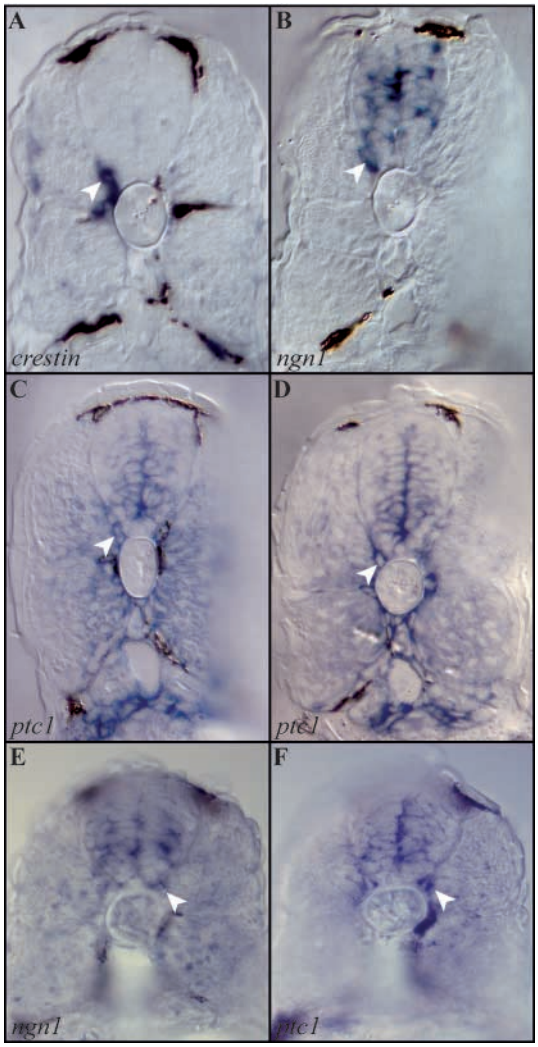

Fig. 7. *ptc1* transcripts are upregulated in presumptive DRG precursors. Wild-type embryos (36 hpf) were processed for whole-mount in situ hybridization, embedded in araldite and transverse sections were cut at trunk levels. (A) *crestin* is expressed in migrating neural crest cells (arrowhead). (B) *ngn1* is expressed in neural crest in the nascent DRG (arrowhead). (C,D) Cells expressing high levels of *ptc1* (arrowheads) are found in positions corresponding to expression of *ngn1* and *crestin*. (E,F) Adjacent serial sections were probed for *ngn1* (E) or *ptc* (F).

hybridized adjacent sections with each probe. Expression of *ngn1* (Fig. 7E) in the same ventrolateral position as where we find expression of *ptc1* in the adjacent section (Fig. 7F) suggests that DRG precursors may be upregulating *ptc* and are therefore capable of responding to Hh signals.

To directly determine which cells must respond to Hh signaling for proper DRG development, we transplanted wild-type cells into mutant embryos that are unable to transduce Hh signals. The *smu* mutation inactivates Smoothened, the transmembrane protein required for positive transduction of Hh signaling (Chen et al., 2001; Varga et al., 2001). The *yot* mutation encodes a C-terminally truncated Gli2 protein that acts as a dominant repressor of Hh signaling and is able to block Gli1-mediated transcriptional activation (Karlstrom et al., 1999; Karlstrom et al., 2003). Because *smu* (*smo*) is required for reception and transduction of Hh signals and *yot* (*gli2*) is a transcriptional effector of the pathway, cells mutant for either of these genes have defective transduction of Hh signals, and only transplanted wild-type cells would be capable of properly responding to Hh in these backgrounds. If the DRG defects in mutant embryos were because of the loss of neural tube or somite signals, then transplanted wild-type cells contributing to either of these tissues should have the potential to rescue adjacent DRG development. In contrast, if Hh signals were needed directly then DRG development would only be restored after transplantation of wild-type sensory precursors.

Significant contributions of wild-type cells to both spinal cord and somitic tissue never rescued adjacent DRG neurons in *smu* or in *yot* mutant embryos (Table 1). Similarly, in reciprocal transplants of *smu* or *yot* cells into wild-type hosts, significant contributions of mutant cells to spinal cord or

somitic tissue never disrupted development of adjacent DRG neurons (Table 1). In contrast, wild-type cells were capable of producing normal DRG neurons in both *smu* and *yot* mutant backgrounds (Fig. 8; Table 1). Although *smu* mutant cells were capable of producing neural crest-derived melanophores in wild-type embryos, they never formed DRG neurons (Table 1). Together, these results suggest that Hh signal transduction must occur directly within DRG precursors for normal DRG development to proceed.

Cyclopamine treatment during somitogenesis reveals a temporal requirement for Hh signaling during DRG development

In mutant embryos Hh signaling is attenuated throughout development, making it difficult to determine when Hh is specifically required for DRG formation. However, we can regulate the timing during which Hh signaling is perturbed by treating wild-type embryos with cyclopamine at specific developmental stages. To precisely determine when Hh signaling is needed during DRG development, cyclopamine was added to the embryo medium at different developmental time points and embryos were incubated until 60 hpf and then fixed and processed to examine Hu expression. Because development proceeds in a rostrocaudal pattern, we scored DRG defects at each segmental level. In embryos treated with cyclopamine beginning before the onset of gastrulation, most segments display a complete absence of DRG neurons (not shown). DRG neurons are absent in most segments in embryos that were treated with cyclopamine starting at 24 hpf; DRG are only found in the most rostral segments (Fig. 9). At this stage, somitogenesis is complete and DRG precursors are already well into their ventral migration. As cyclopamine is added at progressively later stages, the percentage of normal DRG increases in more caudal segments. Finally by 36 hpf, DRG neurons are only lost in the most caudal segments. We conclude from these results that although DRG precursors still require Hh signaling during late stages of their ventral migration, these cells cease to require Hh signaling prior to overt neuronal determination. DRG precursors at the axial level of somites 13-16 become insensitive to loss of Hh signaling somewhere between 28 and 32 hpf. At this time, the precursors have probably already reached the position of the nascent ganglia adjacent to the dorsal edge of the notochord where Hh levels are highest. Interestingly, this time period also correlates well with the onset of *ngn1* expression in DRG precursors, suggesting that Hh may normally influence DRG development through effects on *ngn1* expression.

Table 1. Fates of transplanted cells in mosaic embryos

Phenotypes			Number of DRG neurons/ number of hosts with DRG*	Number of hosts with melanophores
Donor	Host	*n*		
Wild type	Wild type	137	33/26	n.d.
Wild type	*yot*	41	16/11	n.d.
yot	Wild type	25	0/0	n.d.
Wild type	*smu*	14	4/4	n.d.
smu	*nac*	8	0/0	6
Wild type	*nac*	44	n.d./11	28

*Rhodamine dextran-labeled DRG neurons.
n.d., not determined.

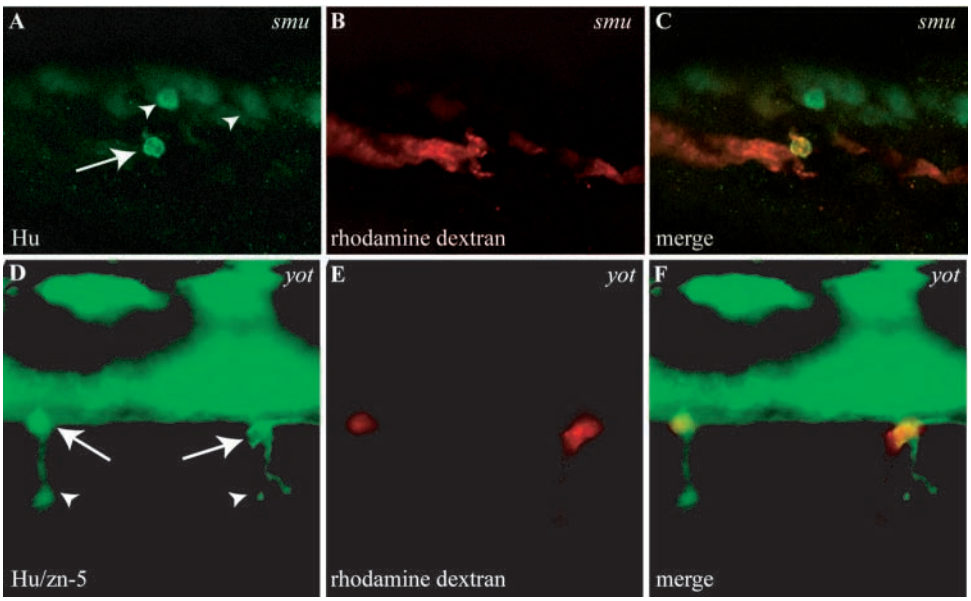

Fig. 8. Transplanted wild-type (WT) DRG precursor cells rescue DRG development in *smu* and *yot* backgrounds. (A) Lateral view of a *smu* host labeled with anti-Hu antibody viewed by confocal microscopy. (B) WT cells are shown in red in the same optical section. (C) Photoshop merge of A and B showing co-localization of labeled WT cells and Hu+ DRG neurons. (D) Lateral views of a *yot* host embryo labeled with anti-Hu and anti-zn5 (both in green). Hu expression can be seen in the spinal cord and in two separate ganglia (arrows) lateral to the spinal cord. zn-5 expression can be seen in secondary motor neuron projections (arrowheads). Notice the motor neuron projections are truncated, consistent with previously reported outgrowth defects in *yot* mutants (Brand et al., 1996). (E) The same *yot* mutant host embryo shown in D showing WT donor-derived cells (red). (F) Photoshop merge of D and E showing co-localization of labeled WT cells and Hu+ DRG neurons.

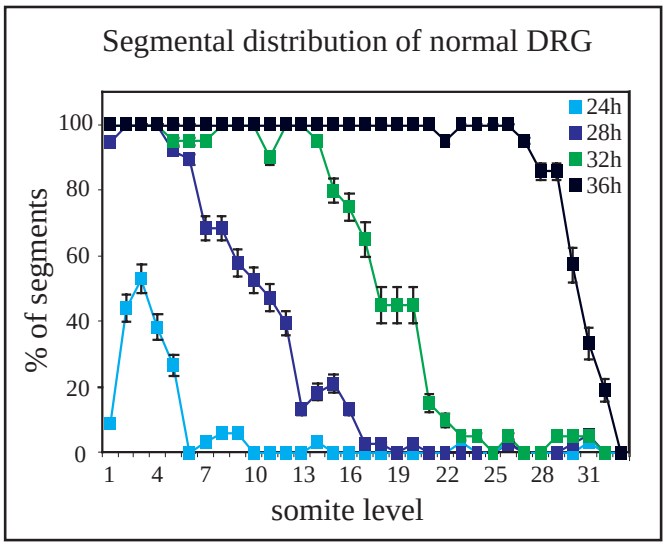

Fig. 9. Temporal sensitivity to cyclopamine. Embryos were treated with 6.0 μg/ml cyclopamine beginning at the indicated times and allowed to develop in the presence of cyclopamine until 60 hpf when they were scored for DRG phenotypes. The X-axis indicates somite level; 1 is the most rostral somite, 33 is the most caudal somite, and the Y-axis shows the percentage of embryos with 'normal DRG'. DRG were scored in at least 20 embryos for each treatment.

Discussion

Our results provide evidence for a role for Hh signaling in promoting the development of neural crest-derived DRG neurons. Analysis of both an allelic series and a comparison of the effects of different concentrations of cyclopamine, reveals a correlation between the severity of the DRG defects observed and the level of Hh signaling. Furthermore, analyses of the timing and tissue requirements for Shh signaling reveal a direct requirement for Hh signal transduction within DRG precursors, and suggest that Shh signaling may act upstream of Ngn1 to promote the specification of DRG neurons. These studies add to the previously demonstrated roles for Shh signaling in other aspects of neural crest development, particularly craniofacial development (Dunn et al., 1995; Ahlgren and Bronner-Fraser, 1999).

In addition to the prevalent loss of DRG in midline/Hh mutants and in cyclopamine-treated embryos, in some segments we observed the appearance of abnormal neuronal clusters ventrolateral to the spinal cord. The expression of the spinal cord neuron marker, Lim-1/2, in these cells in *smu* mutant embryos suggests that these may be cells that have inappropriately exited the spinal cord. It is possible that normal Hh signaling is required for structural integrity of the neural tube. Alternatively, given the specific ventrolateral appearance of these clusters, Hh signaling may be more specifically required for impeding migration of cells out of the motor exit points. Neural crest-derived cells are required to 'cap' the motor exit points and act as a barrier to movement of cell bodies between the CNS and PNS compartments in both mouse and chick embryos (Vermeren et al., 2003). It is possible that Hh signaling, in addition to its requirement for development of DRG precursors, is also required for development of these neural crest-derived border cells. The location of border cap cells at the ventrolateral edge of the spinal cord, adjacent to the sources of Shh, is consistent with a role for Hh in their development. Identifying specific markers for border cap cells would allow testing of this hypothesis.

Previous studies examining the effects of Shh on neural crest suggest several possible cellular mechanisms underlying the DRG phenotypes we observe in zebrafish. Shh signaling has been shown to play an important role in the survival of both neural tube and neural crest cell populations (Dunn et al., 1995; Miao et al., 1997; Ahlgren and Bronner-Fraser, 1999; Charrier et al., 2001). Injection of function-blocking anti-Shh antibody into chick cranial mesenchyme results in a loss of branchial arch structures that is associated with significant cell death in both the neural tube and neural crest (Ahlgren and Bronner-Fraser, 1999). 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). In our experiments, zebrafish migratory trunk neural crest appears normal in embryos in which Hh signaling is blocked. In addition, we do not observe any obvious cell death associated with the DRG in zebrafish Hh signaling mutants (data not shown). Moreover, in both the chick and *Xenopus* studies, loss of Shh signaling had no effect on the survival of trunk neural crest, suggesting that the anti-apoptotic role of Shh appears restricted to cranial neural crest populations. Similarly, although Shh promotes survival of specific CNS neurons following toxic insult, Shh fails to show any neuroprotective effects on DRG neurons in culture (Miao et al., 1997).

Hh signaling may alternatively be involved in regulating the migration of neural crest cells. Dorsally expressed bone morphogenetic proteins (BMPs) have been shown to promote neural crest dispersion through effects on integrins and cadherins (Sela-Donenfeld and Kalcheim, 1999). The work of Testaz et al. suggests that ventrally expressed Hh may play an opposing role in neural crest development by limiting the migration of neural crest cells: in neural tube explants cultured on fibronectin and immobilized N-terminal Shh, neural crest cell dispersion is severely restricted when compared with migration of neural crest cells on fibronectin alone (Testaz et al., 2001). Based on these results, we might predict that loss of DRG neurons in zebrafish Hh signaling mutants reflects the failure to localize DRG precursors to their appropriate positions. Consistent with this idea we see disorganization of trunk neural crest streams in Hh signaling mutants. However, although we observe significant loss of DRG neurons in embryos treated with cyclopamine at 18 hpf, we do not see any obvious effects on neural crest migration in these embryos. Moreover, other trunk neural crest derivatives, such as melanophores, still differentiate in their normal positions in the absence of Hh signaling (data not shown). Together, these results argue against the idea that the effects of Hh signaling on DRG development are mediated through general effects on neural crest migration. Interestingly, Testaz et al. also suggest that the effects of Shh on neural crest migration and adhesion probably involve mechanisms independent of the canonical Patched/Smoothened/Gli signaling cascade because neither forskolin, an activator of protein kinase A (PKA) and known antagonist of Hh signaling, nor cyclopamine block the Shh-mediated inhibition of neural crest cell migration (Testaz et al.,

2001). In contrast, zebrafish *smoothened* and *gli* mutations have the same effect on development of DRG neurons as loss of *shh*, indicating that the canonical Hh signaling pathway is required for normal DRG development.

Another possibility is that Hh signaling is required to directly promote the differentiation of DRG neurons. It is well-established that Shh signaling is both necessary and sufficient for motor neuron differentiation (Roelink et al., 1995; Chiang et al., 1996; Ericson et al., 1996) and promotes differentiation of other neuronal cell-types according to the appropriate axial position (Ericson et al., 1995; Hynes et al., 1995). All of our results are consistent with this role of Shh signaling in neuronal development. Others and ourselves have shown previously that Ngn1 is required for zebrafish sensory neuron specification (Andermann et al., 2002; Cornell and Eisen, 2002), and expression of *ngn1* is specifically absent from DRG precursors in midline/Hh mutants and cyclopamine-treated embryos. Both cell transplantation experiments and *ptc1* expression suggest that DRG precursors receive the Hh signal directly. Timed cyclopamine addition experiments further suggest that the window of requirement for the Hh signal ends at approximately the time *ngn1* is first expressed in DRG precursors. Taken together, these observations suggest the possibility that Hh promotes the determination of DRG precursors by activating *ngn1* expression, a role for Hh that has been suggested previously for other neuronal cell-types in zebrafish (Blader et al., 1997). In these studies, overexpression of Shh expanded the endogenous expression of zebrafish *ngn1* in the neural plate. Conversely, downregulation of Hh signaling by injecting a constitutively active form of PKA abolished neural plate expression of *ngn1*. Together, these results showed that Shh is capable of driving expression of *ngn1*, either directly or indirectly. Similarly, our results, together with previous descriptions of *ngn1* expression and DRG precursor migration patterns, suggest that Hh signaling may normally help regulate expression of *ngn1* in DRG sensory precursors.

Neural crest cells that ultimately populate the DRG migrate ventrally on the medial pathway along with sympathetic and pigment cell precursors. However, at a point adjacent to the notochord, sensory precursors stop and return dorsally to the position of the DRG (Raible and Eisen, 1994) where they begin to express *ngn1* (Cornell and Eisen, 2002; Andermann et al., 2002). The timing of onset of *ngn1* expression suggests that Hh signals emanating from the notochord and/or neural tube may be involved in initiation of *ngn1* expression in DRG precursors. Ngns are known to be sufficient for conferring neuronal identity on uncommitted precursors (Farah et al., 2000; Sun et al., 2001; Nieto et al., 2001). Furthermore, Ngns are thought to reinforce the neuronal program by inhibiting genes necessary for gliogenesis (Sun et al., 2001). This difference in migration behavior between sensory precursors and autonomic and pigment cell precursors further suggests that DRG precursors are already predisposed to respond to Hh signals early in their migration. Rather than biasing neural crest cells toward a sensory fate, Hh signaling may be influencing DRG precursors to adopt a neuronal cell fate by promoting *ngn1* expression.

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References

Ahlgren, S. C. and Bronner-Fraser, M. (1999). Inhibition of sonic hedgehog signaling in vivo results in craniofacial neural crest cell death. *Curr. Biol.* **9**, 1304-1314.

Ahlgren, S. C., Thakur, V. and Bronner-Fraser, M. (2002). Sonic hedgehog rescues cranial neural crest from cell death induced by ethanol exposure. *Proc. Natl. Acad. Sci. USA* **99**, 10476-10481.

Andermann, P., Ungos, J. M. and Raible, D. W. (2002). Neurogenin1 defines zebrafish cranial sensory ganglia precursors. *Dev. Biol.* **125**, 45-58.

Barresi, M. J., Stickney, H. L. and Devoto, S. H. (2000). The zebrafish *slow-muscle-omitted gene* product is required for Hedgehog signal transduction and the development of slow muscle identity. *Development* **127**, 2189-2199.

Beattie, C. E., Hatta, K., Halpern, M. E., Liu, H., Eisen, J. S. and Kimmel, C. B. (1997). Temporal separation in the specification of primary and secondary motoneurons in zebrafish. *Dev. Biol.* **187**, 171-182.

Blader, P., Fischer, N., Gradwohl, G., Guillemont, F. and Strahle, U. (1997). The activity of *neurogenin1* is controlled by local cues in the zebrafish embryo. *Development* **124**, 4557-4569.

Brand, M., Heisenberg, C. P., Warga, R. M., Pelegri, F., Karlstrom, R. O., Beuchle, D., Picker, A., Jiang, Y. J., Furutani-Seiki, M., van Eeden, F. J. et al. (1996). Mutations affecting development of the midline and general body shape during zebrafish embryogenesis. *Development* **123**, 129-142.

Charrier, J. B., Lapointe, F., Le Douarin, N. M. and Teillet, M. A. (2001). Anti-apoptotic role of Sonic hedgehog protein at the early stages of nervous system organogenesis. *Development* **128**, 4011-4020.

Chen, J. K., Taipale, J., Cooper, M. K. and Beachy, P. A. (2002). Inhibition of hedgehog signaling by direct binding of cyclopamine to *smoothened*. *Genes Dev.* **16**, 2743-2748.

Chen, W., Burgess, S. and Hopkins, N. (2001). Analysis of the zebrafish *smoothened* mutant reveals conserved and divergent functions of hedgehog activity. *Development* **128**, 2385-2396.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407-413.

Concordet, J. P., Lewis, K. E., Moore, J. W., Goodrich, L. V., Johnson, R. L., Scott, M. P. and Ingham, P. W. (1996). Spatial regulation of a zebrafish *patched* homologue reflects the roles of sonic hedgehog and protein kinase A in neural tube and somite patterning. *Development* **122**, 2835-2846.

Cooper, M. K., Porter, J. A., Young, K. E. and Beachy, P. A. (1998). Teratogen-mediated inhibition of target tissue response to Shh signaling. *Science* **280**, 1603-1607.

Cornell, R. A. and Eisen, J. S. (2002). Delta/Notch signaling promotes formation of zebrafish neural crest by repressing Neurogenin 1 function. *Development* **129**, 2639-2648.

Dunn, M. K., Mercola, M. and Moore, D. D. (1995). Cyclopamine, a steroidal alkaloid, disrupts development of cranial neural crest cells in *Xenopus*. *Dev. Dyn.* **202**, 255-270.

Dutton, K. A., Pauliny, A., Lopes, S. S., Elworthy, S., Carney, T. J., Rauch, J., Geisler, R., Haffter, P. and Kelsh, R. N. (2001). Zebrafish colourless encodes sox10 and specifies non-ectomesenchymal neural crest fates. *Development* **128**, 4113-4125.

Ericson, J., Thor, S., Edlund, T., Jessell, T. M. and Yamada, T. (1992). Early stages of motor neuron differentiation revealed by expression of homeobox gene Islet-1. *Science* **256**, 1555-1560.

Ericson, J., Muhr, J., Placzek, M., Lints, T., Jessell, T. M. and Edlund, T. (1995). Sonic hedgehog induces the differentiation of ventral forebrain neurons: a common signal for ventral patterning within the neural tube. *Cell* **81**, 747-756.

Ericson, J., Morton, S., Kawakami, A., Roelink, H. and Jessell, T. M. (1996). Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. *Cell* **87**, 661-673.

Ericson, J., Briscoe, J., Rashbass, P., van Heyningen, V. and Jessell, T. M. (1997). Graded sonic hedgehog signaling and the specification of cell fate in the ventral neural tube. *Cold Spring Harbor Symp. Quant. Biol.* **62**, 451-466.

Fan, C. M. and Tessier-Lavigne, M. (1994). Patterning of mammalian somites by surface ectoderm and notochord: evidence for sclerotome induction by a hedgehog homolog. *Cell* **79**, 1175-1186.

Farah, M. H., Olson, J. M., Sucic, H. B., Hume, R. I., Tapscott, S. J. and Turner, D. L. (2000). Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. *Development* **127**, 693-702.

Fashena, D. and Westerfield, M. (1999). Secondary motoneuron axons localize DM-GRASP on their fasciculated segments. *J. Comp. Neurol.* **406**, 415-424.

Goldstein, R. S. and Kalcheim, C. (1991). Normal segmentation and size of the primary sympathetic ganglia depend upon the alternation of rostrocaudal properties of the somites. *Development* **112**, 327-334.

Goldstein, R. S., Teillet, M. A. and Kalcheim, C. (1990). The microenvironment created by grafting rostral half-somites is mitogenic for neural crest cells. *Proc. Natl. Acad. Sci. USA* **87**, 4476-4480.

Griffin, K. J., Amacher, S. L., Kimmel, C. B. and Kimelman, D. (1998). Molecular identification of *spadetail*: regulation of zebrafish trunk and tail mesoderm formation by T-box genes. *Development* **125**, 3379-3388.

Halpern, M. E., Thisse, C., Ho, R. K., Thisse, B., Riggleman, B., Trevarrow, B., Weinberg, E. S., Postlethwait, J. H. and Kimmel, C. B. (1995). Cell-autonomous shift from axial to paraxial mesodermal development in zebrafish *floating head* mutants. *Development* **121**, 4257-4264.

Henion, P. D., Raible, D. W., Beattie, C. E., Stoesser, K. L., Weston, J. A. and Eisen, J. S. (1996). Screen for mutations affecting development of zebrafish neural crest. *Dev. Genet.* **18**, 11-17.

Hidalgo, A. and Ingham, P. (1990). Cell patterning in the *Drosophila* segment: spatial regulation of the segment polarity gene *patched*. *Development* **110**, 291-302.

Hynes, M., Porter, J. A., Chiang, C., Chang, D., Tessier-Lavigne, M., Beachy, P. A. and Rosenthal, A. (1995). Induction of midbrain dopaminergic neurons by Sonic hedgehog. *Neuron* **15**, 35-44.

Incardona, J. P., Gaffield, W., Kapur, R. P. and Roelink, H. (1998). The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction. *Development* **125**, 3553-3562.

Itoh, M., Kim, C. H., Palardy, G., Oda, T., Jiang, Y. J., Maust, D., Yeo, S. Y., Lorick, K., Wright, G. J., Ariza-McNaughton, L. et al. (2003). Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Dev. Cell* **4**, 67-82.

Johnson, R. L., Laufer, E., Riddle, R. D. and Tabin, C. (1994). Ectopic expression of sonic hedgehog alters dorsal-ventral patterning of somites. *Cell* **79**, 1165-1173.

Kalcheim, C. and Le Douarin, N. M. (1986). Requirement of a neural tube signal for the differentiation of neural crest cells into dorsal root ganglia. *Dev. Biol.* **116**, 451-466.

Kalcheim, C. and Teillet, M. A. (1989). Consequences of somite manipulation on the pattern of dorsal root ganglion development. *Development* **106**, 85-93.

Karlstrom, R. O., Trowe, T., Klostermann, S., Baier, H., Brand, M., Crawford, A. D., Grunewald, B., Haffter, P., Hoffmann, H., Meyer, S. U. et al. (1996). Zebrafish mutations affecting retinotectal axon pathfinding. *Development* **123**, 427-438.

Karlstrom, R. O., Talbot, W. S. and Schier, A. F. (1999). Comparative synteny cloning of zebrafish *you-too*: mutations in the Hedgehog target *gli2* affect ventral forebrain patterning. *Genes Dev.* **13**, 388-393.

Karlstrom, R. O., Tyurina, O., Kawakami, A., Talbot, W. S., Sasaki, H. and Schier, A. F. (2003). Genetic analysis of zebrafish *gli1* and *gli2* reveals divergent requirements for *gli* genes in vertebrate development. *Development* **130**, 1549-1564.

Kimmel, C. B., Kane, D. A., Walker, C., Warga, R. M. and Rothman, M. B. (1989). A mutation that changes cell movement and cell fate in the zebrafish embryo. *Nature* **337**, 358-362.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.

Kimmel, C. B., Miller, C. T. and Moens, C. B. (2001). Specification and morphogenesis of the zebrafish larval head skeleton. *Dev. Biol.* **233**, 239-257.

Korzh, V., Edlund, T. and Thor, S. (1993). Zebrafish primary neurons initiate expression of the LIM homeodomain protein Isl-1 at the end of gastrulation. *Development* **118**, 417-425.

Krauss, S., Concordet, J. P. and Ingham, P. W. (1993). A functionally conserved homolog of the *Drosophila* segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75**, 1431-1444.

Lewis, K. E. and Eisen, J. S. (2001). Hedgehog signaling is required for primary motoneuron induction in zebrafish. *Development* **128**, 3485-3495.

Lister, J. A., Robertson, C. P., Lepage, T., Johnson, S. L. and Raible, D. W. (1999). *nacre* encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate. *Development* **126**, 3757-3767.

Luo, R., An, M., Arduini, B. L. and Henion, P. D. (2001). Specific pan-neural crest expression of zebrafish Crestin throughout embryonic development. *Dev. Dyn.* **220**, 169-174.

Ma, Q., Chen, Z., del Barco Barrantes, I., de la Pompa, J. L. and Anderson, D. J. (1998). *neurogenin1* is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. *Neuron* **20**, 469-482.

Ma, Q., Fode, C., Guillemot, F. and Anderson, D. J. (1999). Neurogenin1 and neurogenin2 control two distinct waves of neurogenesis in developing dorsal root ganglia. *Genes Dev.* **13**, 1717-1728.

Marusich, M. F., Furneaux, H. M., Henion, P. D. and Weston, J. A. (1994). Hu neuronal proteins are expressed in proliferating neurogenic cells. *J. Neurobiol.* **25**, 143-155.

Miao, N., Wang, M., Ott, J. A., D'Alessandro, J. S., Woolf, T. M., Bumcrot, D. A., Mahanthappa, N. K. and Pang, K. (1997). Sonic hedgehog promotes the survival of specific CNS neuron populations and protects these cells from toxic insult in vitro. *J. Neurosci.* **17**, 5891-5899.

Neumann, C. J., Grandel, H., Gaffield, W., Schulte-Merker, S. and Nusslein-Volhard, C. (1999). Transient establishment of anteroposterior polarity in the zebrafish pectoral fin bud in the absence of sonic hedgehog activity. *Development* **126**, 4817-4826.

Nieto, M., Schuurmans, C., Britz, O. and Guillemot, F. (2001). Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. *Neuron* **29**, 401-413.

Park, H. C., Kim, C. H., Bae, Y. K., Yeo, S. Y., Kim, S. H., Hong, S. K., Shin, J., Yoo, K. W., Hibi, M., Hirano, T. et al. (2000). Analysis of upstream elements in the HuC promoter leads to the establishment of transgenic zebrafish with fluorescent neurons. *Dev. Biol.* **227**, 279-293.

Perez, S. E., Rebelo, S. and Anderson, D. J. (1999). Early specification of sensory neuron fate revealed by expression and function of neurogenins in the chick embryo. *Development* **126**, 1715-1728.

Raible, D. W. and Eisen, J. S. (1994). Restriction of neural crest cell fate in the trunk of the embryonic zebrafish. *Development* **120**, 495-503.

Raible, D. W., Wood, A., Hodsdon, W., Henion, P. D., Weston, J. A. and Eisen, J. S. (1992). Segregation and early dispersal of neural crest cells in the embryonic zebrafish. *Dev. Dyn.* **195**, 29-42.

Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T. M. et al. (1994). Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. *Cell* **76**, 761-775.

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A. and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* **81**, 445-455.

Rubinstein, A. L., Lee, D., Luo, R., Henion, P. D. and Halpern, M. E. (2000). Genes dependent on zebrafish *cyclops* function identified by AFLP differential gene expression screen. *Genesis* **26**, 86-97.

Sbrogna, J. L., Barresi, M. J. and Karlstrom, R. O. (2003). Multiple roles for Hedgehog signaling in zebrafish pituitary development. *Dev. Biol.* **254**, 19-35.

Schauerte, H. E., van Eeden, F. J., Fricke, C., Odenthal, J., Strahle, U. and Haffter, P. (1998). *Sonic hedgehog* is not required for the induction of medial floor plate cells in the zebrafish. *Development* **125**, 2983-2993.

Sela-Donenfeld, D. and Kalcheim, C. (1999). Regulation of the onset of neural crest migration by coordinated activity of BMP4 and Noggin in the dorsal neural tube. *Development* **126**, 4749-4762.

Stenkamp, D. L. and Frey, R. A. (2003). Extraretinal and retinal hedgehog signaling sequentially regulate retinal differentiation in zebrafish. *Dev. Biol.* **258**, 349-363.

Sun, Y., Nadal-Vicens, M., Misono, S., Lin, M. Z., Zubiaga, A., Hua, X., Fan, G. and Greenberg, M. E. (2001). Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. *Cell* **104**, 365-376.

Talbot, W. S., Trevarrow, B., Halpern, M. E., Melby, A. E., Farr, G., Postlethwait, J. H., Jowett, T., Kimmel, C. B. and Kimelman, D. (1995). A homeobox gene essential for zebrafish notochord development. *Nature* **378**, 150-157.

Teillet, M. A. and Le Douarin, N. M. (1983). Consequences of neural tube

and notochord excision on the development of the peripheral nervous system in the chick embryo. *Dev. Biol.* **98**, 192-211.

Testaz, S., Jarov, A., Williams, K. P., Ling, L. E., Koteliansky, V. E., Fournier-Thibault, C. and Duband, J. L. (2001). Sonic hedgehog restricts adhesion and migration of neural crest cells independently of the Patched-Smoothened-Gli signaling pathway. *Proc. Natl. Acad. Sci. USA* **98**, 12521-12526.

Thisse, C., Thisse, B., Schilling, T. F. and Postlethwait, J. H. (1993). Structure of the zebrafish snail1 gene and its expression in wild-type, *spadetail* and *no tail* mutant embryos. *Development* **119**, 1203-1215.

Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M. and Pfaff, S. L. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. *Cell* **79**, 957-970.

van Eeden, F. J., Granato, M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C. P., Jiang, Y. J., Kane, D. A. et al. (1996). Mutations affecting somite formation and patterning in the zebrafish, *Danio rerio*. *Development* **123**, 153-164.

Varga, Z. M., Amores, A., Lewis, K. E., Yan, Y. L., Postlethwait, J. H., Eisen, J. S. and Westerfield, M. (2001). Zebrafish *smoothened* functions in ventral neural tube specification and axon tract formation. *Development* **128**, 3497-3509.

Vermeren, M., Maro, G. S., Bron, R., McGonnell, I. M., Charnay, P., Topilko, P. and Cohen, J. (2003). Integrity of developing spinal motor columns is regulated by neural crest derivatives at motor exit points. *Neuron* **37**, 403-415.

Westerfield, M. (1994). *The Zebrafish Book*, University of Oregon Press.

Wolff, C., Roy, S. and Ingham, P. W. (2003). Multiple muscle cell identities induced by distinct levels and timing of hedgehog activity in the zebrafish embryo. *Curr. Biol.* **13**, 1169-1181.