Mutations in the **stumpy** gene reveal intermediate targets for zebrafish motor axons

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SUMMARY

Primary motoneurons, the earliest developing spinal motoneurons in zebrafish, have highly stereotyped axon projections. Although much is known about the development of these neurons, the molecular cues guiding their axons have not been identified. In a screen designed to reveal mutations affecting motor axons, we isolated two mutations in the **stumpy** gene that dramatically affect pathfinding by the primary motoneuron, CaP. In **stumpy** mutants, CaP axons extend along the common pathway, a region shared by other primary motor axons, but stall at an intermediate target, the horizontal myoseptum, and fail to extend along their axon-specific pathway during the first day of development. Later, most CaP axons progress a short distance beyond the horizontal myoseptum, but tend to stall at another intermediate target. Mosaic analysis revealed that **stumpy** function is needed both autonomously in CaP and non-autonomously in other cells. **stumpy** function is also required for axons of other primary and secondary motoneurons to progress properly past intermediate targets and to branch. These results reveal a series of intermediate targets involved in motor axon guidance and suggest that **stumpy** function is required for motor axons to progress from proximally located intermediate targets to distally located ones.

Key words: Primary motoneuron, Muscle pioneer, Single-cell transplants, Mosaic analysis, Laser ablation, Genetic mapping

INTRODUCTION

Formation of appropriate connections between motoneurons and their target muscles is essential for animals to carry out their normal behaviors. En route to their targets, motor axons execute a series of intricate steps, including extension out of the spinal cord, navigation on the proper pathway and innervation of correct muscle targets. In vivo observations have revealed that axons extend towards their targets in a highly stereotyped manner, making few navigational errors (Eisen, 1994). The precision of these events is astonishing considering the complex, dynamic environment within a developing embryo and the distances many axons travel to reach their targets.

There is considerable evidence that, as they navigate toward their targets, axons integrate information from various cues that determine when and where to extend (Goodman and Tessier-Lavigne, 1997). Mutagenesis screens in *Drosophila melanogaster*, in which antibody markers were used to visualize pathfinding defects, have uncovered many molecules that function in axon guidance (Goodman, 1996; Chiba and Keshishian, 1996). One of the themes that has emerged from these studies is that motor axons navigate to their muscle targets by extending along pathways punctuated by ‘intermediate targets’, where they pause before branching, turning or extending distally. Thus, both intermediate targets and distant pathway regions are likely to provide guidance information. Formation of stereotyped pathways may also involve inhibition of axons from invading regions adjacent to their normal pathways.

Intermediate targets have also been described in vertebrates. For example, commissural axons navigate to the floor plate, pause and then turn to extend along other specific pathways (Bovolenta and Dodd, 1990; Kuwada et al., 1990). In the forebrain, a population of retinal axons crosses a zone along the midline of the optic chiasm whereas another population fails to cross, suggesting that this region is an intermediate target for retinal axons (Godement et al., 1990; Sretavan et al., 1995). Vertebrate motoneurons also extend axons to intermediate targets, the crural and sciatic plexus in avians (Lance-Jones and Landmesser, 1981; Tosney and Landmesser, 1985) and the muscle pioneer cells that define the horizontal myoseptum in zebrafish (Eisen et al., 1986; Melancon et al., 1997). Unlike studies in insects, however, studies in vertebrates have not yet revealed a series of closely spaced intermediate targets for navigating motor axons.

The zebrafish is an excellent system for elucidating mechanisms of vertebrate motor axon guidance. Each myotome is innervated in a highly stereotyped manner by three or four primary motoneurons, each of which can be individually identified based on morphology and gene expression (Eisen, 1999). Axons from the primary...
motoneurons of each spinal hemisegment exit the spinal cord in the same ventral root and extend ventrally along a common pathway on the medial aspect of the myotome until they reach the muscle pioneers, an intermediate target at the nascent horizontal myoseptum, where they typically pause for up to 2 hours (Eisen et al., 1986; Fig. 1). After pausing, these axons diverge along cell-specific pathways: the Caudal Primary motoneuron, CaP, extends an axon along the ventromedial myotome; the Middle Primary motoneuron, MiP, sprouts a collateral that extends along the dorsomedial myotome and later retracts the original ventral axon; the Rostral Primary motoneuron, RoP, extends an axon laterally, through the horizontal myoseptum; the Variable Primary motoneuron, VaP, does not extend an axon beyond the horizontal myoseptum and typically dies after a few hours (Eisen et al., 1990). During the first day of development, primary motoneurons are the only cells with axons along these pathways; later they are joined by axons of secondary motoneurons (Pike et al., 1992). Although previous work has characterized the outgrowth, morphology, synaptic interactions and pathway regions of primary motor axons (Eisen et al., 1986; Myers et al., 1986; Melancon et al., 1997; Beattie and Eisen, 1997; Bernhardt et al., 1998), very little is known about the molecular mechanisms underlying their stereotyped pathfinding. Many of these mechanisms should be revealed by characterization of mutations affecting zebrafish motor axons (Granato et al., 1996; Zeller and Granato, 1999; Beattie et al., 1999).

To identify genes involved in motoneuron pathfinding, we screened zebrafish mutants using antibodies to uncover defects in axon pathfinding (Beattie et al., 1999). Here we characterize the phenotype of zebrafish embryos homozygous for mutations in the *stumpy* (*sty*) gene. Analysis of these mutants reveals a series of intermediate targets for motor axons. Our data also suggest that *stumpy* may encode part of an attractive signaling system necessary for motor axons to extend beyond these intermediate targets onto distal pathway regions and for them to branch. We suggest that, like insects, vertebrate motor axons navigate to their targets by following a closely spaced series of intermediate targets. Uncovering these aspects of pathfinding paves the way for future molecular dissection of the interactions involved in this stepwise process.

**MATERIALS AND METHODS**

**Fish strains**

Mutant strains were kept as heterozygous (*stumpy*<sup>2393</sup>, *stumpy*<sup>2398</sup>) and homozygous lines (*stumpy*<sup>2393</sup>) in the *AB* background. Homozygous mutant embryos (*stumpy*) were obtained by natural crosses between *stumpy*<sup>2393</sup> and *stumpy*<sup>2398</sup> heterozygotes. Genotypes were assigned based on CaP axon length at 24-26 h, visualized by whole-mount antibody labeling with zn1 and znp1 (see below).

**Genetic mapping**

To map *stumpy*, mutants (*AB* background) were outcrossed to WIK and SJL strains to create polymorphic mapping lines. Parthenogenetic F2 diploid embryos were produced by fertilizing eggs from identified carriers with UV-irradiated sperm (Streisinger et al., 1981). Genomic DNA was isolated from individual embryos designated either wild type or mutant, as determined by antibody labeling. PCR with Simple Sequence Length Polymorphic markers (SSLP markers; Knapik et al., 1996, 1998; Shimoda et al., 1999) was used to determine centromeric linkage (Johnson et al., 1995, 1996). *stumpy*<sup>2398</sup> linkage was confirmed using normal diploid embryos from a *AB × WIK* cross.

**Whole-mount antibody labeling and in situ RNA hybridization**

Whole-mount antibody labeling was performed as described in Eisen et al., (1989) with the following modifications. After fixation, embryos were incubated in water for 30 minutes (24-26 h embryos) to 4 hours (36, 60, 72 h embryos) to increase permeability, placed into PBDT blocking solution (phosphate buffered saline (PBS), 1% dimethylsulfoxide (DMSO), 1% bovine serum albumin (BSA), 0.5% Triton X-100) containing 2.5% goat serum, for 1 hour, incubated overnight at 4°C in antibodies diluted in PBDT containing 2.5% goat serum, and washed in PBS/0.5% Triton X-100 for 1 hour. Antibodies were detected using Clonal-PAP system (Sternberger) with dianimobenzidine (DAB) as a substrate (see Beattie and Eisen, 1997) or FITC-conjugated goat anti-mouse IgG. After antibody labeling, some embryos were embedded in 1.5% agar/5% sucrose and cut into 16 μm transverse cryostat sections. The zn1 and zn1 monoclonal antibodies (mAbs) recognize primary and secondary motor axons (Trevarrow et al., 1990; Melancon et al., 1997) and the 4D9 mAb recognizes zebrafish Engrailed proteins (Patel et al., 1989; Hatta et al., 1991).

Whole-mount in situ RNA hybridization using digoxigenin-labeled anti-sense *islet2* probes (Appel et al., 1995) was performed as described in Beattie et al. (1997).

**Single cell labels**

Individual motoneurons were labeled with rhodamine dextran (3×10<sup>3</sup> MW; Molecular Probes) as described in Eisen et al., (1989). Embryos were mounted in 1.2% agar on a microslide for labeling, then removed from the agar and placed in embryo medium (Westerfield, 1995) containing 50 i.u. penicillin and 5 μg streptomycin at 28.5°C. Labeled cells were visualized using a Zeiss Axioscope. Images were captured with a Photometrics SPOT camera and were colorized using Photoshop (Adobe).

**Single cell and myotome transplants**

Myotome transplants were performed as described in Beattie and Eisen (1997). Single cell transplants were performed as described in Eisen (1991). Briefly, 16 h donor embryos labeled with rhodamine dextran (10×10<sup>3</sup> MW; Molecular Probes) and unlabeled host embryos were mounted side by side in 1.2% agar on a microslide. Individual CaP were transplanted from labeled donors to unlabeled host spinal hemisegments from which the native CaP and VaP (Eisen et al., 1990) motoneurons had been removed. Transplanted cells were visualized using a Zeiss Universal Compound Microscope equipped with a Dark Invader low light level camera. Images were captured using AxoVideo (Axon Instruments) and colorized using Photoshop (Adobe).
Muscle pioneer ablations

Muscle pioneer ablations were performed by laser-irradiation as previously described (Melancon et al., 1997). Muscle pioneers in *stumpy* mutants were visualized under Nomarski DIC optics and ablations were performed in one or two somites at axial levels 7-10 prior to growth cone contact (approximately 17 h). Embryos were allowed to recover until 24-26 h and then processed for anti-engrailed (4D9; Hatta et al., 1991) and znp1 immunoreactivity. As in previous studies, occasionally laser-irradiation created muscle scarring that precluded scoring extension of CaP axons (Melancon et al., 1997).

RESULTS

Mutations in the *stumpy* gene affect motor axons

To identify mutations disrupting motoneurons, we screened 24-26 h progeny from ENU-mutagenized fish with antibodies that recognize motoneurons (Beattie et al., 1999). We found three mutations in which embryos lacked ventral motor axons. Complementation tests suggested that two of these mutations affect the same gene, which we named *stumpy* (Fig. 2; Table 1). The third mutation, *topped*<sup>b393</sup>, complements *stumpy* and will be discussed elsewhere (C. M. Herpolshheimer and C. E. Beattie, unpublished). Both *stumpy*<sup>b393</sup> and *stumpy*<sup>b398</sup> show Mendelian inheritance (Table 1); however, *stumpy*<sup>b398</sup> is a recessive, lethal mutation whereas *stumpy*<sup>b393</sup> is a viable mutation that displays partial dominance; approximately 50% of the embryos from a heterozygous *stumpy*<sup>b392</sup> mating show a phenotype intermediate between the phenotypes of homozygous mutants and wild types (Fig. 3; Table 1). *stumpy*-embryos and larvae have no obvious motility defect; however, we cannot rule out subtle defects that might only be visible using high-speed video microscopy.

As a first step toward molecular identification of *stumpy*, we placed both putative alleles on the zebrafish genome linkage map (Postlethwait et al., 1994, 1998). To map *stumpy*<sup>b393</sup> to a linkage group (Lg), we used Simple Sequence Length Polymorphisms (SSLP; Knapik et al., 1996, 1998) on parthenogenetic diploid embryos generated by suppressing the second meiotic division (Streisinger et al., 1981; Johnson et al., 1995, 1996). Mapping lines were generated by crossing the mutant line, on the *AB* background, to two other parental lines (SJD, WIK) commonly used for mapping because of the high degree of polymorphism with *AB* (Nechiporuk et al., 1999). DNA was amplified by PCR from individual parthenogenetic diploid embryos scored as phenotypically mutant or wild type. Two markers near the centromere of Lg 13, z9995 and z3424, segregated with *stumpy*<sup>b393</sup> in all mutants tested (n=50), indicating that *stumpy* maps to this Lg (data not shown). *stumpy*<sup>b398</sup> was shown to segregate with z9995 using normal diploid embryos (data not shown), further supporting the notion that these two mutations are allelic.

**stumpy** function is required for CaP pathfinding

The striking absence of ventral motor axons at 24 h suggested that *stumpy* specifically affects CaP motoneurons. Antibody labeling showed that CaPs were present in *stumpy* mutants (see white arrowhead in Fig. 3C) and vital dye labeling revealing that *stumpy*- CaP axons extended along the common pathway but stopped at the horizontal myoseptum (Fig. 4A). This defect could result from a requirement for *stumpy* function during either cell-fate specification or pathfinding.

To address whether CaPs were properly specified, we analyzed expression of *islet2*, a LIM homeobox gene expressed in CaP and VaP (Appel et al., 1995; Tokumoto et al., 1995). The distribution of CaPs and VaPs in *stumpy* mutants was indistinguishable from that in wild types (Fig. 4B,C), suggesting that *stumpy*- CaPs were properly specified. Thus, we hypothesized that the mutation affected CaP pathfinding.

To analyze pathfinding, we labeled CaPs in living wild-type and mutant embryos and compared their axon projections over several days (Fig. 5; Table 2). At 22-24 h, wild-type axons had extended past the horizontal myoseptum along a CaP-specific pathway on the ventromedial myotome (Table 2; Fig. 5A). At this stage, many axons had a prominent varicosity adjacent to the ventral aspect of the notochord (* in Fig. 5A). It is also common for axons to branch in this region (Eisen et al., 1986; Myers et al., 1986), thus it appears to be a second intermediate target for CaP axons. By 48 h, axons had reached the ventral aspect of the myotome (Fig. 5B; Table 2) and turned laterally.

Table 1. The *stumpy* mutant phenotype segregates in a Mendelian fashion

<table>
<thead>
<tr>
<th>Phenotypes from heterozygous matings (%)</th>
<th>Wild type</th>
<th>Heterozygous</th>
<th>Homozygous</th>
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<tr>
<td>b393 (n=155)</td>
<td>23.2±1.1</td>
<td>53.6±1.1</td>
<td>23.2±2.0</td>
</tr>
<tr>
<td>b398 (n=187)</td>
<td>74.4±1.2</td>
<td>na</td>
<td>25.6±1.2</td>
</tr>
<tr>
<td>b393 × b398 (n=158)</td>
<td>48.0±5.3</td>
<td>24.7±4.9</td>
<td>27.3±1.5</td>
</tr>
</tbody>
</table>

Embryos were collected from four separate matings of heterozygous *stumpy*<sup>b393</sup>, *stumpy*<sup>b398</sup> and *stumpy*<sup>b392</sup> × *stumpy*<sup>b390</sup> fish. Phenotypes were designated based on axon length, as visualized using whole-mount antibody labeling with zn1 and zn11 at 22-24 h (see Fig. 3). Numbers did not significantly differ from expected Mendelian ratios (χ² test, data not shown).

na, not applicable.

**Fig. 1.** Schematic diagram of primary motor axon pathways. (A) Spinal cord hemisegments and overlying myomeres. CaP (red), MiP (blue) and RoP (green) cell bodies reside in stereotyped spinal cord locations. All primary motor axons leave the spinal cord at the same ventral root (1) and traverse a common pathway (a). At the horizontal myoseptum (dashed line), they pause and then diverge along axon-specific pathways (2). CaP axons extend ventrally along the medial myotome (b). MiP axons branch and extend along the dorsomedial myotome (c). By approximately 26 h (3), MiP has retracted its ventral axon and both the CaP and MiP axons have turned laterally. RoP axons extend mediolaterally through the horizontal myoseptum (d). VaP is not pictured here. Its cell body is directly adjacent to CaP and its axon only extends to the horizontal myoseptum. (B) Cross sectional view showing CaP and MiP (on separate sides for clarity), the common pathway (a), the CaP-specific pathway (b) and the MiP-specific pathway (c). sc, spinal cord; nc, notochord.
By 72 h, axons had extended dorsally along the rostral myotome boundary (arrow in Fig. 5C; Table 2;) and there were numerous branches throughout the ventral myotome. The most prominent of these branches originated at what appears to be a third intermediate target for CaP axons at the ventral aspect of the myotome (* in Fig. 5C; see Eisen et al., 1986; Myers et al., 1986; Liu and Westerfield, 1990).

In contrast to wild types, at 22-24 h CaP axons in stumpyb393 mutants were stalled at the horizontal myoseptum (Table 2; Fig. 5D). Even at 48 h, some axons (27%) remained stalled there (Table 2), while the remainder extended further, to the intermediate target at the ventral aspect of the notochord (Table 2; Fig. 5E). By 72 h, most CaPs that had not extended axons beyond the horizontal myoseptum were dead or dying, as revealed by the cell body and axon breaking apart, unless they made ectopic branches into the ventral muscle (Table 2). Even at this stage the vast majority (88%) of the axons remained stalled at the ventral notochord intermediate target (Table 2; Fig. 5F).

We also labeled CaPs in homozygous stumpyb398 mutants. At 24 h, all CaP axons were either stalled at the horizontal myoseptum (67%) or at the ventral notochord intermediate target (33%; Table 2). However, in contrast to stumpyb393, by 48 h almost half the axons extended to the ventral myotome intermediate target, suggesting that this allele has a less severe phenotype (Table 2). As in stumpyb393 mutants, by 72 h most stumpyb398 CaPs that had not extended axons into the ventral myotome were dead or dying (Table 2). These results suggest that without wild-type stumpy function, CaP axons stall at intermediate targets along their pathway.

**Muscle pioneers may contribute to the stumpy CaP phenotype**

To learn what aspect of pathfinding was affected by stumpy gene function, we considered the myotome regions encountered during outgrowth. CaP axons traverse a common pathway, pause at the horizontal myoseptum and extend along the CaP-specific pathway (Fig. 1). Extension along the common pathway appeared normal in stumpy mutants (Fig. 4A). Although most stumpy CaP axons eventually extended beyond the horizontal myoseptum, they paused there for an excessive amount of time, up to 20 hours, compared with about 2 hours in wild types (Eisen et al., 1986). Thus, we reasoned that stumpy function types might regulate the ability of CaP axons to leave the horizontal myoseptum. Previous studies suggested that the muscle pioneers that define the horizontal myoseptum normally provide a signal that specifically affects MiPs, because ablation of muscle pioneers allowed MiPs to

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**Table 2. CaP axons in stumpy mutants show developmental defects**

<table>
<thead>
<tr>
<th></th>
<th>24 h</th>
<th></th>
<th>48 h</th>
<th></th>
<th>72 h</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>HS</td>
<td>VNC</td>
<td>VM</td>
<td>HS</td>
<td>VNC</td>
<td>VM</td>
</tr>
<tr>
<td>Wild type</td>
<td>0/10 (0)</td>
<td>0/10 (0)</td>
<td>10/10 (100)</td>
<td>0/10 (0)</td>
<td>0/10 (0)</td>
<td>10/10 (100)</td>
</tr>
<tr>
<td>b393</td>
<td>15/15 (100)</td>
<td>0/15 (0)</td>
<td>0/15 (0)</td>
<td>4/15 (27)</td>
<td>11/15 (73)</td>
<td>0/15 (0)</td>
</tr>
<tr>
<td>b398</td>
<td>8/12 (67)</td>
<td>4/12 (33)</td>
<td>0/12 (0)</td>
<td>2/12 (17)</td>
<td>4/12 (33)</td>
<td>6/12 (50)</td>
</tr>
</tbody>
</table>

CaP axon length was determined in living embryos after injecting dye into single CaP somata. Axons were scored at three time points (approximately 24, 48, 72 h) and the position of the most distal part of the axon was noted.

HS, horizontal myoseptum; VNC, myotome region adjacent to the ventral edge of the notochord; VM, distal region of the ventral myotome.

The number of CaPs with axons at a particular location are represented as a fraction with the percentage in parentheses.

*One embryo had a CaP that extended slightly past the HMS and was alive at 72 h while the other three cells were dead at 72 h.

*Four of these cells were dead or dying at 72 h.

*Axon branched within the proximal ventral myotome and did not reach the ventral edge of the myotome.

*Both of these cells were dead at 72 h.

*Two of these cells were dead or dying at 72 h.

*These cells had ectopic branches at the HS.
extend axons slightly beyond the horizontal myoseptum (Melancon et al., 1997). The *stumpy* phenotype could result if wild-type *stumpy* function was required to prevent CaPs from responding to this signal.

To test this idea, we ablated muscle pioneers in *stumpy* b393 mutants and analyzed CaP axons. In non-ablated, control segments, 17% (2/12) of *stumpy* CaP axons had extended beyond the horizontal myoseptum by 26 h. In contrast, after removal of muscle pioneers, 55% (5/9) of *stumpy* CaP axons had extended beyond the horizontal myoseptum by this stage; four of these axons (4/5, 80%) were stopped at the ventral notochord intermediate target (Fig. 6). These results support the idea that *stumpy* CaP axons may respond to a muscle pioneer-derived stop signal that they normally ignore in wild types. Even in experimental segments, however, CaP axons were not normal, suggesting that *stumpy* also functions to promote CaP axon extension along its cell-specific pathway independent of the muscle pioneers.

**Genetic mosaics reveal that *stumpy* function is needed in both CaP and other cells**

To address whether *stumpy* function was required cell-autonomously in CaP, we created genetic mosaics by transplanting *stumpy* b393 CaPs into wild-type hosts and wild-type CaPs into *stumpy* b393 hosts prior to axogenesis. Wild-type CaPs transplanted into wild-type hosts developed normal axon projections (8/8; Fig. 7A). In contrast, in *stumpy* hosts, most wild-type CaP axons stalled at the horizontal myoseptum (86%; 6/7; Fig. 7B), although one CaP appeared wild type. Most *stumpy* CaPs also stalled at the horizontal myoseptum in wild-type hosts (67%; 6/9; Fig. 7C), although three CaPs appeared wild type. We also created genetic mosaics in which *stumpy* b393 myotomes were replaced with wild-type myotomes; these also failed to rescue *stumpy* CaP axons (n=6, data not shown). Together these results suggest that wild-type *stumpy* function is needed both in CaP and in other cells for CaP to extend normally along its cell-specific pathway.

**MiP and RoP axons in *stumpy* mutants display defects in branching**

Because *stumpy* function appears to be needed in surrounding cells for proper extension of CaP axons, we wondered whether it was also needed for proper extension of MiP and RoP axons. To test this possibility, we labeled MiPs and RoPs with vital dye and followed their axon projections in living *stumpy* b393 mutants. Both motoneurons extended axons normally along the common pathway (data not shown). *stumpy* RoP axons looked normal during the first day of development but at 48 h they were less branched than wild-type RoP axons (n=6; Fig. 8A,B). Although antibody labeling revealed that MiP dorsal axons were present in *stumpy* mutants (see Fig. 3), examination of vital dye-labeled cells

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Fig. 3. *stumpy* mutants have short ventral axons. Whole-mount antibody labeling of 26 h *stumpy* b393 wild-type sibling (A), heterozygous (B) and homozygous (C) mutants. Arrows denote the choice point at the horizontal myoseptum; black arrowheads indicate dorsally projecting MiP axons; the asterisk in B indicates neurite extending beyond this choice point; the white arrowhead in C denotes zn1-labeled CaP cell body. Bar, 25 μm.

Fig. 4. *stumpy* function is unnecessary for CaP specification. (A) CaP motoneuron (red) labeled with vital dye in a living *stumpy* mutant, which was then sectioned and processed for immunohistochemistry (zn1 and znpi; green). Arrowhead denotes the CaP cell body and arrows denote the horizontal myoseptum. (B,C) Whole-mount RNA in situ hybridization using a digoxigenin-labeled probe for anti-sense *islet2* (blue) in wild-type (B) and *stumpy* embryos (C) at approximately 24 h. Arrowheads point to CaPs and asterisks denotes CaP/VaP pairs. Bar, 10 μm.
revealed that stumpy− MiP axon projections were variable. Half the labeled MiPs retracted the ventral axon on a normal, wild-type schedule but had a dorsal axon that was less branched and failed to make the normal lateral turn during the second day of development (6/12; Fig. 8C,D). One third (4/12) had a similarly affected dorsal axon but also retained a ventral axon that extended aberrantly through the horizontal myoseptum to the caudal segment boundary and retrogradely on the lateral myotome surface. One of the remaining MiPs had a normal dorsal axon but an aberrant ventral axon and the other MiP looked wild type. In wild types, the MiP dorsal axon often pauses at a specific distal location and forms a caudal branch as it turns laterally (Fig. 8C; Eisen et al., 1986; Myers et al., 1986). In 83% of the cases (10/12), stumpy− MiPs stalled in this region suggesting that it is an intermediate target for MiP axons. These results illustrate that most stumpy− MiP and RoP axons looked similar to wild-type axons during the first day of development, but later showed a variety of defects.

stumpy function is unnecessary for pathfinding by other types of neurons

To learn whether axons of other types of neurons were affected by stumpy mutations, we examined Rohon-Beard, trigeminal and lateral line sensory neurons (Metcalfe et al., 1990) and VeLD (Bernhardt et al., 1990) and Mauthner (Metcalfe et al., 1986) interneurons. Pathfinding by all of these cell types appeared unaffected in stumpy− mutants (data not shown), suggesting that stumpy does not function globally in axon pathfinding, but is specific for motor axons.

stumpy function is required for proper formation of motor nerves

To learn whether stumpy mutations affected other motor axons, we examined wild-type and stumpy− embryos at later developmental stages. The zn1 monoclonal antibody recognizes the axons of early developing primary motoneurons and later developing secondary motoneurons (Melancon, 1994), both of which contribute to the dorsal and ventral motor nerves. We followed these nerves between 36-72 h in the caudal trunk (segments 9-13) and rostral tail (segments 14-18). As with many other aspects of development along the body axis, at a single developmental stage, motor nerves in the caudal trunk were developmentally more advanced than those in the rostral tail. We observed several phases of nerve extension similar to those described previously (Pike et al., 1992). First, nerves extended along characteristic medial pathways to the distal aspects of the ventral and dorsal myotome (see Fig. 9K). Next, nerves extended back toward the horizontal myoseptum along characteristic pathways on the lateral myotome (see Fig. 9K). Finally, nerves branched within the myotome. Wild-type nerves had extended distally (Fig. 9A) and begun to turn laterally (data not shown, see also Pike et al., 1992) by 36 h and projected many branches by 72 h (Fig. 9I).
Development of dorsal and ventral motor nerves was delayed in *stumpy* mutants and these nerves showed aberrant branching patterns. We first examined motor nerves in *stumpy* b393 mutants. In contrast to wild type, most *stumpy* nerves had not reached the distal aspect of the medial pathway by 36 h (Fig. 9A, compare with B). Approximately half the nerves (45±11.2%) were stalled at the ventral notochord intermediate target (Fig. 9B), the same region where CaP stalled (see Fig. 5E). Unlike CaP, however, most nerves had extended to the distal aspect of the medial pathway by 48 h (85±11.2%; Fig. 9C, compare with D), although some axons in the rostral tail remained short (arrow in Fig. 9C). By 60 h, nerves extended to the ventral aspect of the myotome (Fig. 9E, compare with F), but stalled there as well, so turning onto the lateral myotome was dramatically delayed, occurring some time between 48-60 h as compared to 36 h in wild types. Moreover, some nerves also extended along the same aberrant pathway as ventral MiP axons, through the horizontal myoseptum and retrogradely along the lateral myotome (Fig. 9G, compare with H). Not only did these nerves extend through the myotome in an abnormal position, they also extended in abnormal locations along the lateral myotome (Fig. 9G, compare with H), and sometimes crossed segment boundaries, unlike nerves in wild types (data not shown). However, although the proximal portions of the nerves along the lateral myotome were in abnormal locations, the positions were more normal distally. Branching of both dorsal and ventral nerves was also dramatically reduced at 72 h (Fig. 9I, compare with J). We also examined motor nerves in homozygous *stumpy* b398 mutants. We found similar defects; however, the phenotype was markedly less severe than *stumpy* b393 and by 48 h it was difficult to distinguish *stumpy* b398 mutants from wild types (data not shown).

**DISCUSSION**

We describe two mutations in the *stumpy* gene that disrupt pathfinding by zebrafish primary and secondary motoneurons. These mutations were isolated using antibodies to screen for axon defects. It seems unlikely that either allele is a protein null, because one allele is recessive and embryonic lethal and the other allele is partially dominant and viable. Motoneurons of embryos homozygous for the partially dominant allele are affected more dramatically than are those of embryos homozygous for the recessive allele. These mutations reveal previously unrecognized aspects of motoneuron pathfinding and will lead to elucidation of molecules that function during this process.

Pathfinding begins normally in *stumpy* mutants

Normal motoneuron projections require proper cell-fate specification (Eisen, 1998, 1999). *islet2* expression and the normal correspondence of motoneuron cell body positions and axon trajectories reveals that specification of primary motoneurons is normal in *stumpy* mutants. Extension along the
common pathway is also normal, and thus appears to be independent of *stumpy* function; it has recently been shown to depend on function of another gene, *diwanka* (Zeller and Granato, 1999). The *stumpy*\(^{-}\) phenotype is first manifest when axons pause for an abnormally long time at the horizontal myoseptum. This data supports the idea that the horizontal myoseptum is the first intermediate target encountered by motor axons. Although axonal projections beyond the horizontal myoseptum are abnormal, when axons do project they typically select the appropriate pathway, suggesting that any positive and negative cues that distinguish the cell-specific pathways are normally distributed in *stumpy* mutants.

**stumpy is required for motor axons to extend past intermediate targets and for proper axon branching**

Although *stumpy*\(^{-}\) motor axons projected normally along the common pathway, projection distally was marked by stalling at specific locations: the horizontal myoseptum, the ventral aspect of the notochord, and the ventral and dorsal aspects of the myotome. These are regions where wild-type primary motor axons normally pause, make conspicuous varicosities and often branch (Eisen et al., 1986; Myers et al., 1986), suggesting that they may be intermediate targets (Melancon et al., 1997; Goodman and Tessier-Lavigne, 1997). The stalling of *stumpy*\(^{-}\) primary, and in some cases secondary, motor axons in these regions provides strong evidence that these regions are intermediate targets and that *stumpy* function is normally required for axons to leave intermediate targets and extend into more distal territory. One interpretation consistent with these data is that attractive cues that normally prompt motor axons to extend beyond intermediate targets are not present in *stumpy* mutants, or that motor axons are unable to respond to them. Alternatively, *stumpy* may function to destabilize the interaction between growth cones and intermediate targets, thus enabling growth cones to progress beyond these regions; without *stumpy* function, growth cones stall or stop at these regions. There is evidence for this type of anti-adhesive function in *Drosophila* where the gene *beaten path* functions to regulate defasciculation from choice points (Fambrough and Goodman, 1996).

*stumpy* gene function also regulates branching of motor axons at intermediate targets. For example, wild-type MiPs retract their ventral axons following prolonged contact with muscle pioneer cells at the horizontal myoseptum, but in the absence of these cells the ventral axon persists and may extend abnormal branches (Melancon et al., 1997). Most *stumpy*\(^{-}\) MiPs failed to retract the ventral axon, which then branched

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**Fig. 9.** *stumpy*\(^{-}\) motor nerves are aberrant. Wild-type (A,C,E,G,I) and *stumpy* mutant (B,D,F,H,J) embryos labeled with znlp1 at 36 h (A,B; medial focal plane), 48 h (C,D; medial focal plane), 60 h (E,F; medial focal plane), 48 h (G,H; lateral focal plane) and 72 h (I,J; lateral focal plane). Arrows point to axons along the medial myotome, arrowheads point to axons along the lateral myotome and asterisks indicate axons extending laterally through the horizontal myoseptum. (K,L) Schematic drawings of axons in cross section (K, wild type) and (L, mutant) at 48 h. Arrows denote direction of axon growth. For each time point, 40 axons from segments 8-17 were analyzed in 4 embryos. Bars, 100 \(\mu\)m (A-D); 75 \(\mu\)m (E-F,I-J); 50 \(\mu\)m (G-H).

**Fig. 10.** Model for *stumpy* function. In wild-type embryos, primary motor axons extend along the medial myotome and pause or branch at intermediate targets (blue dots). In *stumpy* mutants, primary motor axons stall at proximal intermediate targets and fail to reach distal ones. Thus, *stumpy* may be a molecule important for attracting growth cones to distal pathway regions (green).
aberrantly, suggesting that *stumpy* function is required to sculpt the MiP arbor by preventing the ventral axon from branching and ensuring that it is retracted. *stumpy* function also regulates branching of secondary motor axons, some of which project aberrantly through the horizontal myoseptum in *stumpy* mutants. Because this is similar to the aberrant path taken by some MiP ventral axons, it is unclear whether *stumpy* affects secondary motoneurons directly, or whether this effect is through interactions between primary and secondary motor axons (see Pike et al., 1992).

**CaP survival may depend on axons contacting appropriate muscle**

Survival of vertebrate motoneurons has been linked to receiving proper trophic support from target muscles (Oppenheim, 1996). Interestingly, *stumpy* CaP motoneurons that fail to extend an axon to their normal ventral muscle target typically die by 72 h. In contrast, most *stumpy* CaP motoneurons that extend a short distance along the ventral muscle, either by forming ectopic branches at the horizontal myoseptum or by extending to the ventral notochord intermediate target, survive longer. Previous studies have shown that CaP motoneurons can activate contractions in muscle pioneers (Melancon et al., 1997), suggesting intimate interactions between these two cell types. However, our results suggest that ventral muscle provides trophic support required for CaP motoneuron survival, and that this support cannot be derived from muscle pioneers.

**stumpy mutations do not simply convert CaPs to VaPs**

A superficial interpretation of the *stumpy* phenotype is that CaPs are converted into VaPs. All known molecular markers, as well as transplantation studies, show that CaP and VaP are both specified to be CaP and that interactions between the cells force one of them to adopt the VaP fate (Eisen, 1992). VaPs fail to extend axons beyond the horizontal myoseptum and typically die by 36 h (Eisen et al., 1990). However, although *stumpy* CaPs that fail to extend axons beyond the horizontal myoseptum also die, they survive more than a day longer than wild-type VaPs, a very significant amount of time in zebrafish development. Further, most *stumpy* CaPs do extend axons beyond the horizontal myoseptum, something never observed for VaPs in wild-type embryos. Although we cannot rule out a role for *stumpy* in specifying VaP, the clear differences between VaPs and *stumpy* CaPs suggest that the interactions determining the VaP fate are more complicated than a simple alteration in *stumpy* function.

**stumpy function is required both in CaP and in surrounding cells**

Our genetic mosaic analysis suggests that *stumpy* function is required both in motoneurons and in other cells for normal pathfinding. This idea is supported by previous mosaic analyses which distinguished cell-autonomous and cell-nonautonomous functions required for motoneuron development. For example, individual primary motoneurons transplanted between wild types and *spadetail* mutants always behaved in the fashion appropriate for the genotype of the host, showing that the mutant motoneuron phenotype is cell-nonautonomous (Eisen and Pike, 1991). In contrast, wild-type cells transplanted into *detour* mutant hosts can develop into branchiomotor neurons, which are absent from mutants, suggesting that *detour* function is required cell-autonomously in branchiomotor neurons (Chandrasakhar et al., 1999). Without knowing precisely which other cells require *stumpy* function, it is difficult to predict what kind of molecule might be required both in motoneurons and in other cells for proper pathfinding. One possibility is a homophilic guidance or adhesion molecule, although other possibilities, such as a molecule that disrupts cell-cell interactions, cannot be excluded.

**stumpy functions in motor axon guidance**

Our studies reveal a series of intermediate targets along the pathways traversed by zebrafish motor axons. Studies in insects have shown that axon pathways are broken up into relatively short segments by a series of intermediate targets that provide important guidance information during pathfinding (Goodman and Tessier-Lavigne, 1997). Although intermediate targets have also been described during pathfinding in vertebrate embryos (Goodman and Tessier-Lavigne, 1997), there have not been clear examples in which an individual pathway is divided into short segments. We have shown that the pathways followed by spinal motor axons in embryonic zebrafish are punctuated by intermediate targets at relatively close intervals. Genetic screens (Eisen, 1996) will be important to establish the identities of the cells that define each of these intermediate targets and the specific molecular interactions they mediate.

Axons normally extend directly to intermediate targets and transiently pause there, suggesting that these regions are more attractive than surrounding areas. This idea is supported by experiments showing that removal of at least one of these intermediate targets enhances motor axon extension, in both wild types (Melancon et al., 1997) and in *stumpy* mutants; however, extension of axons beyond these intermediate targets also suggests the presence of other attractive cues located more distally. Together, these observations suggest the following model (Fig. 10). During extension, axons are attracted to intermediate targets. They pause there, but later leave as the intermediate targets become less attractive, because there are more attractive targets located distally, or because attractive interactions are disrupted. Perhaps the simplest way of thinking about *stumpy* gene function is that it encodes part of an attractive system required for axons to extend beyond intermediate targets whose attractiveness is mediated by molecules other than Stumpy. When *stumpy* function is absent, axons do not receive signals that prompt them to extend beyond intermediate targets, and thus they tend to stall; much later the attractive signals at these intermediate targets may diminish, and this allows axons to extend to the next intermediate target. This may also explain the lack of branching; if axons are stalled at an intermediate target, they may be unable to respond to signals that instruct them to branch. Eventual cloning of *stumpy* will help elucidate the molecular cues involved in these steps of motor axon pathfinding.

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