Slow muscle regulates the pattern of trunk neural crest migration in zebrafish

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Summary

In avians and mice, trunk neural crest migration is restricted to the anterior half of each somite. Sclerotome has been shown to play an essential role in this restriction; the potential role of other somite components in specifying neural crest migration is currently unclear. By contrast, in zebrafish trunk neural crest, migration on the medial pathway is restricted to the middle of the medial surface of each somite. Sclerotome comprises only a minor part of zebrafish somites, and the pattern of neural crest migration is established before crest cells contact sclerotome cells, suggesting other somite components regulate the pattern of zebrafish neural crest migration. Here, we use mutants to investigate which components regulate the pattern of zebrafish trunk neural crest migration on the medial pathway. The pattern of trunk neural crest migration is aberrant in *spadetail* mutants that have very reduced somitic mesoderm, in *no tail* mutants injected with *spadetail* morpholino antisense oligonucleotides that entirely lack somitic mesoderm and in somite segmentation mutants that have normal somite components but disrupted segment borders. Fast muscle cells appear dispensable for patterning trunk neural crest migration. However, migration is abnormal in Hedgehog signaling mutants that lack slow muscle cells, providing evidence that slow muscle cells regulate the pattern of trunk neural crest migration. Consistent with this idea, surgical removal of adaxial cells, which are slow muscle precursors, results in abnormal patterning of neural crest migration; normal patterning can be restored by replacing the ablated adaxial cells with ones transplanted from wild-type embryos.

Key words: Myotome, *Danio rerio*, *spadetail*, *beamter*, *fused somites*, *sonic hedgehog*, *sonic-you*, *slow muscle omitted*, Smoothened, *unplugged*, *you too*, *after eight*, *deadly seven*, Notch pathway mutants

Introduction

Although all vertebrates have a similar body plan, the early cell movements that establish that body plan can differ considerably in different vertebrate subgroups. For example, neural crest cells, a defining feature of vertebrates, migrate from their origin in the region of the dorsal neural tube throughout the body and differentiate into a well-characterized set of derivatives (Le Douarin and Kalcheim, 1999; Kalcheim, 2000; Eisen and Weston, 1993). The pathways of neural crest migration are highly regulated, but differ between amniotes, such as avians and mice, and anamniotes, such as zebrafish. The neural crest medial migration pathway in zebrafish is restricted to the middle of the medial surface of each somite (Fig. 1) (Raible et al., 1992); thus, trunk neural crest cells migrate in a pattern of 'streams' in which there is a single stream medial to the middle of each somite. By contrast, the equivalent migration pathway in avians and mice is restricted to the anterior half of each somite. Despite these differences in migration path in amniotes and anamniotes, early-migrating crest cells in both groups generate similar neuronal derivatives (Le Douarin and Kalcheim, 1999; Kalcheim, 2000; Christiansen et al., 2000).

The pattern of trunk crest migration is likely to be established by different somite components in amniotes and anamniotes. In avian and mammalian embryos, trunk neural crest migration is regulated by environmental cues thought to be derived from somites, and especially from sclerotome. Many studies provide evidence that neural crest migrates through the anterior half sclerotome of each somite, and several molecules expressed in posterior half sclerotome repel crest migration (Newgreen et al., 1990) (reviewed by Le Douarin and Kalcheim, 1999; Krull, 2001; Kalcheim, 2000; Kuan et al., 2004). In contrast to amniote embryos, the majority of cells in the somites of anamniotes such as zebrafish and frogs are myotomal, rather than sclerotomal (Fig. 1) (Morin-Kensicki and Eisen, 1997; Keller, 2000). In zebrafish, sclerotome precursors are first seen at the most ventromedial region of the somite. These cells migrate dorsally and eventually meet neural crest cells, but not until after the segmental pattern of neural crest migration streams on the medial pathway is already established. Thus, the mechanisms that initiate the segmental pattern of neural crest migration streams in zebrafish are currently unknown.

Myotomal cells are present at the right time and place to establish the segmental pattern of migration of zebrafish trunk neural crest on the medial pathway. Studies of zebrafish sclerotome showed that, in contrast to avian embryos, sclerotome was not required for formation of dorsal root
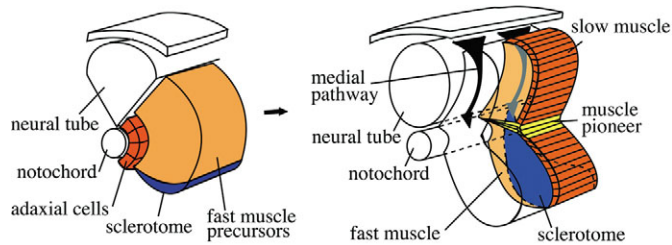
Fig. 1. Neural crest migration and somite development in zebrafish. Myotome is the major component of zebrafish somites. Slow muscle precursors (red), called adaxial cells, are located adjacent to notochord before segmentation (left). During segmentation stages they become a single-layered sheet of fully extended muscle cells along the anteroposterior axis and then migrate through the somite to the lateral surface (right). A subset of these cells, called muscle pioneers, remain medial and define the horizontal myoseptum (yellow). The major part of each somite is composed of fast muscle cells (orange). Sclerotome cells (blue) arise in ventromedial somite and migrate dorsally. Neural crest migrates on a medial pathway in the middle of the medial aspect of each somite (arrow).

ganglia or for pathfinding by motor axons, leading Morin-Kensicki and Eisen (Morin-Kensicki and Eisen, 1997) to suggest that zebrafish myotome contains the patterning information attributed to avian sclerotome. This idea is supported by the observation that as neural crest cells begin to migrate along the medial pathway, myotome cells are the first cells they encounter, and the onset of neural crest migration coincides with contact between the two cell types (Raible et al., 1992). The myotomal precursors of zebrafish slow and fast muscle fibers can be recognized very early in development. Slow muscle precursors, called adaxial cells, can be distinguished before segmentation occurs as a sheet of cuboidal cells in the segmental plate adjacent to the notochord (Devoto et al., 1996). Shortly after somite formation, adaxial cells start to elongate on the medial myotome surface as a monolayer of muscle fibers. These cells then migrate as elongated muscle fibers through the myotome to the lateral surface, where they form a monolayer of slow muscle fibers (Devoto et al., 1996; Cortés et al., 2003). Adaxial cell migration away from the medial myotome surface begins shortly after neural crest cells enter the medial migration pathway [compare time courses of adaxial cell migration in Devoto et al. (Devoto et al., 1996) and neural crest migration onset in Raible et al. (Raible et al., 1992)]. A subset of adaxial cells becomes muscle pioneer cells that extend through the myotome from the notochord to the lateral surface and define the position of the horizontal myoseptum that separates dorsal and ventral myotome regions (Halpern et al., 1993). Fast muscle precursors are initially lateral to adaxial cells but later become medial muscle fibers, after adaxial cell migration (Fig. 1) (Devoto et al., 1996; Blagden et al., 1997) (reviewed by Stickney et al., 2000).

In this paper, we explore how somites affect the restricted migration of trunk neural crest cells in streams along the middle region of the medial surface of each zebrafish somite, with an emphasis on how this pattern is initially established. Analysis of two kinds of somite mutants, ones with reduced somitic mesoderm and ones with segmentation defects, shows that proper somite patterning is required for normal patterning

of neural crest migration. Neural crest forms normal migration streams in embryos that lack fast muscle, showing that this somite component is not required to pattern these streams. By contrast, neural crest migration is disrupted in Hedgehog (Hh) signaling mutants that have normal somite boundaries but reduced numbers of slow muscle cells (van Eeden et al., 1996; Stickney et al., 2000), suggesting that slow muscle regulates the pattern of neural crest migration on the medial pathway. We tested this hypothesis by examining neural crest migration in embryos after surgical removal and replacement of adaxial cells. The pattern of neural crest migration streams is abnormal in somites that lack slow muscle; normal patterning can be restored by transplantation of wild-type slow muscle cells. These data support the hypothesis that slow muscle regulates the pattern of neural crest migration on the medial pathway in zebrafish.

Materials and methods

Animals

Embryos were obtained from natural spawnings of a wild-type colony (AB) or crosses of identified carriers heterozygous for specific mutations. Fish were maintained in the University of Oregon Zebrafish Facility on a 14 hour light/10 hour dark cycle at 28.5°C and embryos staged according to Kimmel et al. (Kimmel et al., 1995) by number of somites or hours post fertilization (hpf) at 28.5°C. Mutant embryos were generated by crossing two heterozygous adult carriers. The following mutant alleles were used in this study; spt^{b104} (Griffin et al., 1998), ntl^{b195} (Schulte-Merker et al., 1994; Amacher et al., 2002), bea^{tm98}, fss^{te314} (van Eeden et al., 1996; van Eeden et al., 1998; Nikaido et al., 2002), syu^{t4} (Schauerte et al., 1998), smu^{b641} (Varga et al., 2001), aei^{tm223}, des^{te322b} (Holley et al., 2000; Holley et al., 2002) and yot^{ty17} (Karlstrom et al., 1999). At least 30 embryos were examined for each mutation.

RNA in situ hybridization and immunohistochemistry

RNA in situ hybridization was performed as described previously (Appel and Eisen, 1998). The *crestin* antisense riboprobe was detected by Fast Fast Red (Sigma) or NBT/BCIP (Roche). DIG-labeled or fluorescein-labeled antisense RNA probes (Roche Diagnostics) for RNA in situ hybridization were generated from plasmids as follows: *crestin* plasmid (Luo et al., 2001) was cut with *Eco*RI and transcribed with T7 polymerase; and *myod* plasmid (Weinberg et al., 1996) was cut with *Xba*I and transcribed with T7 polymerase. F59 monoclonal antibody (Crow and Stockdale, 1986) was used at a 1:10 dilution to detect slow muscle cells (Devoto et al., 1996); EB165 monoclonal antibody (Blagden et al., 1997) was used at 1:5000 dilution to detect fast muscle cells; and anti-HuC antibody (16A11) was used at 1:1000 dilution (Marusich et al., 1994; Henion et al., 1996). Antibody staining was carried out after RNA in situ hybridization. Alexa-488- or Alexa-546-conjugated goat anti-rabbit polyclonal antibody was used as secondary antibody. Fluorescence was visualized using a confocal laser scanning microscope (Biorad Radiance 2100). Fluorescent images shown in Figs 2-6 represent *z*-projections.

mRNA and morpholino microinjection

Embryos were injected with 2-5 nl diluted RNA solution (50 ng/ml) or morpholino antisense oligonucleotides (MOs) into the yolk at the one-cell stage. *spt* MO mix [*spt* MO 1 1.2 mg/ml; *spt* MO 2 0.5 mg/ml; MO sequences reported by Lewis and Eisen (Lewis and Eisen, 2004)] were injected into ntl^{b195} mutant embryos. mRNA was transcribed as described by Lewis and Eisen (Lewis and Eisen, 2001). Pipettes were pulled on a Sutter Instruments Micropipette puller (Model P-2000). Injections were performed with an air injection apparatus (ASI).

Removal and transplantation of adaxial cells

Adaxial cell removal

At the one- to three-somite stage, ~20 adaxial cells were removed from wild-type embryos by gentle suction with a micropipette, as described by Eisen and Pike (Eisen and Pike, 1991). Briefly, embryos were embedded in 5% methylcellulose, a micropipette inserted approximately five somite widths posterior to the most-recently formed somite and ~20 adaxial cells removed from a two- to three-somite wide region by gentle suction. We carried out these experiments under Nomarski optics, which allows adaxial cells to be readily discerned from neighboring non-adaxial cells (Hirsinger et al., 2004), and later confirmed by staining with F59 antibody, which labels slow muscle fibers, that adaxial cells were removed. Only embryos whose adaxial cells were removed were fixed at 21-24 hpf to examine slow muscle and neural crest; embryos in which adaxial cells were still present were discarded. Adaxial cells were removed from 39 embryos. In 20 cases, slow muscle appeared normal; it is unclear whether the ablation did not successfully remove all adaxial cells or whether adaxial cells were replaced as we have seen in previous muscle ablation experiments (J.S.E., unpublished). These animals served as controls; neural crest migration was normal in 17/20. In 19 cases, slow muscle was reduced or absent from the target somites. We examined these embryos to learn whether neural crest migration was affected.

One- to three-somite stage adaxial cell transplantation

For transplantation, wild-type donor embryos were labeled by injection of a mixture of 2.5% rhodamine dextran (lysinated, 3×10^3 M_r; Molecular Probes) and 2.5% fluorescein dextran (lysinated, 3×10^3 M_r; Molecular Probes) in 0.2 M KCl into the yolk cell at the one-cell stage. Adaxial cells were removed as described above from donor and host embryos. Adaxial cells from dye-labeled, wild-type donors were transplanted into unlabeled smu^{b641} mutant hosts. smu mutants with transplanted adaxial cells were fixed at 21-24 hpf. The neural crest was visualized by in situ hybridization with $crestin$ probe, and the transplanted cells were visualized with anti-fluorescein antibody. Later, these embryos were stained with F59 antibody to confirm that transplanted cells were slow muscle cells. Adaxial cells were transplanted in 63 embryos. Of these, 47 were +/+ or smu–/+; we did not score these. In 17 cases, hosts were smu–/–; 11 of these had transplanted wild-type slow muscle cells.

Shield stage adaxial cell transplantation

Transplantation was performed as described by Maves et al. (Maves et al., 2002). In brief, wild-type donors were labeled at the one-cell stage as described above. Cells were removed by using a pulled glass micropipette as a knife and donor tissue (about 50-100 cells) was excised and then inserted into an unlabeled smu^{b641} mutant host. Cells were transplanted ~40° from the shield, 10-15 cells from the edge and from the deepest layer of cells. smu mutants with transplanted adaxial cells were fixed at 21-24 hpf and processed as described above.

Results

Somites play an essential role in patterning zebrafish neural crest migration

Somites are necessary to pattern trunk neural crest migration in amniote vertebrates (Bronner-Fraser and Stern, 1991; Tosney, 1988) (reviewed in Kuan et al., 2004). To learn whether this is also the case in zebrafish, we examined neural crest migration in two kinds of somite defect mutants: (1) mutants in T-box genes that have little or no somitic mesoderm; and (2) mutants that have segmentation defects leading to improperly formed somites. We visualized migrating neural crest cells by RNA in situ hybridization with a riboprobe for $crestin$, a gene
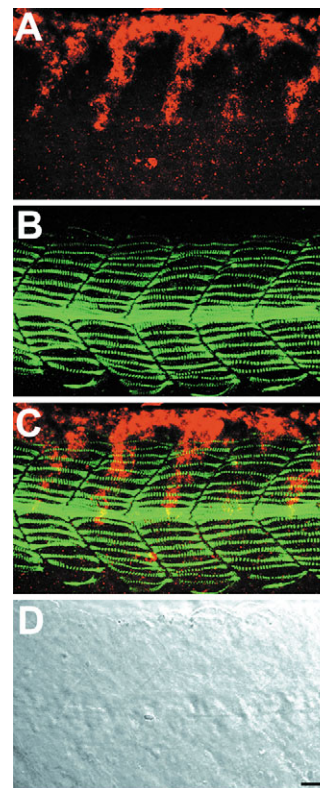
Fig. 2. Neural crest migration in wild-type embryos. Whole-mount staining of 21 hpf wild-type embryo with $crestin$ riboprobe (red) and F59 antibody (green) to reveal trunk neural crest and slow muscle cells. (A) Neural crest alone, (B) slow muscle alone, (C) the merged image, (D) a Nomarski differential interference contrast (DIC) image. Neural crest migrates in the middle of the medial aspect of the myotome in one- to two-cell wide streams. Slow muscle fibers extend throughout the anteroposterior axis of each somite. At this stage of development, midtrunk level neural crest cells are in a more medial focal plane than slow muscle fibers, but they are seen together in this z-projection. Similar z-projections are shown in subsequent figures. Lateral view, anterior toward the left. Scale bar: 20 μm.

expressed in almost all zebrafish trunk neural crest cells (Luo et al., 2001), and slow muscle by staining with F59 antibody that recognizes slow muscles and also reveals somite boundaries (Devoto et al., 1996). Double-labeling revealed that in wild-type embryos, neural crest migration on the medial pathway is restricted to a single stream of cells in the middle of the medial surface of each somite (Fig. 2). Slow muscle fibers are initially located on the medial surface of the somite. They then migrate through the somite and, by the stage shown in Fig. 2, they form a monolayer of slow muscle fibers on the lateral surface of each somite (Devoto et al., 1996).

T-box mutants

$spadetail$ (spt) encodes zebrafish Tbx16 (Griffin et al., 1998) (see http://zfin.org for nomenclature). In spt mutants, somitic mesoderm is reduced or absent (Molven et al., 1990; Ho and Kane, 1990) and there are very few slow muscle cells, as shown by F59 staining (Fig. 3). Neural crest migrates in spt mutants, but migration is entirely unrestricted and there is no pattern of segmental streams (Fig. 3). Because muscle fibers do form in

some *spt* mutants, we decided to examine embryos lacking function of both *spt* and another T-box gene, *no tail* (*ntl*), by injecting *spt* MOs into *ntl* mutants. These embryos entirely lack trunk mesoderm and never make any muscle fibers (Lewis and Eisen, 2004; Amacher et al., 2002), as shown by lack of expression of *myod* (Fig. 3). As in *spt* mutants, in *ntl* mutants injected with *spt* MOs, trunk neural crest cells migrated, but their migration was entirely unrestricted and there was no segmental pattern (Fig. 3). Thus, we conclude that somites are unnecessary for neural crest motility, but they are necessary to restrict neural crest migration into coherent, segmental streams.
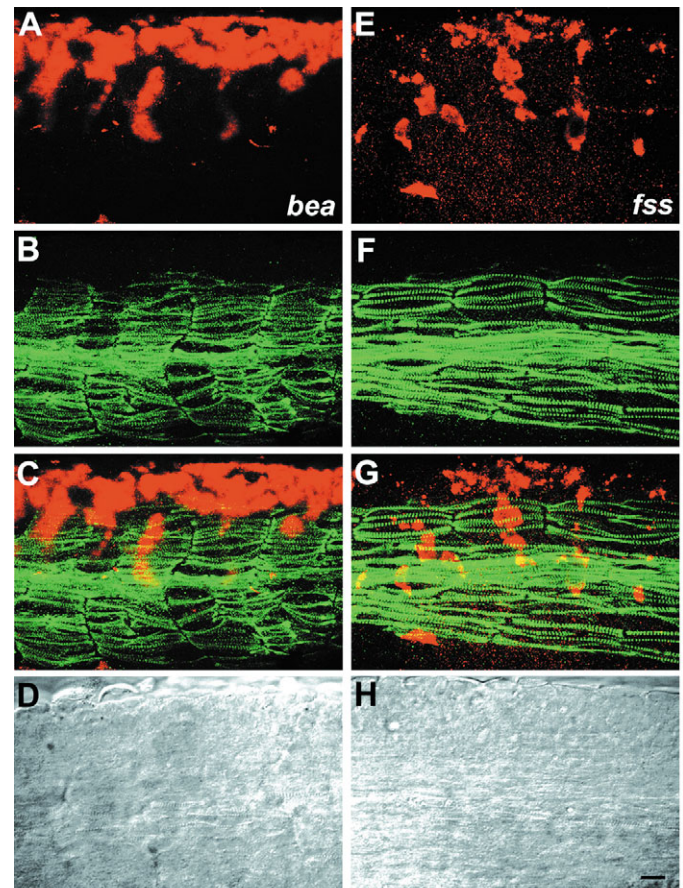
Segmentation mutants

The lack of segmentally patterned trunk neural crest migration in *spt* mutants and *ntl* mutants injected with *spt* MOs suggested that normal somite segmentation might be required to establish a normal pattern of crest migration on the medial pathway. To test this hypothesis, we examined neural crest migration in embryos with mutations in genes required for proper somite segmentation, including *beamter* (*bea*) (van Eeden et al., 1996), *fused somites* [*fss* (*tbx24* – Zebrafish Information Network)] (Nikaido et al., 2002), *after eight* [*aei* (*deltaD* – Zebrafish Information Network)] (Holley et al., 2000) and *deadly seven* [*des* (*notch1a* – Zebrafish Information Network)] (Holley et al., 2002). In *bea* mutants, normal somite boundaries form only for the most anterior 5-7 somites; no normal somite boundaries form at all in *fss* mutants. Although there is an overall segmental pattern to neural crest migration on the medial pathway in these mutants, the pattern is abnormal (Figs 4 and 5). Streams of neural crest cells are closer together or farther apart than in wild types, in correlation with the abnormal size and shape of somites in these mutants. In most cases, the streams were appropriately located in the middle of the medial aspect of the myotome, consistent with our hypothesis that somites pattern trunk neural crest migration. However, in some cases the streams of neural crest cells branched and neural crest cells migrated in abnormal positions.
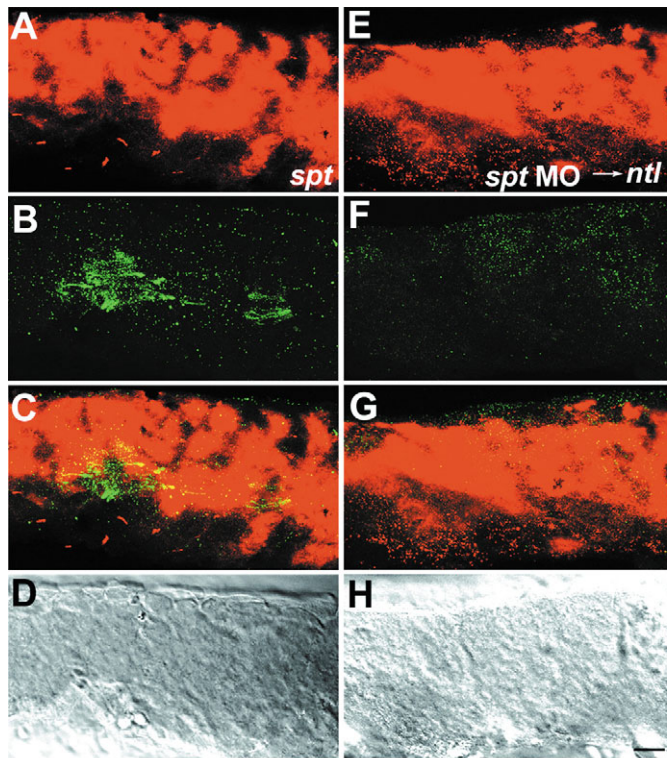

Fig. 3. Neural crest cells migrate in an unrestricted pattern in *spt* mutants and *ntl* mutants injected with *spt* MOs. (A-D) Whole-mount staining of 21 hpf *spt* mutant with *crestin* riboprobe (red) and F59 antibody (green). (A) Neural crest alone, (B) slow muscle alone, (C) the merged image, (D) a DIC image. F59 antibody staining reveals that *spt* mutants have few slow muscle cells. Neural crest cells migrate in *spt* mutants, but migration is not restricted to a specific pathway, thus there are no migration streams. (E-H) Whole-mount staining of 21 hpf *ntl* mutant injected with *spt* MOs. (E) Neural crest alone, (F) slow muscle alone, (G) the merged image, (H) a DIC image. F59 antibody staining reveals that *ntl* mutants injected with *spt* MOs have no muscle. As in *spt* mutants, neural crest migrates in these embryos, but migration is not restricted to a specific pathway and there are no migration streams in *ntl* mutants injected with *spt* MOs that have no muscle cells, as shown by absence of *myod* expression (H). Scale bar: 20 μm.

Fig. 4. Disruption of neural crest migration is correlated with segmentation defects. Whole-mount staining with *crestin* riboprobe (red) and F59 antibody (green) of *bea* (A-D; posterior of somite 9) and *fss* mutants (E-H) at 21 hpf. Streams of neural crest cells (red, A,C,E,G) are present, but less regular than in wild types and the streams show some branching, consistent with abnormal somite shape and size. However, neural crest still migrates generally in the middle of the medial aspect of the myotome in both mutants. (D,H) DIC images of *bea* and *fss* mutants, respectively. Scale bar: 20 μm.

Fig. 5. In segmentation mutants, neural crest migration is disrupted in the same somites in which segmentation is disrupted. Whole-mount staining with a *crestin* riboprobe of wild-type (A) *fss* (B), *bea* (C) and *smu* mutants (D) at 21 hpf to reveal neural crest cells. (A) In wild-type embryos, neural crest cells migrate in segmental streams along the entire AP axis. (B) Neural crest migration is abnormal from the first somite in *fss* mutants. (C) In *bea* mutants, neural crest migration is disrupted posterior to somite 6. (D) In *smu* mutants there is no clear segmental pattern of neural crest migration; in addition, neural crest cells stall at the level of the dorsal aspect of the notochord. (E,F) EB165 antibody staining reveals that in *smu* mutants (F) somites are smaller along the DV axis, but a significant amount of fast muscle is still present, although there may be less than in wild types (E). Scale bar: 60 μm in A-D; 25 μm in E,F.
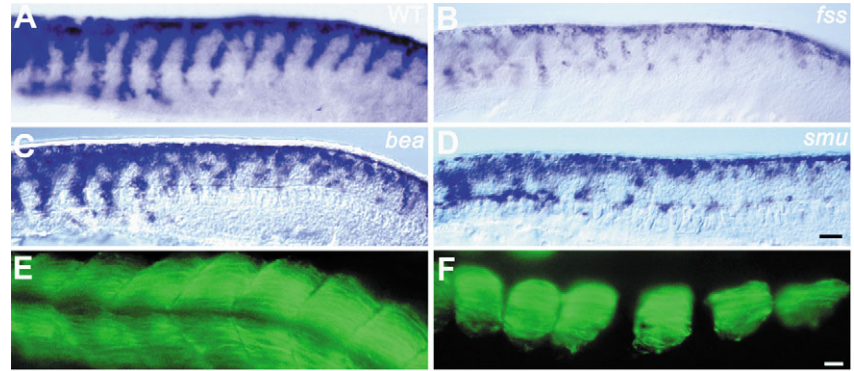
These patterning defects occurred only where somite segmentation was disturbed, i.e. posterior to somite 7 in *bea* mutants and from the first somite in *fss* mutants (Fig. 5). Thus, the neural crest migration patterning defect is precisely correlated with the region of defective segmentation. We observed the same phenotype in *aei* and *des* mutants (data not shown). Together these experiments show that where somites are absent or their patterning is disrupted, trunk neural crest cells migrate aberrantly on the medial pathway, supporting the idea that somite-derived signals regulate neural crest migration.

Fast muscle cells are not required for formation of normal neural crest migration streams

To learn which somite components are involved in patterning trunk neural crest migration, we first considered which components are in close proximity to neural crest cells as they begin to migrate on the medial pathway. Although many studies in amniote embryos have implicated sclerotome in patterning neural crest migration (Newgreen et al., 1990) (reviewed by Le Douarin and Kalcheim, 1999; Krull, 2001; Kalcheim, 2000), in zebrafish, neural crest cells do not encounter sclerotome cells until the segmental pattern of neural crest migration streams is already established (Morin-Kensicki

and Eisen, 1997). Thus, it is unlikely that sclerotome participates in the initial patterning of neural crest migration, although we cannot rule out a role of sclerotome in later stages of neural crest migration, particularly migration ventral of the horizontal myoseptum. Previous studies showed that the first cells encountered by zebrafish neural crest cells are myotomal and that the onset of crest migration coincides with contact between the two cell types (Raible et al., 1992). This suggests that fast or slow muscle cells, or their precursors, establish the initial pattern of neural crest migration streams. To test whether fast muscle cells are important for patterning neural crest migration, we injected *sonic hedgehog* (*shh*) mRNA into one- to two-cell stage embryos. Overexpression of *shh* mRNA results in expansion of slow muscle cells at the expense of fast muscle cells (Du et al., 1997; Barresi et al., 2000); thus, *shh* mRNA-injected embryos have more slow muscle cells but few or no fast muscle cells. In *shh* mRNA-injected embryos, somite shapes were disrupted so that there were spaces between adjacent somites dorsal of the horizontal myoseptum (Fig. 6). Some neural crest migrated in these spaces. As we showed earlier for *spt* mutants and *ntl* mutants injected with *spt* MOs, trunk neural crest cells are motile in embryos lacking somitic muscle; however, the migration of those cells is not

Fig. 6. Fast muscle is not essential for neural crest migration on the medial pathway. (A-D) Whole-mount staining of *shh* mRNA-injected embryo at 21 hpf. (A) *crestin* RNA expression (red) in neural crest cells. (B) F59 protein localization (green) in slow muscle. (C) Merged image. (D) DIC image. In the absence of fast muscle, the somites do not adhere to one another, creating a somite-free space in the intersomitic cleft. Neural crest migrates in this region, presumably because neural crest cells are motile in the absence of somites. However, neural crest also migrates in normal streams medial to the middle of somites (arrows in C). (E,F) Cross-section of *shh* mRNA-injected embryo. The somite on the left side of this embryo has no fast muscle and excess slow muscle (all the muscle is slow, as indicated by F59 staining in F); however, neural crest cells (*crestin* riboprobe, purple in E) have migrated to the same extent as on the right side, where fast muscle is present and slow muscle has migrated to the myotome periphery, as in wild type. Scale bar: 20 μm.
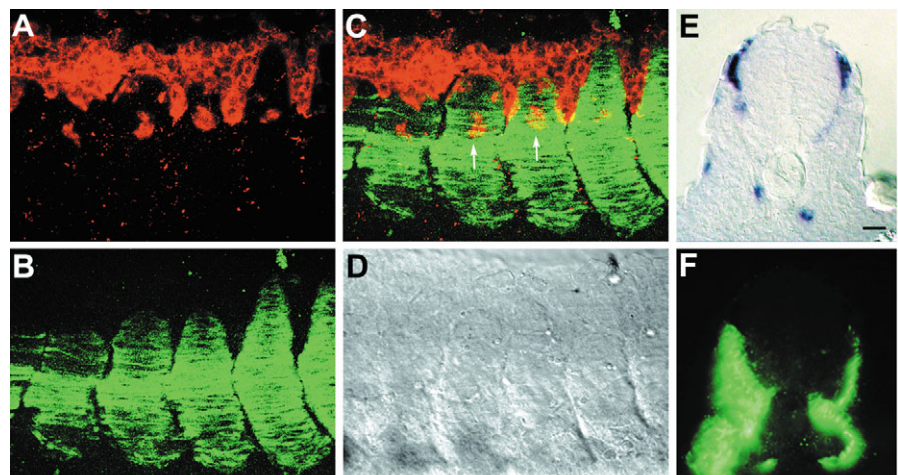
segmentally patterned. Thus, we interpret the migration of trunk neural crest cells in the spaces between adjacent dorsal somites to be unpatterned migration because of lack of somitic mesoderm. In addition to this unpatterned migration, neural crest cells also migrated in normal streams along the middle of the medial aspect of each somite (Fig. 6). This suggests that fast muscle cells are necessary for adjacent somites to remain in contact along their boundaries. However, fast muscle cells are not essential for patterning the streams of neural crest migration on the medial pathway. This result raises the possibility that this patterning role falls to slow muscle cells or their precursors.
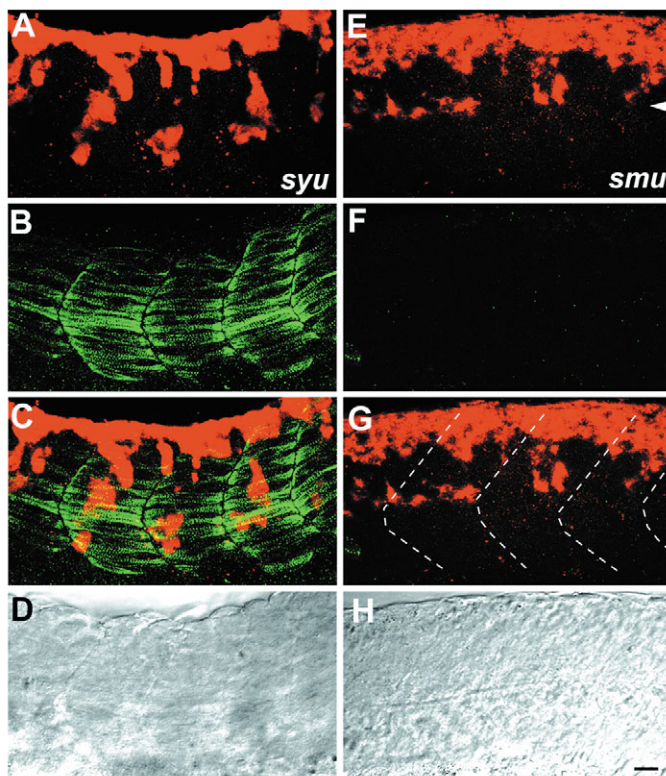
Neural crest migration is disrupted in mutants that lack slow muscle cells

To determine whether slow muscle cells regulate the pattern of trunk neural crest migration on the medial pathway, we examined mutants in which slow muscle cell formation was disrupted. Hedgehog (Hh) signaling is required for specification of slow muscle cells; thus, we focused on mutants affecting the Hh pathway. These mutants have normal somite boundaries but lack muscle pioneer cells and have few or no slow muscle cells; fast muscle cells are

Fig. 7. Neural crest migration is disrupted in Hedgehog signaling mutants. Whole-mount staining (A-C,E-G), and DIC image (D,H) of *syu* (A-D) and *smu* mutants (E-H) at 21 hpf with *crestin* riboprobe and F59 antibody. *syu* mutants have a significant number of slow muscle cells. Consistent with this, neural crest migration is fairly normal, although the streams are less regular than in wild types. By contrast, *smu* mutants entirely lack slow muscle and neural crest cells are abnormally patterned and stall at the level of the dorsal aspect of the notochord (arrowhead in E). Somite boundaries are shown as broken lines in G. Scale bar: 20 μm.

unaffected (Fig. 5E,F) (Hirsinger et al., 2004; van Eeden et al., 1996) (reviewed by Stickney et al., 2000). We investigated neural crest migration in embryos with mutations in three different genes in the Hh signaling pathway: *sonic you* (*syu*), which encodes Sonic hedgehog; *slow muscle omitted* (*smu*), which encodes Smoothend, an essential component of the Hh signaling pathway; and *you-too* (*yot*), which encodes Gli2, a downstream effector of Hh signaling. *syu* mutants have a significant number of slow muscle cells in most somites (Fig. 7B,C). Consistent with this, the general pattern of trunk neural crest migration on the medial pathway was normal, although the streams of cells were much less regular than in wild types (Fig. 7A-C). *smu* mutants essentially lack slow muscle cells (Fig. 7F) (van Eeden, 1996) because of an autonomous requirement for Smoothened activity for their formation (Barresi et al., 2000) and neural crest migration was very abnormal (Fig. 7E-G) (see also Ungos et al., 2003). Although a significant number of neural crest cells migrated, they did not migrate along the middle of the medial aspect of each somite, nor did they migrate in coherent streams. Instead, many of these cells migrated under the overlying somite boundaries (Fig. 8F). In addition, in these mutants, neural crest migration did not extend ventral of the dorsal aspect of the notochord (Fig. 7E-G); thus, neural crest cells tended to pile up at the level of the notochord. This is in contrast to wild-type embryos, in which trunk neural crest cells migrate much farther ventrally (Fig. 5A, Fig. 8E). *yot* mutants showed a phenotype similar to *smu* mutants (data not shown). All of these Hh signaling mutants have normal somite segmentation, suggesting that normal segmentation is not sufficient to define the normal pattern of trunk neural crest migration. Together, these data suggest that slow muscle cells are required to regulate the segmental streams of neural crest migration on the medial pathway. In addition, slow muscle cells may also be necessary for later aspects of migration along the pathway.

Wild-type adaxial cells can restore normal neural crest migration patterning in smu mutants

To further test the hypothesis that slow muscle patterns trunk neural crest migration, we carried out two types of experiment. In the first experiment, we surgically removed adaxial cells from wild-type embryos at the one- to three-somite stage and examined neural crest migration on the medial pathway by in situ hybridization with a *crestin* riboprobe between the 22-26 somite stages. In control experiments, we first removed adaxial cells then immediately replaced them. This had no effect on the pattern of neural crest migration on the medial pathway (data not shown). In 20 out of 39 removal experiments, slow muscle still formed, although it was not entirely clear weather the number of slow muscle cells was decreased relative to unmanipulated embryos. At the 22-26 somite stage, neural crest migration appeared normal in these embryos (Fig. 8D). By contrast, in 19 out of 39 removal experiments, slow muscle was absent (Fig. 8A,B). In the somites lacking slow muscle cells, the streams of migrating neural crest were abnormal. In some cases, the streams were not restricted to the middle of the medial aspect of the somite (Fig. 8A,B; *n*=7); in other cases, there were two streams in one somite (*n*=4; data not shown); and in some cases, streams were branched (*n*=1; data not shown). In some cases (*n*=7),

Fig. 8. Slow muscle regulates neural crest migration. (A-C) Removal of adaxial cells (black box in A; absence of orange stain) results in disruption of neural crest cell (purple) migration. F59 staining reveals slow muscle cells (orange). (B,C) Higher magnification views of where adaxial cells were removed (B, black box in A) and normal slow muscle cells are present (C, blue box in A). (D) Control experiment in which adaxial cells were removed, but slow muscle cells are present at 21 hpf and neural crest migration is essentially normal. (E) Nomarski DIC image of wild-type embryo shows neural crest migrates in the middle of somite. (F-H) Transplantation of adaxial cells in *smu* mutants. (F,G) Nomarski DIC images show neural crest migration (*crestin* riboprobe; purple) and transplanted slow muscle cells (red). Somite boundaries are shown as broken lines. (H) F59 staining (green) of the same embryo shows wild-type adaxial cells. (G) Higher magnification of F. Wild-type adaxial cells transplanted dorsal of the notochord partially restored neural crest migration in the middle of the somite, whereas neural crest migrates on the boundaries of somites without slow muscle. Scale bar: 85 μm in A; 75 μm in B,C; 70 μm in D-F; 40 μm in G,H.
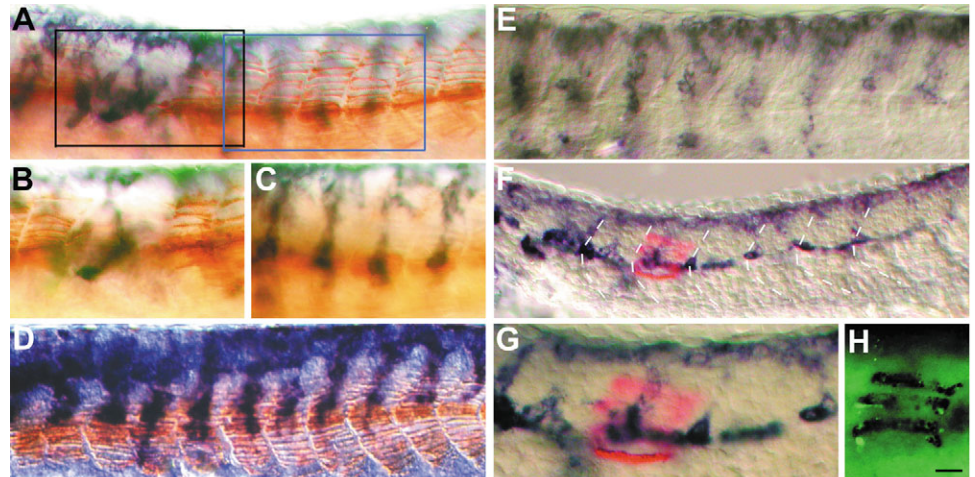
neural crest streams both migrated in an inappropriate position and were branched, or there were two streams in one somite and they both branched.

In the second experiment, we transplanted wild-type adaxial cells into *smu* mutants. Adaxial cells were transplanted from shield stage or 1-3 somite stage, dye-labeled, wild-type donor embryos into unlabeled *smu* mutant hosts of the same developmental stage. Host embryos were fixed between the 22- and 26-somite stages, and examined by in situ hybridization with a *crestin* riboprobe. Transplantation of adaxial cells into the somite dorsal of the level of the notochord partially restored the normal pattern of neural crest migration (Fig. 8F-H). In these somites, neural crest cells migrated as a coherent stream that tended to be in the middle of the medial aspect of the somite (Table 1). By contrast, transplantation of adaxial cells into the somite ventral of the level of the notochord did not restore a normal pattern of neural crest migration (Table 1). Together these results provide strong support for the hypothesis that slow muscle cells or their precursors pattern trunk neural crest migration into coherent streams on the medial pathway.

The migration pathway is crucial for proper development of some neural crest derivatives

To test the role of the migration pathway in formation of neural crest derivatives, we examined dorsal root ganglion (DRG) neurons by HuC antibody staining in embryos whose adaxial cells were surgically removed. At 72 hpf, clusters of DRG neurons are aligned along the neural tube, typically in a single cluster per somite (Fig. 9A,B,E). By contrast, following adaxial cell removal, DRG neurons are often found in two clusters per somite that are spatially segregated along the dorsoventral axis (Fig. 9C,D,F), suggesting that proper migration of neural crest cells is essential for proper development of at least some derivatives.

Discussion

Our key finding is that slow muscle cells or their precursor adaxial cells establish the segmental pattern of trunk neural crest migration on the medial pathway in zebrafish and that this is important for proper formation of at least some derivatives. Although adaxial cells are initially located medially within

Table 1. Quantification of neural crest migration in the absence of slow muscle

				One- to three-somite stage transplants		Shield stage transplants	
	Wild type[†]	*smu* mutants[†]	Adaxial cell removal[‡]	Transplants in the dorsal myotome[§]	Transplants in the ventral myotome[§]	Transplants in the dorsal myotomes[¶]	Transplants in the ventral myotomes[**]
Somites with migrating neural crest streams*	10/10 (100%)	2/8 (25%)	3/10 (30%)	5/7 (71%)	1/7 (14%)	8/11 (72%)	14/25 (56%)
Streams in the middle of medial somite	10/10 (100%)	0/2 (0%)	0/3 (0%)	4/5 (80%)	0/1 (0%)	8/8 (100%)	1/14 (0.07%)

*Only 1-3 cell wide coherent migrating neural crest cells connected from dorsal aspect of somite to dorsal aspect of notochord were counted as a stream.
[†]Counts were carried out in two somites of each of four or five embryos.
[‡]Counts were carried out in two somites lacking slow muscle of each of five embryos.
[§]Counts were carried out in two somites of each of three embryos and one somite of one embryo in which transplanted adaxial cells were present in the indicated position.
[¶]Counts were carried out in all somites of five embryos in which transplanted cells were present in the indicated position.
[**]Counts were carried out in all somites of eight embryos in which transplanted cells were present in the indicated position.

each somite, they elongate and move laterally through the myotome at approximately the same time that neural crest cells are migrating on the medial pathway (Raible et al., 1992; Devoto et al., 1996; Cortés et al., 2003). How might adaxial or slow muscle cells regulate the pattern of neural crest migration on the medial pathway? From their studies of motor axon pathfinding, Zhang and Granato (Zhang and Granato, 2000) proposed that slow muscle might express a guidance molecule and leave it behind on the medial myotome surface as it migrates through the myotome to the lateral surface. *unplugged* and *diwanka* mutants have defects in motor axon pathfinding along this pathway; these defects can be rescued by transplantation of a small number of wild-type slow muscle cells just dorsal to the muscle pioneers that form the horizontal myoseptum, even though slow muscle cells are already migrating toward the lateral myotome surface by the time motor axons extend out of the spinal cord (Zeller and Granato, 1999; Zhang and Granato, 2000). It is possible that similar mechanisms restrict neural crest migration.
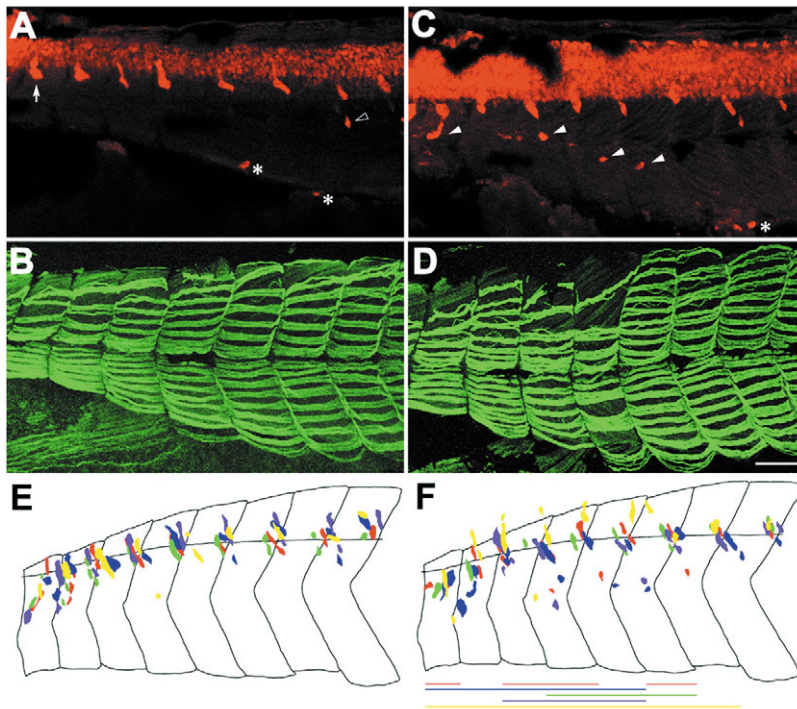
Myotome may establish the initial pattern of migration of

Fig. 9. Slow muscle removal results in aberrant DRGs. (A-D) Views of a single 72 hpf embryo: (A,B) control side; (C,D) experimental side. All figures are printed with anterior toward the left and dorsal toward the top for ease of comparison. (A,C) Anti-HuC antibody staining (red) reveals clusters of DRG neurons adjacent to the neural tube. DRGs are indicated only in the first segment in A (arrow). Enteric neurons are indicated with asterisks. (B,D) F59 staining reveals slow muscle cells (green). (A,B) There is a single DRG per somite in most segments, although one segment (open arrowhead) has two DRG neuron clusters. (C,D) Adaxial cells were removed from the experimental side and F59 staining shows less slow muscle. In many segments there are two DRG neuron clusters per somite; the second cluster is often much further ventral than the normal DRG position (arrowheads in C). (E,F) Schematic showing DRG formation in somites 3-10 of five embryos from which adaxial cells were removed from somites on one side; each color represents a different embryo. (E) Control side. (F) Experimental side; the colored lines represent the somites with reduced or missing slow muscle. Scale bar: 20 μm.

neural crest in avian embryos. There is considerable evidence that sclerotome plays a role in regulating neural crest migration in avian and mammalian embryos (Bronner-Fraser, 1986; Loring and Erickson, 1987; Teillet et al., 1987; Newgreen et al., 1990); however, the role of myotome in initial patterning of neural crest migration has received less attention. Many studies document neural crest migration on the anterior half-sclerotome of each somite, and several molecules expressed in posterior half-sclerotome have been shown to repel neural crest migration (Newgreen et al., 1990) (reviewed by Le Douarin and Kalcheim, 1999; Krull, 2001; Kalcheim, 2000). However, Tosney et al. (Tosney et al., 1994) provided evidence that neural crest cells prefer to migrate on the myotome basal lamina rather than on sclerotome. They found that neural crest cells invade sclerotome only when they fail to contact the myotome basal lamina. Moreover, during the onset of neural crest dispersal from the migration staging area in chick, it appears that neural crest cells contact epithelial somite cells that will become myotome (Loring and Erickson, 1987; Teillet et al., 1987; Kahane et al., 1998; Kiefer and Hauschka, 2001).

This is similar to the situation in zebrafish, in which neural crest migration does not begin until neural crest cells contact myotomal cells (Raible et al., 1992). In avians, neural crest cells do not appear to contact sclerotome until later (Loring and Erickson, 1987; Teillet et al., 1987). This suggests that in avian embryos, as in zebrafish embryos, myotome is responsible for the initial pattern of neural crest migration.

Sclerotome is not present at the right place and time to pattern the initial migration of zebrafish neural crest. We have previously shown that neural crest cells and sclerotome migrate in opposite directions along the same pathway (Morin-Kensicki and Eisen, 1997). However, these two cell types only encounter one another when they both reach the level of the horizontal myoseptum. Thus, the initial segmentally restricted pattern of migration of both cell types is already established before the two cell types meet, raising the possibility that the myotome is responsible for the migration pattern of both neural crest and sclerotome. However, it remains likely that interactions with sclerotome cells regulate aspects of zebrafish neural crest migration along more ventral regions of the migration pathway.

Are the molecular mechanisms that regulate neural crest migration conserved between zebrafish, avian and mammalian embryos? In avian and mammalian embryos, neural crest migrates on the anterior half sclerotome of each somite. Interactions between Ephrins and their Eph receptors have been implicated in patterning neural crest migration in amniotes (Le Douarin and Kalcheim, 1999; Krull, 2001; Kalcheim, 2000; Halloran and Berndt, 2003). Ephrin B proteins are expressed in posterior half sclerotome and repel neural crest migration, presumably through interactions with Eph proteins expressed in neural crest cells (Krull et al., 1997; Wang and

Anderson, 1997). Interestingly, the same Ephrin B proteins expressed in the posterior half of the sclerotome are also expressed in dermamyotome (Wang and Anderson, 1997) and thus might also mediate interactions between neural crest and dermamyotome. In zebrafish, slow muscle, not sclerotome, establishes the initial pattern of neural crest migration. Thus, molecules involved in patterning neural crest migration should be expressed in slow muscle. Zebrafish Eph family members, such as *epha4a*, *ephrin-A-L1* and ephrin B genes are segmentally expressed early in development, and have been implicated in somite formation (Cooke et al., 1997; Durhin et al., 1998; Chan et al., 2001). However, they are diffusely expressed in somites after they differentiate, but the pattern does not resemble that described for amniotes (Cooke et al., 1997; Durhin et al., 1998; Chan et al., 2001). In addition, no Eph family members have been reported to be expressed in zebrafish trunk neural crest cells. Thus, if Eph family members regulate neural crest migration patterning in zebrafish, the molecular mechanisms are likely to be distinct from those of avian and mammalian embryos, because in zebrafish neural crest migration occurs in the middle of the medial myotome surface, rather than through the anterior half somite. In addition to Eph family members, several other proteins have been implicated in patterning neural crest migration in amniotes, including tenascin C, laminins and F-spondin (Le Douarin and Kalcheim, 1999; Krull, 2001; Kalcheim, 2000). In zebrafish, tenascin C proteins are expressed in somite boundaries intensely, throughout somites weakly and also within neural crest cells (Tongiorgi, 1999). Because tenascin C is expressed both in the region of the neural crest migration pathway and in the region between adjacent neural crest migration pathways, its expression pattern is not consistent with a role in patterning neural crest migration on the medial pathway. *laminin γ1* (*lamc1* – Zebrafish Information Network) mRNA is expressed throughout somites and *laminin β1* (*lamb1* – Zebrafish Information Network) mRNA is expressed throughout embryos weakly in zebrafish (Parsons et al., 2002). The gene encoding F-spondin and the related genes *mindin 1* (*spon2a* – Zebrafish Information Network) and *mindin 2* (*spon2b* – Zebrafish Information Network) are also not expressed in slow muscle (Higashijima et al., 1997). So far in zebrafish, no molecules have expression patterns that make them good candidates for establishing the neural crest migration pattern on the medial pathway. Thus the molecular mechanisms that regulate patterning of neural crest migration in zebrafish remain to be resolved.

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