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# The zebrafish *unplugged* gene controls motor axon pathway selection

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## SUMMARY

**En route to their targets, motor axons encounter choice points at which they select their future path. Experimental studies predict that at each choice point specialized cells provide local guidance to pathfinding motor axons, however, the identity of these cells and their signals is unknown. Here, we identify the zebrafish *unplugged* gene as a key component for choice point navigation of pioneering motor axons. We show that in *unplugged* mutant embryos, motor neuron growth cones reach the choice point but make inappropriate pathway decisions. Analysis of chimeric embryos demonstrates that *unplugged* activity is produced by a selective group of mesodermal cells located adjacent to the choice point. As the first motor growth cones approach the choice point, these mesodermal cells migrate away, suggesting that *unplugged* activity influences growth cones by a contact-independent mechanism. These data suggest that *unplugged* defines a somite-derived signal that elicits differential guidance decisions in motor growth cones.**

Key words: Spinal cord, Motor axon, Axon guidance, Choice point, Pathway selection, Neural development, Adaxial cell, Somite, Zebrafish

## INTRODUCTION

During embryogenesis motor neurons extend axonal growth cones to establish precise and stereotyped neural connections with the body musculature. A major challenge for an individual motor growth cone is to select its appropriate axonal path among many possible paths present for similarly searching growth cones. This process of axonal pathfinding is facilitated by intermediate targets and choice points, often located at positions where axonal pathways diverge. At intermediate targets and choice points, specialized cells or extracellular matrix provide a mixture of guidance cues controlling two features of axonal pathfinding: growth cone guidance between intermediate targets (or choice points) and pathway selection at the choice point. Specialized cells at the midline of flies, worms and vertebrates control such growth cone behavior through a sophisticated system of attractive and repellent signals (Terman and Kolodkin, 1999; Van Vactor and Flanagan, 1999). In contrast, the identity of local signals and the guidance capacity of cells at motor pathway choice points are less clear.

In the *Drosophila* embryo, motor neuron growth cones of the segmental and intersegmental nerves encounter multiple well-characterized choice points before reaching their muscle targets (Jacobs and Goodman, 1989; Thomas et al., 1984; Van Vactor et al., 1993). Some of these choice points are associated with identified mesodermal cells, reminiscent of guidepost cells in other organisms (Bate et al., 1991; Van Vactor et al., 1993). Classical and molecular genetic studies have identified numerous genes controlling many steps of this process, including cell surface molecules (e.g. Sema I, Yu et al., 1998; Plexin A, Winberg et al., 1998; DLAR, Desai et al., 1996; Krueger et al., 1996), transcription factors (e.g. *lim3*, Thor et al., 1999) and intracellular signaling molecules (e.g. Drac1, Kaufmann et al., 1998), all of which function on or within motor neurons. Surprisingly, none of the known *Drosophila* choice point genes encodes a cue provided by mesodermal cells at the choice point and capable of controlling pathway selection.

In the vertebrate embryo, different populations of spinal motor neurons initially share common paths, but diverge at specific choice points. For example, in the avian hindlimb, motor growth cones extend within a common path to a plexus region at the base of the limb, where individual axonal populations diverge into the appropriate trunk nerve path, and then diverge further into the correct muscle nerves (Tosney, 1991). When lumbosacral motor neurons were displaced by two segments along the neuraxis, they exited the spinal cord via the adjacent segmental nerve and selected their appropriate path within the plexus region (Lance-Jones and Landmesser, 1980). These and other elegant manipulations have provided evidence that motor growth cones respond to environmental cues that might be located within the plexus region (Lance-Jones, 1988; Tosney, 1991). Recent progress has identified factors that act within vertebrate motor neurons to control cell specification and target selection (Appel, 1999; Eisen, 1998; Pfaff and Kintner, 1998), as well as intrinsic factors converting external cues into growth cone motility (Lanier et al., 1999). In contrast, little is known about guidance cues that direct pathway selection of motor growth cones locally at choice points.

The zebrafish embryo is an excellent model system in which to study pathway selection of spinal motor neurons (Eisen, 1994, 1998). As in other vertebrates, zebrafish spinal motor neurons have distinct identities and targets. Each somitic hemisegment is typically innervated by three pioneering motor neurons: the caudal primary (CaP), middle primary (MiP) and rostral (RoP) primary neurons (Fig. 1A). They first project their axons in common, segmentally reiterated nerves, and then pioneer individual paths towards their somitic muscle targets (Eisen et al., 1986; Myers et al., 1986; Westerfield et al., 1986). All three motor growth cones initially extend on a common path along the medial surface of the somites (Bernhardt et al., 1998; Eisen et al., 1986). Migration of all three motor growth cones along this common path is dependent on *diwanka* gene activity. In *diwanka* mutant embryos, primary motor axons are able to navigate correctly within the spinal cord, but fail to project on the common path from the spinal cord to the somites (Zeller and Granato, 1999). At the distal end of the common path, CaP, MiP and RoP growth cones contact a group of specialized cells called the muscle pioneers (Felsenfeld et al., 1991; Melançon et al., 1997). At this choice point, they pause before selecting cell-type-specific paths to ventral, dorsal and medial myotomal regions, respectively (Fig. 1A). Thus, the region where motor growth cones commonly pause before they pioneer independent pathways might provide signals for such divergent pathway decisions. Laser ablation experiments have shown that muscle pioneer cells are not critical for pathway selection (Melançon et al., 1997), suggesting that other cells may guide the decisions of motor axons at the choice point.

We examined a collection of mutants with presumed defects in the neural circuitries underlying coordinated body movements in order to identify genes controlling motor axon pathfinding (Granato et al., 1996; M. G., unpublished results). Here, we show that *unplugged* embryos display axonal guidance defects specific for motor neurons. We focused on the development of primary motor axons because they can be individually identified and because they pioneer the peripheral motor nerves (Eisen et al., 1986; Myers et al., 1986; Westerfield et al., 1986). In *unplugged* mutants, pathway selection of two motor neurons, CaP and RoP, is affected while pathway selection of MiP neurons appears unaffected. In mutant embryos, CaP and RoP growth cones navigate correctly from the spinal cord to the somitic choice point, but fail to select their subsequent cell-type-specific path. We show that *unplugged*-dependent pathway selection is provided by a small and selective population of somitic cells, and we identify these cells as dorsal adaxial cells, flanking the choice point. Moreover, the discovery that motor growth cones require guidance cues from adaxial cells, both for their migration from the spinal cord to the choice point (Zeller and Granato, 1999) and for pathway selection, suggests a central role of adaxial cells in motor axon guidance. Our results identify the *unplugged* gene as generating or encoding one of the first external cues that controls motor axon pathway selection. We hypothesize that *unplugged* is part of a local signaling mechanism that evokes different pathway decisions in CaP and RoP growth cones.

## MATERIALS AND METHODS

### Fish strains and breeding

Zebrafish were raised and maintained as described by Mullins et al. (1994). A single *unplugged* allele, *unp*$^{te314b}$, was identified in a large scale genetic screen (Granato et al., 1996). This allele was used in a subsequent non-complementation screen (van Eeden et al., 1999), which yielded six additional *unplugged* alleles: *unp*$^{tbr307}$, *unp*$^{tbo81}$, *unp*$^{tbt187}$, *unp*$^{tbb72}$, *unp*$^{tbr160}$ and *unp*$^{tdp253}$. Experiments described here were performed using the *unp*$^{te314b}$ allele in a mixed TL/WIK genetic background or the stronger allele *unp*$^{tbr307}$ in a mixed TL/Tü genetic background. The p$^4$ allele is a γ-ray-induced deficiency, which fails to complement the *unplugged* phenotype. Homozygous p$^4$ embryos lack chromosomal markers to both sides of the *unplugged* locus, suggesting that the p$^4$ allele lacks the entire *unplugged* gene. The phenotypic and molecular characterization of the p$^4$ allele will be published elsewhere.

### Labeling of individual primary motor axon trajectories

Individual CaP, MiP and RoP motor neurons were labeled by single blastomere injection, as described in Zeller and Granato (1999) and by cell transplantation (see below). We took advantage of the fact that *unplugged* is required cell non-autonomously for motor axon pathfinding (see Table 2A). Thus, when genotypically wild-type cells developed in mutant *unplugged* host embryos, they formed mutant CaP and RoP trajectories, indistinguishable from those obtained by single blastomere injection of *unplugged* mutant embryos. Embryos with motor neurons and adaxial cells derived from transplanted, genotypically wild-type cells were not included in this analysis. CaP, MiP and RoP motor neurons were identified by (a) their dorsoventral position within the spinal cord, (b) their rostrocaudal position within a spinal hemisegment, (c) the size of their soma, and (d) by their axonal trajectories before reaching the choice point. Other ventral neurons present at 27 hours postfertilization (hpf) include secondary motor neurons, KA neurons and VeLD neurons (Bernhardt et al., 1992; Kuwada et al., 1990a,b; Myers et al., 1986; Pike et al., 1992). Using the criteria given above, we can distinguish these cell types from mutant primary motor neurons. The secondary motor neurons can be distinguished by their smaller soma size (~1/2 the size of primary motor neurons, Myers et al., 1986) and the more ventral position of their somata in the spinal cord (Myers et al., 1986). KA neurons are located close to the floorplate and extend their axons rostrally (Bernhardt et al., 1992). Although VeLD neurons are located in the same region as the primary motor neurons, at 26 hpf VeLD growth cones have extended up to 10 segments caudally (Kuwada et al., 1990). In about 50% of the wild-type hemisegments an fourth primary motor neuron with a variable fate, VaP, is present (Eisen et al., 1990). The soma of VaP is adjacent to the CaP soma and like CaP neurons, they extend a growth cone ventrally towards the choice point (Eisen et al., 1990). Wild-type CaP and VaP can be distinguished because at 20 hpf CaP growth cones have extended into the ventral somite, while VaP growth cones never extend ventrally from the choice point (Eisen et al., 1990). This may lead to the conclusion that the presumed CaP growth cones that aberrantly stop at the choice point and branch, are in deed VaP growth cones. This seems unlikely because, in *unplugged* mutant embryos, the vast majority of potential CaP or VaP growth cones stall or branch aberrantly at the choice point (85%, Table 1), while we observe the normal number of VaP neurons in *unplugged* mutant hemisegments (50%, Fig. 3F).

### Labeling of other spinal cord trajectories

Axonal trajectories of identified spinal neurons were labeled as follows: Rohon-Beard mechanosensory neurons, DoLA, VeLD and CoSA by cell transplantion and antibody stainings using an anti-acetylated tubulin antibody (Piperno and Fuller, 1985), KA neurons by cell transplantation and antibody staining using an anti-L-GABA antibody (Bernhardt et al., 1992), CoPA neurons by cell transplantation and antibody staining using the 3A10 antibody (Hatta, 1992), and the hindbrain commissural Mauthner neurons by antibody staining using the 3A10 antibody (Hatta, 1992). Labeled neurons were

examined at 26-27 hpf and their identities were determined by (a) their dorsoventral position within the spinal cord, (b) the size of their soma, and (c) by the path of their axonal trajectories as previously described by Bernhardt et al. (1990) and Kuwada et al. (1990a,b). For each neuronal class, we analyzed at least 7 axonal trajectories in at least 7 embryos.

### Antibody staining

Antibody staining was performed essentially as described by Zeller and Granato (1999). The following primary antibodies were used: anti-acetylated tubulin antibody (1:1000; Piperno and Fuller, 1985; Sigma), znp-1 (1:200; Trevarrow et al., 1990; Antibody Facility, University of Oregon), 3A10 (1:50; Hatta, 1992; kindly provided by T. Jessell), prox-1 (1:2000; Glasgow and Tomarev, 1998; a gift from S. Tomarev), 4D9, which recognizes zebrafish Engrailed proteins (1:200; Hatta et al., 1991; Developmental Studies Hybridoma Bank, University of Iowa), zn-5, which recognizes DM-GRASP (1:500; Fashena and Westerfield, 1999; Trevarrow et al., 1990; Antibody Facility, University of Oregon), and F59 (1:10; Crow and Stockdale, 1986; Devoto et al., 1996; kindly provided by F. Stockdale).

### Whole-mount in situ hybridization

In situ hybridization was performed as previously described in Odenthal and Nusslein-Volhard (1998) using the following probes: *islet-1, islet-2* (Appel et al., 1995; kindly provided by I. David), *twist* (Morin-Kensicki and Eisen, 1997; kindly provided by B. Riggleman), and *wnt 11* (Makita et al., 1998; kindly provided by H. Takeda). Stained embryos were dehydrated, viewed and documented as described below.

### Chimeric embryos

Chimeric embryos were generated as previously described in Zeller and Granato (1999). Analysis of chimeric embryos was performed as previously described by Zeller and Granato (1999), with the following modification for the simultaneous analysis of chimeric cells, motor axons and adaxial cells. After F59 antibody staining, chimeric embryos were fixed at room temperature for 1 hour in 4% PFA and rinsed three times with PBST before further processing.

### Microscopy of stained embryos

Stained embryos were dehydrated through a MeOH series (10% / 30% / 50% / 70% / 90% / 100%), cleared in a mixture of benzylbenzoate and benzylalcohol (2:1), mounted in Canada Balsam/methyl salicylate (10:1) and viewed using Nomarski optics on a Zeiss Axiophot microscope. DIC images were acquired via a digital camera (Progres 3012, Kontron), saved on a Macintosh computer and processed with Adobe Photoshop 4.0.1 software. For fluorescent microscopy, stained embryos were sectioned, mounted in Vectashield (Vector Laboratories) and analyzed using a Zeiss Axiophot microscope. For DIC combined with fluorescent microscopy, stained embryos were sectioned, mounted in Vectashield (Vector Laboratories) and analyzed with a Hamamatsu Color Chilled 3CCD camera. Simultaneous visualization of transplanted cells and adaxial cells (Fig. 6) was performed on a Leica confocal microscope.

## RESULTS

### Mutations in the *unplugged* gene cause motor axon defects

We used a motor axon-specific antibody (znp-1; Trevarrow et al., 1990) to examine the trajectories of spinal motor neurons in *unplugged* mutant embryos. At 27 hpf, the znp-1 antibody labels CaP, MiP and RoP axons along their common path, from the spinal cord to the choice point, as well as CaP and MiP axons along their cell-type-specific path into ventral and dorsal somites, respectively (Melançon, 1994; Melançon et al., 1997). In over 97% of the somitic hemisegments that we examined in wild-type embryos, primary motor axons projected along the common path from the spinal cord to the choice point and continued along their cell-type-specific path (segments 6-15, *n*=600, Fig. 1B). In *unplugged* mutant embryos, znp-1-positive axonal trajectories from the spinal cord towards the choice point appeared unaffected whereas, in the region of the choice point, over 84% of the motor axon projections were severely

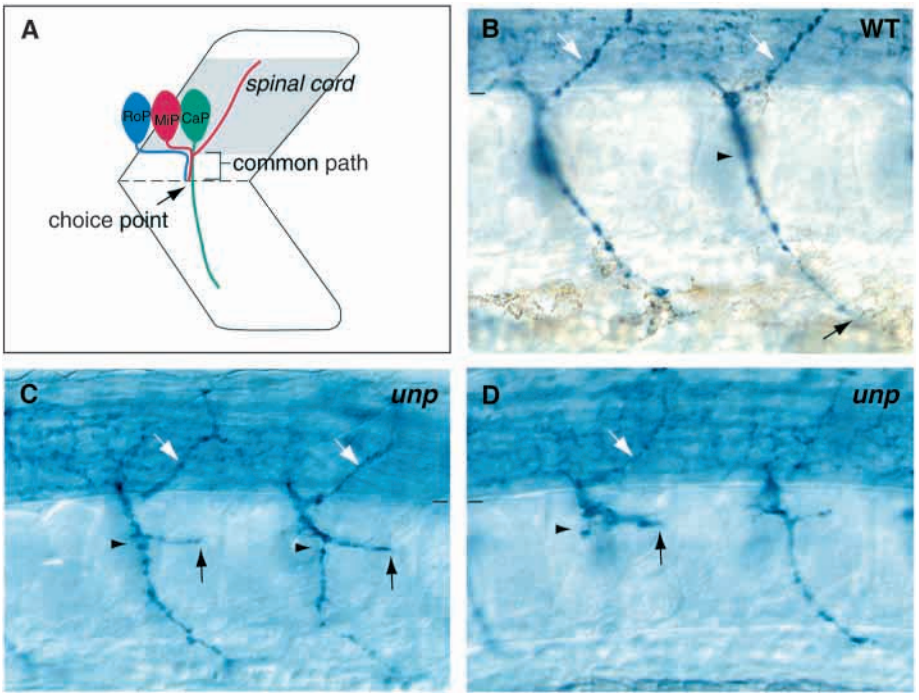


**Fig. 1.** *Unplugged* mutant embryos display motor axon defects. (A) Schematic drawing illustrating the soma positions of RoP, MiP and CaP and their axonal projections in a hemisegment at 27 hpf (lateral view). Wild-type (B) and *unplugged* mutant (C,D) embryos at 27 hpf were stained with znp-1 antibody. (B) Wild-type CaP axons, after reaching the choice point (arrowhead) extended further into the ventral myotome (black arrow), and MiP axons projected a dorsal collateral branch (white arrows) at a position close to the ventral extent of the spinal cord (black line). (C) In *unplugged* mutant embryos, motor axons extended into the ventral myotome but formed multiple aberrant branches (black arrows) at the choice point (arrowheads), or (D) they stopped and branched at the region of choice point (arrow). Presumptive MiP axons projected normally (white arrows).

**Table 1. Motor axon defects in *unplugged* mutants**

| | CaP wild-type axonal projections | *CaP axon stalled at the choice point | ‡CaP axon branching at choice point | ∑ of aberrant CaP axons | RoP wild-type axonal projections | RoP axons into ventral myotome | §RoP axon branching at choice point | ∑ of aberrant RoP axons | MiP wild-type axonal projection | ∑ of aberrant MiPs | ∑ of aberrant CaPs& RoPs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Wild-type | 36/39¶ | 1/39 | 2/39 | 3/39 | 52/52 | 0/52 | 0/52 | 0/52 | 19/19 | 0/19 | 3/90 |
| | 92.3% | 2.6% | 5.1% | 7.7% | 100% | 0% | 0% | 0% | 100% | 0% | 3.4% |
| *unplugged* | 2/14 | 10/14 | 2/14 | 12/14 | 5/13 | 3/13 | 5/13 | 8/13 | 4/4 | 0/4 | 20/27 |
| | 14.3% | 71.4% | 14.3% | 85.7% | 38.5% | 23% | 38.5% | 61.5% | 100% | 0% | 74% |

Primary motor neurons were labeled by injecting single blastomeres with lineage tracer before the 128-cell stage or by transplanting wild-type blastomeres into *unplugged* mutant host embryos. Labeled motor neurons in 23 mutant and 62 sibling embryos were examined at 27 hpf and no significant difference was observed between the labeling techniques. See Materials and Methods for identification of neuronal cell types.

*CaP axons stalled at the region of the choice point and often formed short lateral branches.

‡Branching occurred at the choice point, whereby one trajectory extended into the ventral myotome and a second extended caudally or rostrally at the region of the choice point.

§Branching occurred at the choice point, whereby one trajectory extended rostrally and sometimes a second trajectory extended caudally at the region of the choice point.

¶A small fraction (3/39) of CaP axons in wild-type embryos projected abnormally. In two cases, a main branch extended into the ventral somite and a second branch formed at the choice point. In one case, the neuron extended an axon only to the choice point, thus resembling VaP trajectories (Eisen et al., 1990).

affected (*n*=539, Fig. 1C,D). In the majority of the cases (76%), a CaP-like axonal projection invaded the ventral somite and ectopic caudal and/or rostral projections emerged at the choice point (Fig. 1C). In about 7% of the mutant hemisegments, znp-1-positive motor axons failed to extend into the ventral somite, but formed ectopic caudal and rostral branches at the choice point (Fig. 1D). Conversely, in all mutant hemisegments analyzed, pathway selection of presumptive MiP neurons into the dorsal somite appeared unaffected (Fig. 1C,D). Thus, znp-1 antibody stainings in *unplugged* mutants revealed pathfinding defects predominantly at the choice point, indicating a potential role for *unplugged* in axonal pathway selection.

## The *unplugged* gene controls CaP and RoP pathway selection

To define the role of the *unplugged* gene in more detail, we asked which of the znp-1-positive primary motor neurons in *unplugged* mutants were affected and how their trajectories were altered. Given that the znp-1 epitope is expressed on CaP, MiP and RoP axons, znp-1 stainings did not allow us to determine which of these three motor axons contributed to the mutant axonal phenotype. To visualize individual CaP, MiP and RoP trajectories, we labeled these neurons in wild-type and *unplugged* mutant embryos by transplanting vital-dye-labeled cells and by single blastomere injections (Fig. 2, Table 1; for details see Materials and Methods). Labeling of CaP, MiP and RoP neurons in wild-type embryos revealed their well-described stereotypic axonal trajectories (Fig. 2; Eisen et al., 1986; Myers et al., 1986; Westerfield et al., 1986). At 26 hpf, CaP and MiP growth cones in *unplugged* siblings extended into ventral and dorsal somites, respectively, while RoP growth cones always stopped and paused at the choice point (Fig. 2A,D,E).

In *unplugged* mutant embryos, MiP growth cones always selected their correct path, while CaP and RoP growth cones failed to make appropriate pathway decisions at the choice point. Labeled CaP growth cones navigated correctly to the choice point but instead of migrating into the ventral somite, they stalled at the choice point, where they often formed short, aberrant branches (71%; Fig. 2B; Table 1). In a small fraction of *unplugged* mutants, we observed CaP axons forming two long and distinct branches at the choice point (14%; Fig. 2C; Table 1). One branch extended past the choice point into the ventral somite, while a second branch extended caudally or rostrally at the level of the choice point (Fig. 2C). Conversely, labeled RoP growth cones did not pause at the choice point, but formed aberrant branches at the choice point (38%; Fig. 2G; Table 1) or even invaded the ventral somite (23%; Fig. 2F; Table 1). This observation is significant since, in wild-type embryos, RoP growth cones never extend into the ventral somite (*n*=52, compare Fig. 2E to 2F). Overall, in *unplugged* mutant embryos, more than 70% of CaP and RoP growth cones failed to make their appropriate pathway choice at the choice point. Moreover, at 26 hpf 71% CaP growth cones made a RoP-like pathway choice and 23% of RoP growth cones made a CaP-like pathway choice at the choice point (Fig. 2H; Table 1). Together, these results demonstrate that *unplugged* is essential for CaP and RoP growth cones to select their cell-type-specific path at the choice point.

## *unplugged* function is restricted to spinal motor axons

To investigate if mutations in the *unplugged* gene affect axonal guidance and pathway selection throughout the nervous system or specifically affect pathway selection of CaP and RoP motor neurons, we examined axonal trajectories of various neuronal cell types. We first asked whether axonal trajectories of four identified spinal neurons, Rohon-Beard mechanosensory neurons (RB; Kuwada et al., 1990), dorsal longitudinal ascending (DoLA; Kuwada et al., 1990), ventral longitudinal descending (VeLD; Kuwada et al., 1990) and Kolmer Agduhr (KA; Bernhardt et al., 1992) neurons were affected in *unplugged* mutant embryos. Axonal trajectories of these neurons were visualized by antibody staining or by transplantation of vital-dye-labeled cells (for details see Materials and Methods; *n* refers to the number of axonal trajectories analyzed). In *unplugged* embryos, axonal projections of RB (*n*=81), DoLA (*n*=22), VeLD (*n*=7) and KA (*n*=10) neurons appeared indistinguishable from those in wild-type embryos (data not shown).

We then asked whether mutations in the *unplugged* gene affect axon guidance of commissural neurons at the central nervous system (CNS) midline. Floor plate cells at the vertebrate CNS midline serve as intermediate targets to attract growth cones of commissural neurons and to repel those of non-crossing interneurons (Bovolenta and Dodd, 1991; Kennedy et

al., 1994; Nguyen Ba-Charvet et al., 1999). We reasoned that, if *unplugged* participates in general axonal pathway selection, then axon guidance of commissural interneurons at the CNS midline might also be affected. Analysis of commissural Mauthner neurons in the hindbrain ($n$=10; Metcalfe et al., 1986), spinal commissural primary ascending neurons (CoPA; $n$=10; Kuwada et al., 1990) and spinal commissural secondary ascending neurons (CoSA; $n$=65; Kuwada et al., 1990) in *unplugged* mutant embryos revealed axonal trajectories indistinguishable from those in wild-type embryos (Fig. 3A,B and data not shown). Together, these results strongly suggest that *unplugged* is not required for general axonogenesis or general pathway selection, but is essential for guiding CaP and RoP growth cones at the choice point.

### Specification of motor neurons and somitic cells at the choice point appears unaffected in *unplugged* mutants

In *unplugged* mutant embryos, a significant number of neurons with soma positions consistent with CaP neurons made a RoP-like pathway decision and, conversely, neurons with soma positions consistent with RoP neurons made a CaP-like pathway choice at the choice point (Fig. 2). To examine if this defect is caused by a switch in cell type identity between CaP and RoP neurons, we analyzed the expression of the LIM domain genes *islet-1* and *islet-2*. Similar to other vertebrates, individual subpopulations of zebrafish spinal motor neurons express specific combinations of LIM homeobox genes (Appel et al., 1995; Eisen, 1998). Moreover, transplantation of individual motor neurons to new spinal cord positions resulted in the initiation of a new program of LIM gene expression, as well as axonal trajectories characteristic for their new positions, suggesting that LIM gene expression reflects the specification of individual motor neurons (Appel et al., 1995). In wild-type embryos two adjacent cell bodies (RoP and MiP) at the rostral somite boundary express *islet -1* (see Fig. 3C), whereas a single cell body (CaP), located in the center of the somite, expresses *islet-2* (Fig. 3E). In *unplugged* mutant embryos, we observed wild-type expression patterns of *islet-1* and *islet-2* mRNA, demonstrating that *unplugged* CaP and RoP neurons express their specific complement of LIM genes (Fig. 3D,F).

We also examined the number, position and specification of muscle pioneer cells located at the choice point. Muscle pioneer cells are a specialized group of somitic cells that may influence motor axon pathway selection (Melancon, 1994). To determine if muscle pioneer cells were present and properly specified in *unplugged* mutants, we examined the expression of engrailed and DM-GRASP, two molecules characteristic for muscle pioneer cells (Fashena and Westerfield, 1999; Hatta et al., 1991; Kanki et al., 1994; Trevarrow et al., 1990). The expression levels and patterns of Engrailed and DM-GRASP proteins in *unplugged* muscle pioneer cells were indistinguishable from those in wild-type muscle pioneer cells (Fig. 3G,H and data not

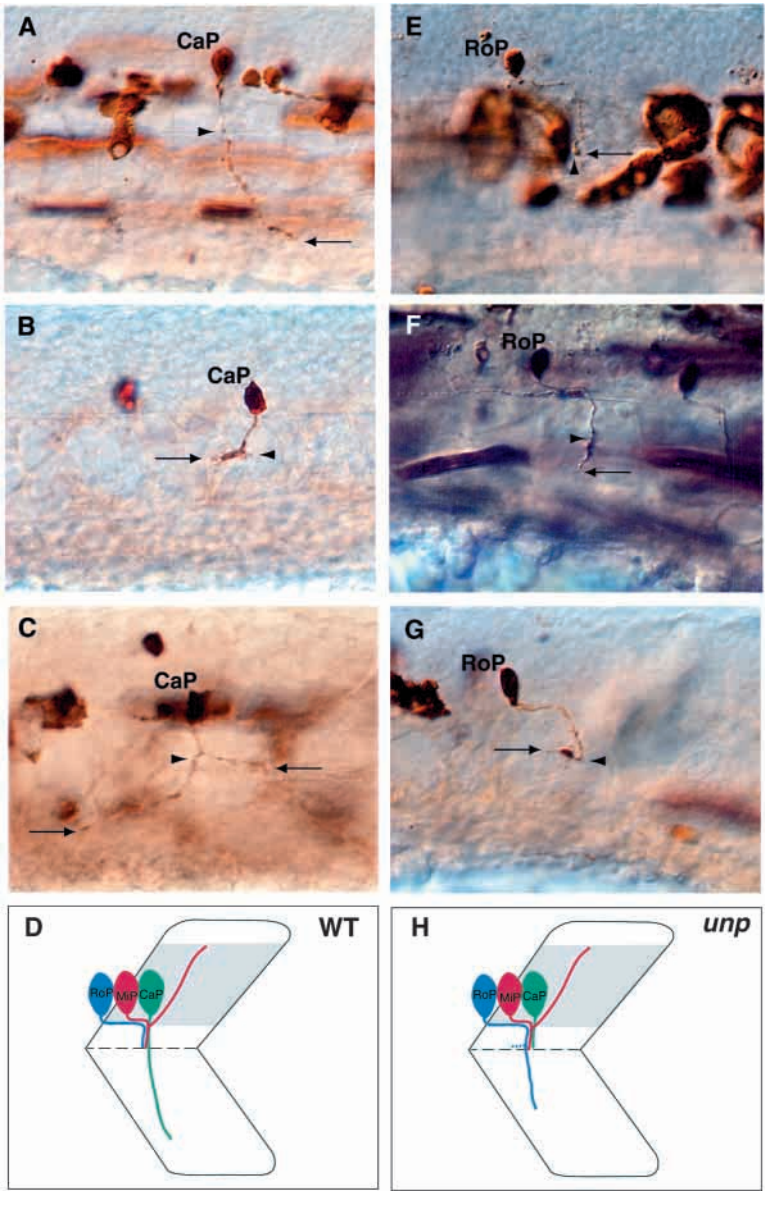


**Fig. 2.** Axonal trajectories of *unplugged* CaP and RoP motor neurons exhibit pathway selection defects. Labeling of individual motor neurons revealed the path of CaP (A-C) and RoP (E-G) in wild-type siblings embryos (A,E) and *unplugged* mutant (B,C,F,G). In all panels, an arrow points to the tip of the axon and an arrowhead points to the choice point. (A) At 27 hpf, wild-type CaP growth cones had always extended into the ventral myotome. (B) In *unplugged* mutants, most CaP growth cones stalled at the choice point, where they often formed short branches at the level of the choice point. Occasionally one long branch extended at the level of the choice point and a second branch projected into the ventral somite (C). In wild-type embryos, RoP growth cones always stopped and paused at the choice point (E), while in *unplugged* mutant embryos RoP growth cones either extended into the ventral myotome (F) or formed aberrant branches at the region of the choice point (G; see also Table 1). Axonal trajectories of all three motor neurons in a wild-type hemisegment (D) are compared to those in *unplugged* mutant embryos (H). Individual motor neurons were labeled by transplanting genotypically wild-type neurons into *unplugged* mutants (B,C,F,G) or into wild-type siblings (A,E). Similar results were obtained by single blastomere injections into *unplugged* mutants.

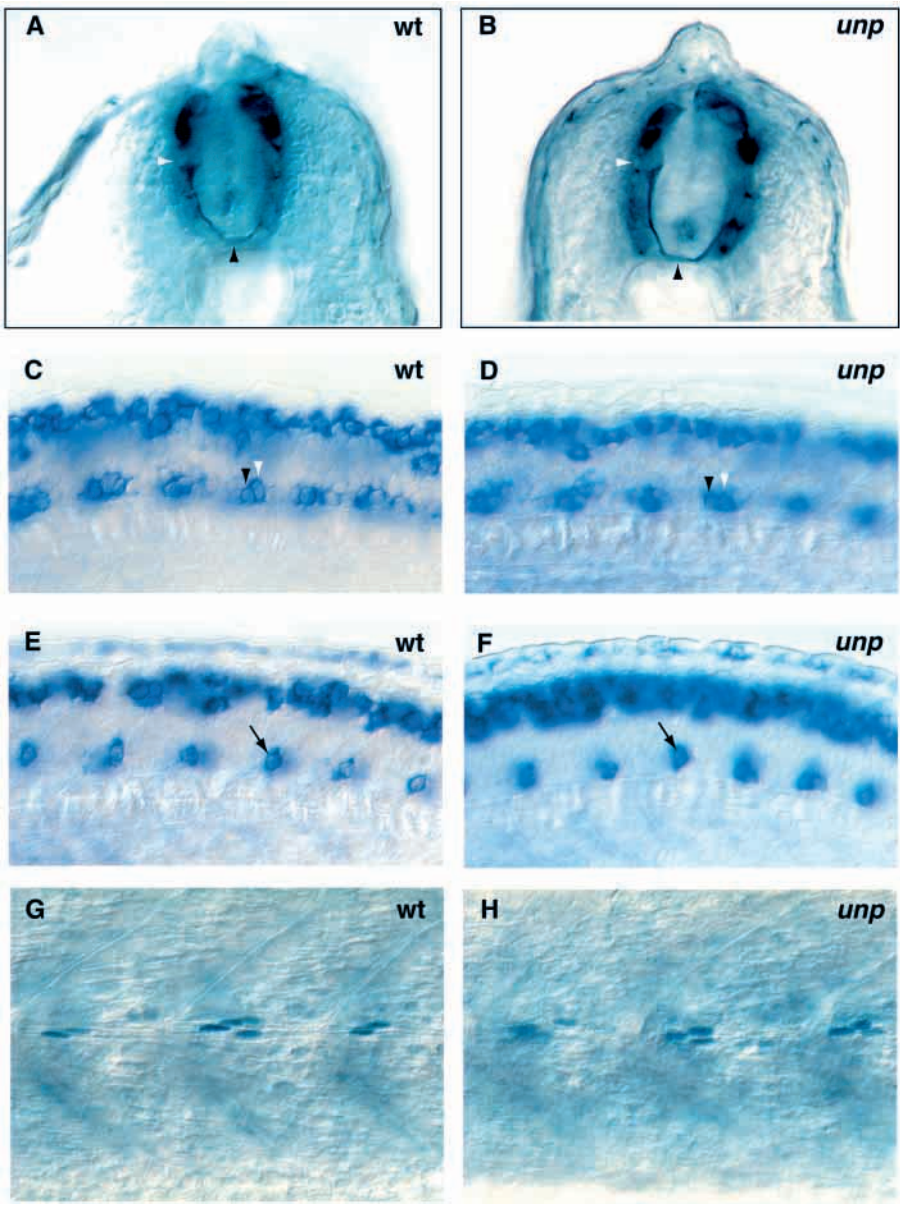


**Fig. 3.** Spinal axonal trajectories, motor neuron specification, and muscle pioneer development appear unaffected in *unplugged* mutant embryos. (A,B) Cross-section of 27 hpf embryos stained with anti-acetylated tubulin. In the wild-type (A) and the mutant embryo (B), a commissural primary ascending (CoPA) soma (white arrowhead) extends an axon ventrally to the midline (black arrowhead) and across the midline. Staining with *islet-1* antisense probe (C,D) or *islet-2* antisense probe (E,F) of a 27 hpf wild-type (C,E) and an *unplugged* mutant embryo (D,F), lateral views. Black and white arrowheads point to *isl-1* positive cells (C,D) in the ventral spinal cord. The position of these two cells is consistent with the position of RoP and MiP neurons, respectively. Arrows in E and F point to *isl-2* positive cells in the ventral spinal cord, consistent with the position of CaP neurons. In about 50% of the wild-type and *unplugged* hemisegments, a second motor neuron, VaP, is stained by *islet-2* (Appel et al., 1995; Eisen et al., 1990). RoP, MiP and CaP motor neurons were identified by their dorsoventral and rostrocaudal position within the spinal cord, their position relatively to the somite boundary (not in focus) and their soma size (see Materials and Methods for details). Lateral view of a 27 hpf wild-type (G) and an *unplugged* mutant embryo (H) stained with 4D9, which recognizes the nuclear Engrailed epitope in muscle pioneer cells. About 2-6 muscle pioneer cells are present per hemisegment, at the level of the horizontal myoseptum (Hatta et al., 1991). The number and position of 4D9 positive muscle pioneer cells in *unplugged* mutant embryos is indistinguishable from those in wild-type embryos.

shown). Thus, our analysis shows that mutations in the *unplugged* gene do not overtly affect motor neuron or muscle pioneer specification, supporting the idea that *unplugged* functions primarily in motor axon pathway selection.

### *unplugged* acts cell non-autonomously in somitic cells to guide motor axons

In order to understand the biological mechanism of how *unplugged* influences axonal pathway selection, we asked whether *unplugged* activity is provided by motor neurons themselves, or by surrounding cells. Pathway selection requires the specific interaction of axonal growth cones with a multitude of external cues (Goodman and Tessier-Lavigne, 1996). Accordingly, genes controlling this event can be divided into two main groups depending on where they function: in neurons or in cells of the embryonic environment. In the zebrafish, this question can be addressed through the analysis of chimeric embryos (Ho and Kane, 1990). To generate chimeric embryos, we transplanted genotypically wild-type, dye-labeled cells at the dome stage into age-matched embryos derived from heterozygous *unplugged* fish. At 27 hpf, we identified *unplugged* mutant embryos by their motility phenotype (Granato et al., 1996) and examined these embryos for the presence of labeled, genotypically wild-type CaP and RoP neurons. In *unplugged* host embryos, genotypically wild-type CaP and RoP motor neurons developed mostly mutant motor axonal trajectories (Table 2A). Reciprocally, we transplanted dye-labeled cells from homozygous mutant *unplugged* embryos into wild-type embryos. In these host embryos, genotypically mutant CaP and RoP motor neurons developed wild-type-like axonal trajectories (Fig. 4B,D; Table 2A). These results demonstrate that the *unplugged* gene is not required in CaP and RoP motor neurons, but instead functions cell non-autonomously in the embryonic environment to mediate correct pathway selection.

To identify the cell type(s) that provides *unplugged* activity, we generated a second set of chimeric embryos and examined

**Table 2A. *unplugged* activity is not required in motor neurons**

| Genotype of donor cells | Genotype of host embryo | CaP | RoP | MiP |
|---|---|---|---|---|
| +/+ | +/+ or +/– | 36/39 (92.3%) | 52/52 (100%) | 19/19 (100%) |
| +/+ | –/– | 2/14 (14.3%) | 5/13 (38%) | 4/4 (100%) |
| +/+ or +/– | +/+ | 65/73 (89%) | 35/35 (100%) | 19/19 (100%) |
| –/– | +/+ | 24/28 (85%) | 8/8 (100%) | 3/3 (100%) |

At 27 hpf host embryos were examined for the presence of transplanted, labeled motor neurons. Indicated is the fraction of donor-derived CaP, RoP and MiP motor neurons with wild-type-like axonal trajectories. TL wild-type fish were mated to each other to produce wild-type embryos with genotypically +/+ donor cells or to produce +/+ host embryos. Heterozygous *unplugged* fish were mated to each other to generate homozygous mutant *unplugged* –/– embryos. These embryos were used as –/– host embryos as well as to obtain genotypically –/– donor cells. Mating between heterozygous *unplugged* fish also generated 75% of phenotypically wild-type embryos which were used as +/+ or +/– host embryos and to obtain genotypically +/+ or +/– donor cells. –/– donor embryos and –/– host embryos were identified at 27 hpf by their motility phenotype.

**Table 2B. *unplugged* activity is provided by lateral somite cells**

| Cell type | N* | Floor plate | Noto chord | Floor plate & notochord cells | Neural crest cells | Muscle pioneer cells | Epithelial cells | Somite cells | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Medial‡ | Medial+Lateral | Lateral‡ |
| rescue/cases | 151/1874 | 2/20 | 1/21 | 0/14 | 0/6 | 4/34 | 1/17 | 1/91 | 22/64 | 34/73 |
| % | 8% | 10% | 4.8% | 0% | 0% | 11.8% | 5.9% | 1.1% | 34.4% | 46.6% |

A total of 143 chimeric embryos were analyzed at 26 hpf. Indicated is the number of rescued CaP motor axon trajectories per total number of cases in which genotypically wild-type cells contributed to the indicated cell type. Only cell types that could be identified unambiguously by their morphology and/or position are considered here. A total of 346 somitic hemisegments with wild-type cells were identified, of which 228 were sectioned in order to analyze the spatial distribution of the cells within the somites. Only cases in which znp-1-positive CaP motor axons had extended beyond the ventral aspect of the notochord on their cell-type-specific pathway similarly to wild-type motor axons were considered as rescued. The znp-1 antibody has been shown to also stain secondary motor axons (Melancon, 1994), which project on the paths pioneered by primary motor axons. However, at 26 hpf secondary motor axons in somitic segments have not yet extended beyond the ventral aspect of the notochord (Melancon, 1994).

*We observed wild-type like CaP motor axon projections without observing any wild-type cell within the same hemisegment in 8% of the somitic hemisegments examined. This is comparable to the frequency with which wild-type-like projections are observed in homozygous mutant *unplugged* embryos (16% at 27 hpf, see text).

‡We classified somitic cells as either medial, lateral or medial & lateral. Accordingly, each somitic hemisegment was divided into a medial (2/3) and a lateral part (1/3).

cell types in the vicinity of motor neurons for their ability to restore pathway selection of *unplugged* motor axons. In this set of experiments, wild-type cells were transplanted at the dome stage into *unplugged* embryos and, at 26 hpf, chimeric embryos were stained with znp-1 antibody to visualize motor axonal projections. We then correlated the identity of wild-type-derived cells with their ability to restore motor pathway selection. It was important to analyze a large number of chimeric *unplugged* embryos because in non-chimeric homozygous mutant *unplugged* embryos, we observed that 16% of the somitic hemisegments contained motor axonal projections indistinguishable from those in wild-type embryos. Consistent with this observation, in chimeric host embryos 8% of all somitic hemisegments lacked wild-type-derived cells but displayed wild-type-like axonal trajectories. Given the incomplete penetrance of the *unplugged* phenotype, we therefore only considered values well above 16% to be significant for the ability of a particular cell type to provide *unplugged* activity.

Analysis of 143 chimeric *unplugged* embryos revealed that

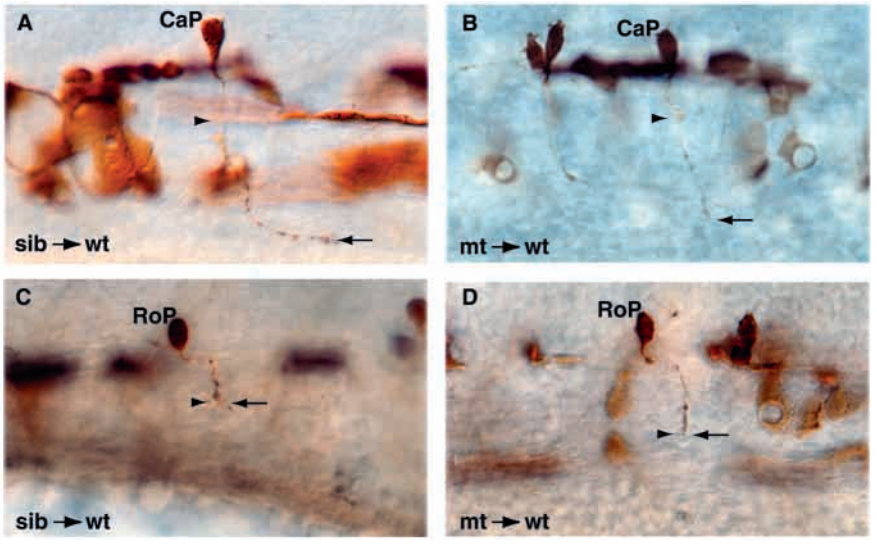


**Fig. 4.** *unplugged* acts cell non-autonomously in CaP and RoP pathfinding. (A, C) In wild-type embryos, transplanted +/+ or *unplugged*/+ cells developed into CaP (A) and RoP (C) neurons with normal axonal trajectories (see also Table 2A). (B,D) Similarly, transplanted *unplugged*/*unplugged* CaP (B) and RoP (D) neurons developed normal axonal trajectories in wild-type embryos (see also Table 2A). Arrowheads indicate the position of the choice point and arrows point to the tip of the axon. When transplanted into *unplugged*/*unplugged* embryos, genotypically wild-type CaP and RoP neurons developed mutant trajectories (see Table 2A).

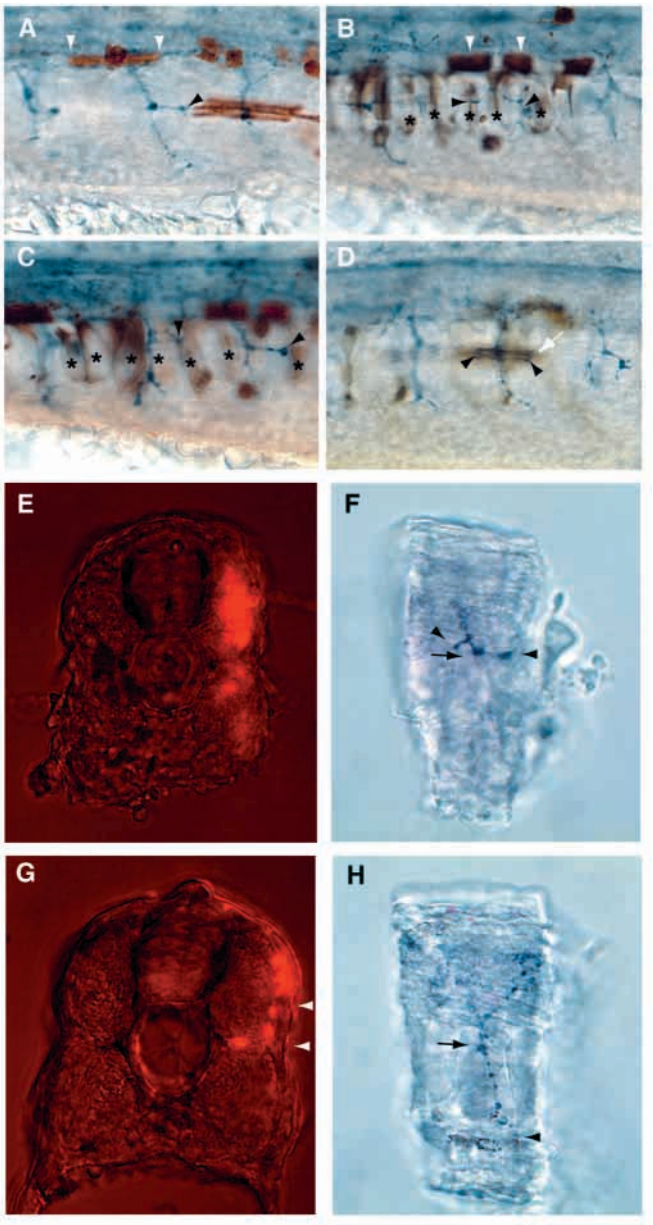


**Fig. 5.** Analysis of chimeric embryos reveals that lateral somitic cells provide *unplugged* gene activity to CaP and RoP motor neurons. Biotin dextran- or Rhodamine dextran-labeled wild-type cells (brown in A-D and red in E and G) were transplanted into *unplugged* embryos and at 26 hpf chimeric embryos were stained with znp-1 antibody to visualize motor axonal projections (blue in A-D and F, H). (A-D) The presence of wild-type-derived cells in tissues surrounding motor neurons did not rescue the *unplugged* motor axon phenotype. Aberrant axons are labeled with black arrowheads. (A) A row of six adjacent wild-type-derived floorplate cells (delineated by white arrowheads); (B) notochord (black asterisks) and floorplate cells (white arrowheads); (C) six adjacent notochord cells (black asterisks), and (D) muscle pioneer cells (white arrow). The lineage tracer in the muscle pioneer cells partially masks the two aberrant axonal trajectories in D (arrowheads). Note that at this stage notochord cells are highly vacuolated and that the brown lineage tracer is restricted to a small strip of remaining cytoplasm. (E,G) Cross-section of mosaic *unplugged* embryos using fluorescent illumination to reveal the positions of rhodamine dextran-labeled wild-type cells. (F,H) Sections E, G were rotated to yield lateral views of the same somitic hemisegments. DIC illumination now reveals znp-1-stained motor axon trajectories; black arrows indicate the position of the choice point, and black arrowheads point to the tip of the motor axons. (E) Large groups of genotypically wild-type muscle cells in the medial part of the somite failed to restore the motor axon defect in this hemisegment (F). (G) In contrast, small groups of genotypically wild-type muscle cells in the lateral part of the somite restored the motor axon defect (H). White arrowheads in G delineate the region between the ventral extent of the spinal cord (upper arrowhead) and the choice point (lower arrowhead). Rescuing cells were located in this region.

wild-type-derived notochord, floorplate, neural crest and epithelial cells failed to rescue the axonal phenotype (Fig. 5A-C; Table 2B). Even when genotypically wild-type cells populated combinations of tissues, such as the notochord and floorplate ($n$=14; Table 2B), they failed to restore motor axon trajectories (Fig. 5B). In contrast, genotypically wild-type cells located in the somites were able to rescue the axonal phenotype, although to a variable degree, depending on their positions. Medially located somitic cells were not able to rescue the axonal phenotype (Fig. 5E,F; Table 2B), while approximately 34% of the somitic hemisegments with wild-type cells populating medial and lateral regions were capable of restoring axonal trajectories (Table 2B). Moreover, when wild-type cells were predominately located in lateral regions of the somite, rescue of the axonal phenotype was evident in 46% of the hemisegments (Fig. 5G,H; Table 2B). Interestingly, wild-type-derived muscle pioneer cells, located at the motor axon choice point, did not rescue the axonal phenotype (Fig. 5A,D; Table 2B). Our results demonstrate that cells in the lateral somite can provide *unplugged* activity to motor neuron growth cones.

## A subset of adaxial cells is crucial for *unplugged* mediated axonal pathway selection

The position of the somitic cells capable of rescuing the *unplugged* motor axon phenotype is consistent with the position of adaxial cells (Devoto et al., 1996). Adaxial cells are initially located in the medial region of the somite, adjacent to the notochord. After somite formation, adaxial cells organize into a single cell layer along the medial surface of the somite and later migrate radially through the somites to form a single layer of slow muscle fibers (Devoto et al., 1996). During radial migration, adaxial cells maintain their dorsoventral positions, and by 26 hpf, they have migrated to the lateral portion of the somite (Devoto et al., 1996). A subset of adaxial cells, the muscle pioneers, do not migrate to the lateral surface of the somite, but instead span the depth of the somite at the region of the future choice point (Devoto et al., 1996). To determine if the lateral somitic cells capable of restoring pathway selection in *unplugged* mutants were adaxial cells, we generated a third set of chimeric embryos by transplanting wild-type cells into *unplugged* mutant host embryos. In these chimeric embryos, we monitored simultaneously the position of the transplanted, genotypically wild-type cells (rhodamine dextran labeled, red in Fig. 6), the axonal path of primary motor axons (znp-1 antibody labeled, blue in Fig. 6), and the position of adaxial cells using an adaxial cell-specific antibody, F59 (Devoto et al., 1996; green in Fig. 6).

In 100 chimeric *unplugged* embryos, we examined a total of 148 somitic hemisegments in which wild-type-derived cells

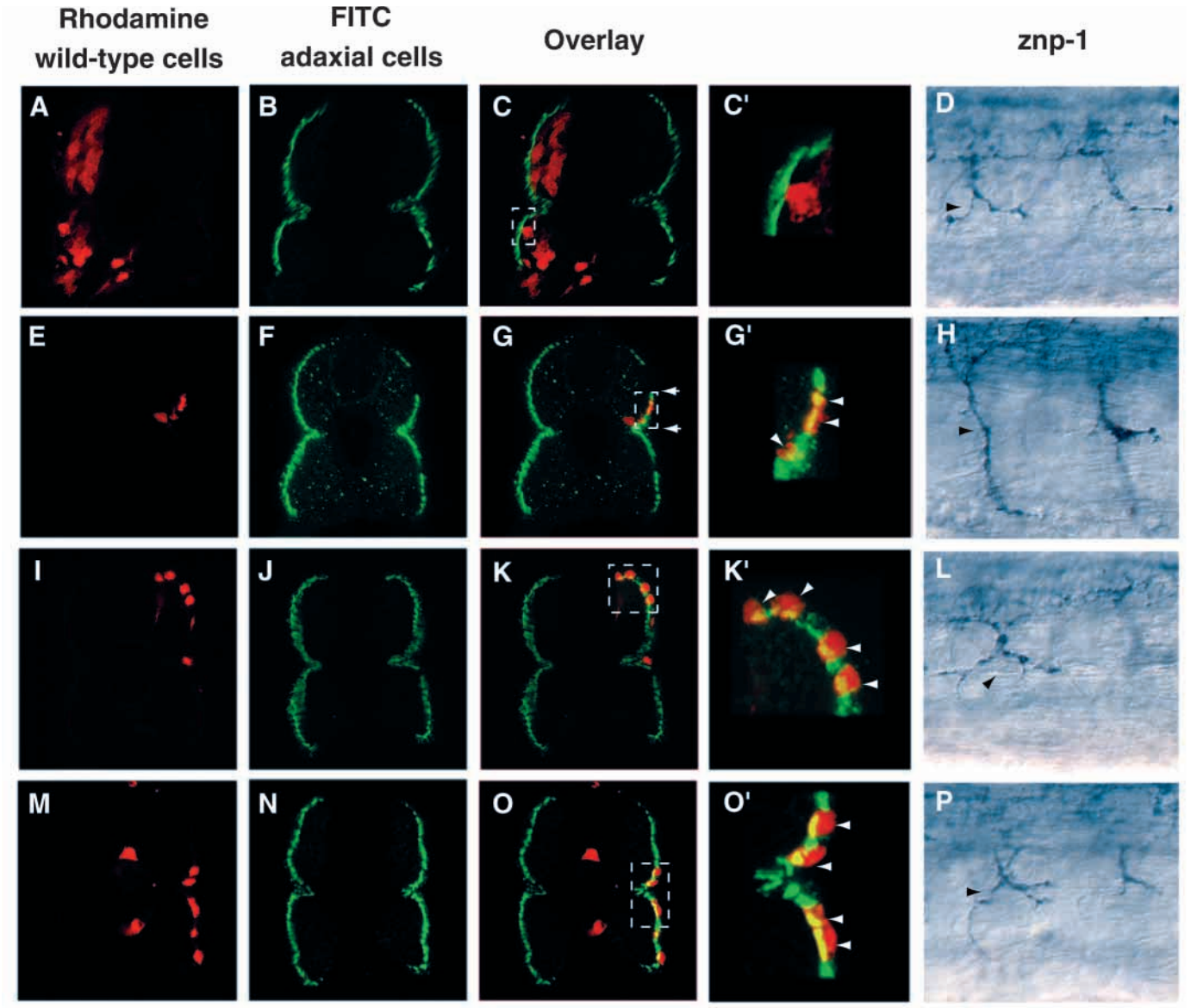


**Fig. 6.** *unplugged* activity is provided by an exclusive group of dorsal adaxial cells. Labeled wild-type cells were transplanted into *unplugged* mutant host embryos. In cross sections (A-C′,E-G′,I-K′,M-O′), the positions of the rhodamine dextran-labeled transplanted wild-type cells (A,E,I,M) and the positions of antibody-labeled adaxial cells (B,F,J,N) were determined. (C,G,K,O) Overlay of the rhodamine and FITC images, and (C′,G′,K′,O′) enlargement of the areas boxed in C,G,K,O, respectively. White arrowheads point to doubly labeled cells, i.e. wild-type derived adaxial cells. (D,H,L,P) Lateral view of the corresponding somitic hemisegments, stained with znp-1 antibody and viewed using DIC optics to reveal motor axon trajectories. Black arrowhead indicates the position of the choice point. Groups of non-adaxial somitic cells (A-C′), or adaxial cells located either dorsally (I-G′) or ventrally (M-O′) of the critical region failed to restore the motor axon defect (D,L,P). Only the presence of three or more adaxial cells in a dorsal domain located adjacent to the choice point (E-G′) rescued the motor axon defect in *unplugged* mutant embryos (H). Note that the presence of only two wild-type-derived adaxial cells located in this dorsal domain (O′, upper two arrowheads) was not sufficient to restore pathway selection (P). (G) White arrows delineate the dorsal domain, which encompasses 6-7 adaxial cells, adjacent to the choice point. This domain of adaxial cells, extending from the ventral spinal cord to the region of the choice point, appears critical for *unplugged*-dependent pathway selection.

contributed to somitic tissue. In 108 hemisegments, wild-type-derived cells were distributed throughout the somite, but did not contribute to adaxial cells (Fig. 6A-C′). In the vast majority of these hemisegments (89/108, 82%), we observed the characteristic *unplugged* phenotype (Fig. 6D), showing that non-adaxial somitic cells were unable to restore pathway selection. In contrast, wild-type cells that developed into three or more dorsal adaxial cells were able to rescue the *unplugged* axonal phenotype very efficiently (16/17, 94%; Fig. 6E-H). These rescuing adaxial cells were located in a dorsal domain, adjacent to the muscle pioneers and the choice point, encompassing a region of about 6 to 7 cells (arrows in Fig. 6G). Wild-type-derived adaxial cells located further dorsally or in the ventral half of the somite failed to rescue the mutant phenotype (1/23, 4%; Fig. 6I-L). Moreover, we find that, in addition to their positions, the number of wild-type-derived

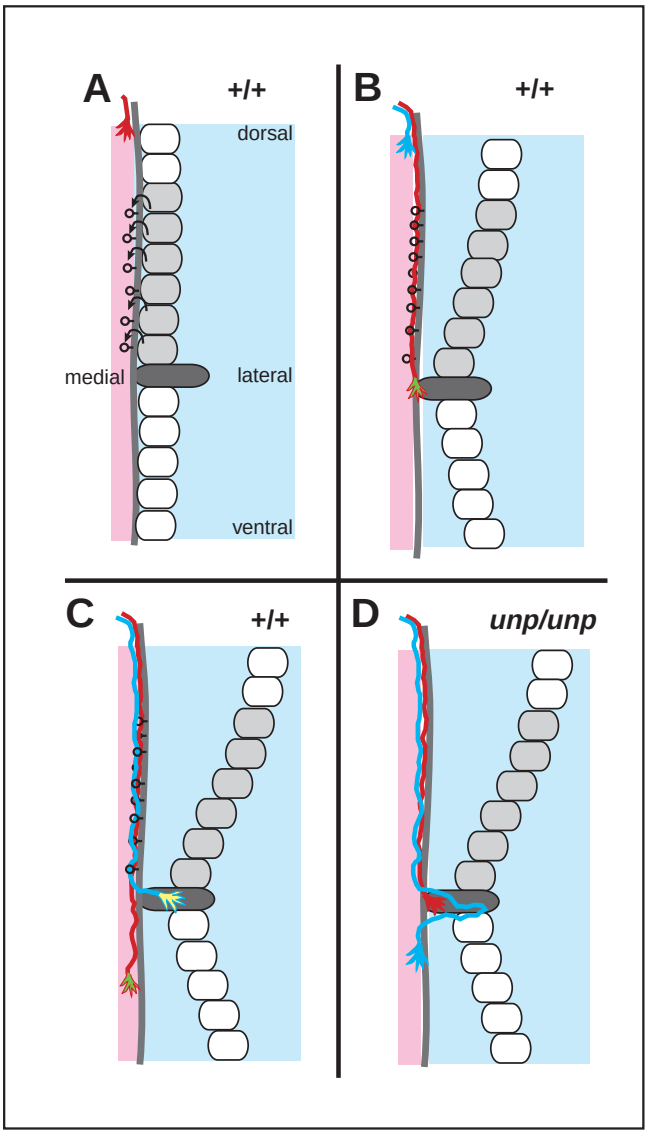


**Fig. 7.** *unplugged* gene is part of a local, mesodermal guidance mechanism. (A) In this model, a CaP growth cone (red) and later a RoP growth cone (blue), approach the choice point. *unplugged* activity (small circles) is deposited on the extracellular matrix by adaxial cells (gray), adjacent to the choice point (black). (B) In wild-type embryos, the CaP and later the RoP growth cone are exposed to *unplugged* gene activity located on the medial somite surface, while the adaxial cells have migrated laterally. (C) *unplugged* functions as a bifunctional cue, eliciting different responses in CaP and RoP growth cones (indicated by the change of color in CaP and RoP growth cones). It instructs CaP growth cones to select a path into the ventral somite, but prevents RoP growth cones from inappropriately crossing the choice point. (D) In *unplugged* mutants, the activation of specific receptor mechanisms and/or downstream effectors within CaP and RoP growth cones does not occur (indicated by the lack of color change in CaP and RoP growth cones). As a result, CaP growth cones stall at their intermediate target and RoP growth cones explore the adjacent region or project occasionally into the ventral somite.

dorsal adaxial cells was equally important. The presence of only one or two wild-type-derived adaxial cells within the dorsal domain was insufficient to rescue the mutant phenotype (3/17; 17%; Fig. 6M-P), while three or more cells in the same domain were sufficient to rescue the mutant phenotype (16/17, 94%; Fig. 6E-H). In summary, our chimeric analyses demonstrate that a small group of dorsal adaxial cells, confined to a region adjacent to the choice point, is crucial for *unplugged*-mediated pathway selection of motor axons.

## DISCUSSION

We used the well-characterized neuromuscular system of the zebrafish embryo to study pathway selection of motor growth cones. Our phenotypic analyses implicate the *unplugged* gene in a local, rather than a global mechanism specialized in motor axon guidance. We demonstrate that an exclusive set of adaxial cells in the zebrafish embryo provides *unplugged*-dependent signaling, essential for two different subtypes of motor growth cones to select their appropriate paths. To our knowledge, this represents a unique example in vertebrates where a group of identified somitic cells influences divergent growth cone decisions at a motor axon choice point. Our studies identify the *unplugged* gene as generating or encoding one of the first external cues required for pathway selection of motor axons. We propose a model in which *unplugged* activity governs pathway selection by eliciting different responses in CaP and RoP growth cones.

### The *unplugged* gene specifically controls CaP and RoP pathway selection

A total of seven recessive mutant *unplugged* alleles were isolated in a genetic screen for zygotic mutations affecting zebrafish embryogenesis and in a subsequent screen for non-complementation of the *unplugged* phenotype (Granato et al., 1996; van Eeden et al., 1999). Mutants of the different *unplugged* alleles are readily identifiable by their reduced motility between 26 and 48 hpf (Granato et al., 1996). Analysis of embryos from all seven ENU-induced *unplugged* alleles reveals variation in the penetrance of the axonal phenotype (Table 1; data not shown), possibly reflecting the presence of residual *unplugged* activity in mutant embryos. However, embryos carrying one ENU-induced allele, $unp^{tbr307}$, and the p4 allele in which the *unplugged* locus is entirely deleted, display a similar range of motor axon defects as observed in $unp^{tbr307}/unp^{tbr307}$ mutant embryos, indicating that the $unp^{tbr307}$ allele is a strong hypomorphic or amorphic allele (data not shown).

Analysis of axonal trajectories of identified spinal cord interneurons, sensory neurons and primary motor axons indicates that the *unplugged* gene specifically acts in the guidance decision of CaP and RoP growth cones at the choice point. In *unplugged* mutant embryos, axonal growth and pathfinding of all sensory and interneuronal cell types examined appear normal (e.g. Fig. 3A,B). Furthermore, in *unplugged* mutant embryos MiP motor neurons always select their appropriate path, while CaP and RoP motor neurons fail to make their appropriate pathway selection at the choice point (Fig. 2; Table 1). As in wild-type embryos, *unplugged* CaP growth cones navigate correctly to the choice point. However, *unplugged* mutant CaP growth cones stall at the choice point, while wild-type CaP growth cones extend into the ventral somite (Fig. 2; Table 1). Often mutant CaP growth cones extend multiple small branches at the region of the choice point, reminiscent of transient CaP branches observed in 20 hpf wild-type embryos (see Fig. 5 in Liu and Westerfield, 1990).

In wild-type embryos CaP motor neurons initially form one or two short branches at the region of the choice point, but these branches are later retracted (Liu and Westerfield, 1990; Myers et al., 1986; Westerfield et al., 1986). By 27 hpf, CaP branches at the choice point are not detectable in wild-type and *unplugged* sibling embryos (see Figs 2A, 4A), while they are present in *unplugged* mutant embryos (Fig. 2B). Thus, it is possible that, in mutant embryos, transient CaP branches at the choice point persist or even extend, reflecting a random search for alternative cues in the absence of *unplugged*-dependent guidance.

While wild-type RoP growth cones always stop and pause at the choice point, in *unplugged* mutant embryos, RoP growth cones explore the region around the choice point or extend far ventrally into the somite. Thus, *unplugged* activity enables CaP growth cones to pioneer the ventral somite path and prevents RoP growth cones from inappropriately crossing the choice point. Our results demonstrate that the *unplugged* gene specifically controls growth cone behavior of CaP and RoP neurons at the choice point. Moreover, the *unplugged* mutant phenotype suggests that pathway selection of primary motor neurons requires different cues at the choice point. While CaP and RoP neurons depend on *unplugged* activity to make their appropriate guidance decisions, MiP neurons appear to utilize a separate, yet unknown signaling mechanism, to direct the MiP growth cone towards the dorsal somite.

### The role of mesodermal cells in motor pathway selection

En route to their synaptic partners, pioneering growth cones encounter specialized cells that serve as intermediate targets or flank choice points. Specialized cells at the CNS midline provide a well-studied repertoire of cues controlling both axonal guidance towards the midline and pathway selection at the choice point (for reviews see: Chien, 1998; Flanagan and Van Vactor, 1998; Terman and Kolodkin, 1999; Van Vactor and Flanagan, 1999). In contrast, the guidance capacity of cells at choice points along motor pathways is less clear (Tosney, 1991). In the chick embryo, the plexus region at the base of the limb bud serves as an intermediate target, where individual motor neuron populations make their first pathway selection (Lance-Jones and Landmesser, 1981; Tosney and Landmesser, 1985a,b). In the absence of all limb bud tissue distal to the plexus, motor growth cones correctly navigate through the plexus region, suggesting that the cues for specific pathway decisions are target independent and, thus, may reside within the plexus region (Tosney and Landmesser, 1984). In the zebrafish, all primary motor growth cones contact a group of muscle pioneer cells at the choice point, suggesting that these cells may provide important signals for proper pathway selection. However, in somitic hemisegments in which all muscle pioneer cells were ablated, CaP and MiP growth cones select their appropriate pathways (Melançon et al., 1997). These results raise the intriguing possibility that other cells near the choice point are important for zebrafish motor neuron pathway selection. In summary, many studies suggest that cells at motor choice points provide cues for pathway selection; however, the identity of such cues and the mechanisms by which they control pathway selection have remained elusive.

Our analyses of chimeric embryos revealed that only a selective group of adaxial cells is capable of restoring *unplugged*-dependent pathway selection. About 20 adaxial cells are present in each somitic hemisegment. They first elongate to span the entire length of the somite and then migrate radially through the somites to form a single layer of superficial myotomal cells (Devoto et al., 1996). A subset of adaxial cells, the muscle pioneer cells, do not migrate radially, but extend from the notochord to the surface of the myotome, at the level of the future choice point (Devoto et al., 1996). Consistent with previous studies in which laser ablation of muscle pioneers did not severely affect pathway selection (Melançon et al., 1997), muscle pioneer cells were unable to rescue *unplugged*-dependent pathway selection (Table 2B). Adaxial cells capable of restoring pathway selection in *unplugged* mutants were confined to a dorsal domain encompassing 6 to 7 cells, adjacent to muscle pioneer cells and the choice point (Fig. 6).

Our chimeric analysis of the *unplugged* gene identifies exactly the same set of adaxial cells that are crucial for motor axon guidance towards the choice point (Zeller and Granato, 1999). We had previously shown that in *diwanka* mutant embryos, CaP, MiP and RoP growth cones failed to migrate along their common path from the spinal cord to the choice point and that *diwanka* activity is provided by a defined set of adaxial cells (Zeller and Granato, 1999). Our chimeric analysis of the *unplugged* gene identifies the same group of adaxial cells to be important for pathway selection. While the presence of only one or two wild-type-derived adaxial cells is sufficient to rescue the axonal *diwanka* phenotype, at least three of the six or seven adaxial cells had to be of wild-type origin to correct the *unplugged* phenotype. Our previous results showed that these adaxial cells prefigure the common path to the choice point (Zeller and Granato, 1999). As the first motor growth cone approaches, these adaxial cells migrate away, suggesting that they do not simply provide a substratum for migrating growth cones, but provide a source of guidance information (Zeller and Granato, 1999). Together, our results define two important roles for dorsal adaxial cells in controlling growth cone behavior. They provide local guidance information required for all primary motor growth cones to exit the spinal cord and to migrate towards the choice point (Zeller and Granato, 1999). Secondly, they control pathway selection of both CaP and RoP growth cones at the choice point. Thus, dorsal adaxial cells adjacent to the choice point might play dual roles in axonal guidance, similar to specialized cells governing growth cone behavior at the CNS midline.

### How does *unplugged* gene activity control pathway selection?

We demonstrate that pathway selection of CaP and RoP growth cones at the choice point is guided by *unplugged* gene activity emanating from an identified group of mesoderm-derived adaxial cells. These adaxial cells delineate the common motor path before growth cone extension and start their radial migration as the first motor growth cone (CaP) pioneers the common path (Zeller and Granato, 1999). By the time RoP growth cones arrive at the choice point, these adaxial cells have reached the lateral surface of the somite. Based on these observations, we conclude that adaxial cells do not influence pathway selection through extensive cell-cell contacts with CaP and RoP motor growth cones, but might provide a source of guidance information, enabling motor axons to select their appropriate paths. What information could this be?

We can exclude several simple models. First, *unplugged* activity might exclusively control CaP pathway selection while RoP pathfinding defects are only a secondary consequence of CaP growth cones failing to select their appropriate path. For example, in the absence of CaP growth cones invading the ventral somite, RoP growth cones might extend into this territory simply because it is vacant. This is unlikely, because in the absence of CaP neurons, RoP growth cones select their appropriate path (Eisen et al., 1989; Pike and Eisen, 1990). Alternatively, *unplugged* might play a role in dorsoventral somite patterning. Such patterning might confer polarity to the ventral somite, rendering it permissive for CaP growth cones and repulsive to RoP growth cones. We find that *wnt11r* (Makita et al., 1998) and *twist* (Morin-Kensicki and Eisen, 1997) mRNA, which are both predominately localized to the ventral somite of wild-type embryos, are properly expressed in *unplugged* mutant embryos, suggesting that *unplugged* does not affect overall somite polarity (data not shown). Finally, our experiments show that CaP and RoP pathway selection requires *unplugged* gene activity, provided by a selective group of adaxial cells. This raises the possibility that mutations in the *unplugged* gene affect specification or migration of these adaxial cells, thereby affecting pathfinding of CaP and RoP motor axons. Analysis of two different adaxial cell-specific markers (prox-1, Glasgow and Tomarev, 1998; and F59), did not reveal any difference in the total number of adaxial cells or in their radial migration pattern, suggesting that in *unplugged* mutant embryos proliferation, migration and specification of adaxial cells are unaffected (data not shown).

How, then, does *unplugged* influence CaP and RoP pathway selection at the choice point? Our results show that adaxial cells located dorsal to the choice point are responsible for *unplugged*-dependent pathway selection, suggesting that *unplugged* activity influences CaP and RoP growth cones before they reach the choice point and make their pathway decision. One attractive model that takes these findings into account is that the dorsal adaxial cells adjacent to the choice point deposit *unplugged* activity on the extracellular matrix of the overlying somite surface (Fig. 7A). This *unplugged* activity generates or functions as a bifunctional cue, eliciting different responses in CaP and RoP growth cones (Fig. 7B). Consistent with our results, *unplugged* activity may instruct CaP growth cones to extend into the ventral somites while preventing RoP growth cones from inappropriately crossing the choice point (Fig. 7C). Such bifunctionality could be achieved if *unplugged* activates a single receptor mechanism that is coupled to different downstream effectors present in CaP and RoP growth cones. Alternatively, *unplugged* could activate different types of receptors in CaP and RoP growth cones. From biochemical and genetic studies, both types of mechanisms have been shown to operate (Hong et al., 1999 and references therein). Regardless of the exact mechanism, mutations in the *unplugged* gene abolish its bifunctional effects and consequently the activation of downstream effectors within CaP and RoP growth cones. As a result, *unplugged* CaP growth cones are not directed into the ventral somite and stall at their intermediate target (Fig. 7D). Similarly, RoP growth cones are not restricted to the choice point and explore adjacent regions (Fig. 7D). Thus, it is conceivable that *unplugged* is part of a somite-derived signaling mechanism evoking differential pathway decisions in CaP and RoP growth cones.

Our studies demonstrate that a selective group of mesodermal cells provides *unplugged*-dependent guidance at a motor pathway choice point. To our knowledge, this analysis of the *unplugged* mutant phenotype provides one of the few examples demonstrating the potential of external cues in controlling pathway selection at motor neuron choice points. Our analysis does not allow us to distinguish if *unplugged* acts indirectly (e.g. as transcriptional regulator in adaxial cells), or directly with CaP and RoP growth cones (e.g. as a secreted cue). To address this question, we have undertaken a positional cloning approach to identify the gene mutated at the *unplugged* locus, which ultimately will reveal the mechanism by which *unplugged* activity guides the decision of motor growth cones at the choice point.

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