

The zebrafish *diwanka* gene controls an early step of motor growth cone migration

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SUMMARY

During vertebrate embryogenesis different classes of motor axons exit the spinal cord and migrate on common axonal paths into the periphery. Surprisingly little is known about how this initial migration of spinal motor axons is controlled by external cues. Here, we show that the *diwanka* gene is required for growth cone migration of three identified subtypes of zebrafish primary motoneurons. In *diwanka* mutant embryos, motor growth cone migration within the spinal cord is unaffected but it is strongly impaired as motor axons enter their common path to the somites. Chimera analysis shows that *diwanka* gene activity is required in a small set of myotomal cells, called adaxial cells. We identified a subset of the adaxial cells to be

sufficient to rescue the *diwanka* motor axon defect. Moreover, we show that this subset of adaxial cells delineates the common axonal path prior to axonogenesis, and we show that interactions between these adaxial cells and motor growth cones are likely to be transient. The studies demonstrate that a distinct population of myotomal cells plays a pivotal role in the early migration of zebrafish motor axons and identify the *diwanka* gene as a somite-derived cue required to establish an axonal path from the spinal cord to the somites.

Key words: Spinal cord, Motor axon, Axon guidance, Neural development, Adaxial cells, Somite, Zebrafish

INTRODUCTION

The nervous system and the body musculature are connected by centrally located motor neurons whose growth cones exit the central nervous system during embryogenesis and migrate to their appropriate muscle targets. A striking feature of this pathfinding process in vertebrates and in insects is that different populations of motor growth cones initially share common axonal paths. For example, in the *Drosophila* embryo, distinct motoneuron populations with dorsal or ventral muscle destinations project their axons on a common path, the intersegmental nerve, into the periphery (Landgraf et al., 1999; Thor and Thomas, 1997). Similarly, in vertebrates different subtypes of motoneurons extend through a common, segmental nerve to the adjacent somite (Appel et al., 1995; Sharma et al., 1998; Tsuchida et al., 1994). Despite substantial information regarding intrinsic factors controlling motoneuron subtype specification and target specificity (for reviews see Appel, 1999; Eisen, 1998; Pfaff and Kintner, 1998), external cues controlling the initial migration of vertebrate motor growth cones into the periphery remain largely unknown.

The zebrafish embryo is an excellent model system in which to study the cellular and genetic mechanisms controlling motor axon pathfinding (for review see Eisen, 1994, 1998). As in other vertebrates, spinal motoneurons with distinct identities and targets project their axons on common, segmentally reiterated nerves into the periphery (Eisen et al., 1986; Myers

et al., 1986; Westerfield et al., 1986). The segmental motor nerve is shared by axons from primary motoneurons, which pioneer the nerve path, and by axons from secondary motoneurons, which develop later and whose growth cones extend along axons of primary motoneurons (Eisen et al., 1986; Myers et al., 1986; Westerfield et al., 1986). Each somitic hemisegment is innervated by three primary motoneurons: the caudal primary (CaP), middle primary (MiP) and rostral (RoP) primary neurons (Fig. 1A). Each of these primary motoneurons can be identified by their stereotyped soma position in the spinal cord, their unique combinatorial expression of LIM homeobox genes and their stereotypic axonal trajectories (Appel et al., 1995; Eisen et al., 1986). Axonogenesis of CaP, MiP and RoP neurons to their axial muscle targets occurs in a well-documented sequence (Eisen et al., 1986). CaP growth cones are the first to pioneer a common path from the spinal cord to the nascent horizontal myoseptum. Within 2 hours after CaP neurons start axonogenesis, MiP and then RoP growth cones migrate caudally within the spinal cord towards the CaP soma (Eisen et al., 1989). When they encounter the CaP axon they turn ventrally, exit the spinal cord and follow on the medial surface of the somites, the path taken by the CaP growth cone (Bernhardt et al., 1998; Eisen et al., 1986; Fig. 1B). Once they reach the distal end of the common path, termed the choice point, all motor growth cones pause before continuing on divergent paths to ventral, dorsal and medial myotomal regions (Fig. 1A). Although CaP axons pioneer the common path,

elegant laser ablation experiments have shown that each of the three motor axons has the potential to pioneer the common path and to select their appropriate cell-type-specific pathway (Eisen et al., 1989; Pike and Eisen, 1990).

We used a genetic approach in the zebrafish to identify cues essential for spinal motor axon pathfinding. In a large genetic screen, we previously identified a unique collection of mutants with presumed defects in the neural circuitries underlying coordinated body movements (Granato et al., 1996). We screened this mutant collection with a motor-axon-specific antibody for axonal pathfinding defects (M.G., unpublished results). Here, we show that embryos mutant for the *diwanka* gene display migration defects specific for motor axons. We focused on the development of primary motor axons because of the ease with which individual primary motor axons can be anatomically identified and because they pioneer the peripheral motor nerves (Eisen et al., 1986; Myers et al., 1986; Westerfield et al., 1986). In *diwanka* mutant embryos, primary motor axons are able to navigate within the spinal cord, but fail to project on the common path from the spinal cord to the somites. Analysis of chimeric embryos shows that *diwanka* gene activity is required in a distinct group of myotomal cells, the adaxial cells. We demonstrate that adaxial cells delineate the future common path just before the first motor growth cone pioneers this path and they migrate away as the first growth cones approach. We propose that the *diwanka* gene functions as an adaxial-cell-derived signal, required for motor growth cones to establish the segmental motor nerve path.

MATERIALS AND METHODS

Fish maintenance and breeding

Zebrafish were raised and maintained as described by Mullins et al. (1994). All experiments were performed using the *diw^{iv205a}* allele in the Tübingen or the TL, AB genetic background.

Blastomere injections for motor axon labeling

Injection of blastomeres was performed as previously described by Myers et al. (1986), with the following modifications. Embryos in their chorions were placed in agar molds (1.5%) and injected between the 16- and 128-cell stage with a mixture of 2.5% biotin-dextran and 2.5% rhodamine dextran (both M_r 10,000; Molecular Probes) in 0.1 M KCl using a pressure injector. At 26 hours of development *diwanka* mutant embryos were sorted by their reduced motility (Granato et al., 1996) and fixed in 4% paraformaldehyde. Fixed embryos were washed several times with phosphate buffer (0.1 M, pH 7.4), dehydrated through a MeOH series (10%/30%/50%/70%/90%/100%), permeabilized for 5 minutes in acetone at -20°C , and rehydrated with incubation buffer (0.2% BSA, 0.5% Triton in 0.1 M phosphate buffer, pH 7.4). Biotin-labeled cells were detected using the ABC Vectastain kit (Vector Laboratories according to manufacturer's instructions) with 0.15 mg/ml diaminobenzidine and 0.001% H_2O_2 .

Antibody staining

Embryos were fixed in 4% paraformaldehyde, dehydrated through a MeOH series (see above), permeabilized for 5 minutes in acetone at -20°C , and rehydrated directly with incubation buffer (0.2% BSA, 0.5% Triton in 0.1 M phosphate buffer, pH 7.4). 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 Dr T. Jessell), prox-1 (1:2000; Glasgow and Tomarev, 1998; a gift from S. Tomarev), and F59 (1:10; Crow and Stockdale, 1986; Devoto et al., 1996; kindly provided by Dr F. Stockdale). Embryos were incubated

for 16 hours with diluted primary antibody, and the bound antibody was detected using the ABC Vectastain kit and the SG substrate kit (both Vector Laboratories, according to manufacturer's instructions). For double labeling with znp-1 and F59, znp-1 immunoreactivity was first visualized using diaminobenzidine with 0.15 mg/ml diaminobenzidine and 0.001% H_2O_2 , resulting in a brown precipitate. Stained embryos were fixed for 1 hour in 4% paraformaldehyde, incubated with diluted F59 supernatant, and immunoreactivity was then detected with the ABC Vectastain kit and the SG substrate kit, resulting in a blue precipitate. Stained embryos were dehydrated, viewed and documented as described below.

Whole-mount in situ hybridization

In situ hybridization was performed as previously described in Odenthal and Nusslein-Volhard (1998). The following probes were used: *islet-1*, *islet-2*, *lim-3* (Appel et al., 1995; kindly provided by Dr I. Dawid), *sonic hedgehog* (Krauss et al., 1993) and *type II collagen* (Yan et al., 1995). Stained embryos were dehydrated, viewed and documented as described below.

Chimeric embryos

To generate chimeric embryos, cell transplantations between wild-type and mutant embryos were performed as previously described (Ho and Kane, 1990; van Eeden et al., 1996), with modifications as described in Nguyen et al. (1998). Donor embryos for the transplants were injected with the vital dye rhodamine-dextran (M_r 10,000; Molecular Probes) and the fixable tracer biotin-dextran (M_r 10,000; Molecular Probes; each 2.5% in 0.1 KCl). Cells were transplanted before shield stage and *diwanka* mutant embryos were identified at 26 hpf by their reduced motility (Granato et al., 1996). Biotin-labeled cells were detected using the ABC Vectastain kit (Vector Laboratories according to the manufacturer's instructions) with 0.15 mg/ml diaminobenzidine and 0.001% H_2O_2 . To detect the biotin-labeled transplanted cells and motor axons simultaneously, embryos were refixed for 1 hour at room temperature in 4% paraformaldehyde and incubated with znp-1 antibody (1:200). znp-1 immunoreactivity was then detected using the ABC Vectastain kit, but using the SG substrate kit (Vector Laboratories according to manufacturer's instructions), resulting in a blue precipitate. Stained embryos were dehydrated, viewed and documented as described below (microscopy of stained embryos). To visualize chimeric cells, motor axons and adaxial cells simultaneously, donor embryos were injected with 5% fixable rhodamine-dextran (M_r 10,000; Molecular Probes). Chimeric embryos were fixed and incubated with znp-1 antibody (1:200), which was detected using the ABC Vectastain kit and the SG substrate, resulting in a blue precipitate. Embryos were then incubated with F59 supernatant (1:10). F59-positive cells were detected using a fluorescein-conjugated anti-mouse antibody (Jackson Laboratories).

Microscopy of stained embryos

Stained embryos were dehydrated through a MeOH series (10%/30%/50%/70%/90%/100%), cleared in a mixture of benzyl benzoate and benzyl alcohol (2:1), mounted in Canada Balsam/methyl salicylate (10:1) and viewed using Nomarski optics on a Zeiss Axiophot microscope. 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 confocal microscope (Zeiss).

RESULTS

Mutations in *diwanka* cause motor axon defects

Three independent *diwanka* alleles were isolated in a genetic screen for zygotically affecting zebrafish embryogenesis

(Granato et al., 1996). Mutant *diwanka* embryos can readily be identified at 23 hours post fertilization (hpf) by their motility defect (Granato et al., 1996). All three mutant alleles are embryonic lethal mutations and display similar phenotypes (J. Z. and M. G., unpublished observation). We used the monoclonal antibody *znp-1* to examine motor axonal trajectories in *diwanka* mutants. In 23 hpf wild-type embryos, the *znp-1* antibody labels the common axonal path of CaP, MiP and RoP from the spinal cord to the choice point, as well as the cell-type-specific projections of CaP and MiP axons into ventral and dorsal somite territories, respectively (Melançon et al., 1997; Trevarrow et al., 1990; Fig. 1C: RoP axons migrate later along their rostral-caudal cell-type-specific path). At 23 hpf in over 90% of the somitic hemisegments examined, wild-type primary motor axons have completed the common path and extended along their cell-type-specific path (segments 6-15, $n=230$, Fig. 1C). In contrast, in *diwanka* mutant embryos, *znp-1*-positive motor axons were abnormal in over 95% of the somitic hemisegments ($n=230$). *diwanka* mutant embryos displayed a range of phenotypes, from the absence of motor growth cones on the common path (Fig. 1D), to growth cones stalled along the common path (Fig. 1E) and growth cones projecting beyond the distal end of the common path (Fig. 1F). From this analysis, it is apparent that migration of *znp-1*-positive motor axons is defective, suggesting that the *diwanka* gene plays an important role in motor axon development. To study the mutant phenotypes in more detail we examined the axonal trajectories of individual motoneurons (see below).

Motoneuron differentiation and survival as well as notochord and floorplate development appear unaffected in *diwanka* mutants

To determine if the axonal phenotype in *diwanka* mutant embryos is caused by lack of cell fate specification or progressive degeneration of motoneurons, we examined cell death, as well as the expression of several motoneuron-specific LIM domain genes, such as *islet-1*, *islet-2* and *lim-3* mRNA (Appel et al., 1995) in wild-type and mutant embryos. As shown in Fig. 2, expression of *islet-1* mRNA in *diwanka* MiP and RoP motor neurons was indistinguishable from wild-type siblings (Fig. 2A,B). Similarly, no differences were observed between the

expression pattern of *islet-2* or *lim-3* in wild-type and *diwanka* mutant embryos (data not shown). In addition, cell death assays in wild-type and *diwanka* mutant embryos did not reveal significant differences in the amount or pattern of cell death (data not shown). We also examined two surrounding tissues crucial for normal development of spinal motoneurons, the notochord and the floorplate (Beattie and Eisen, 1997; Beattie et al., 1997; Yamada et al., 1993). Analysis of molecular markers expressed in the notochord and the floorplate, such as *sonic hedgehog* (Krauss et al., 1993; data not shown) and *type*

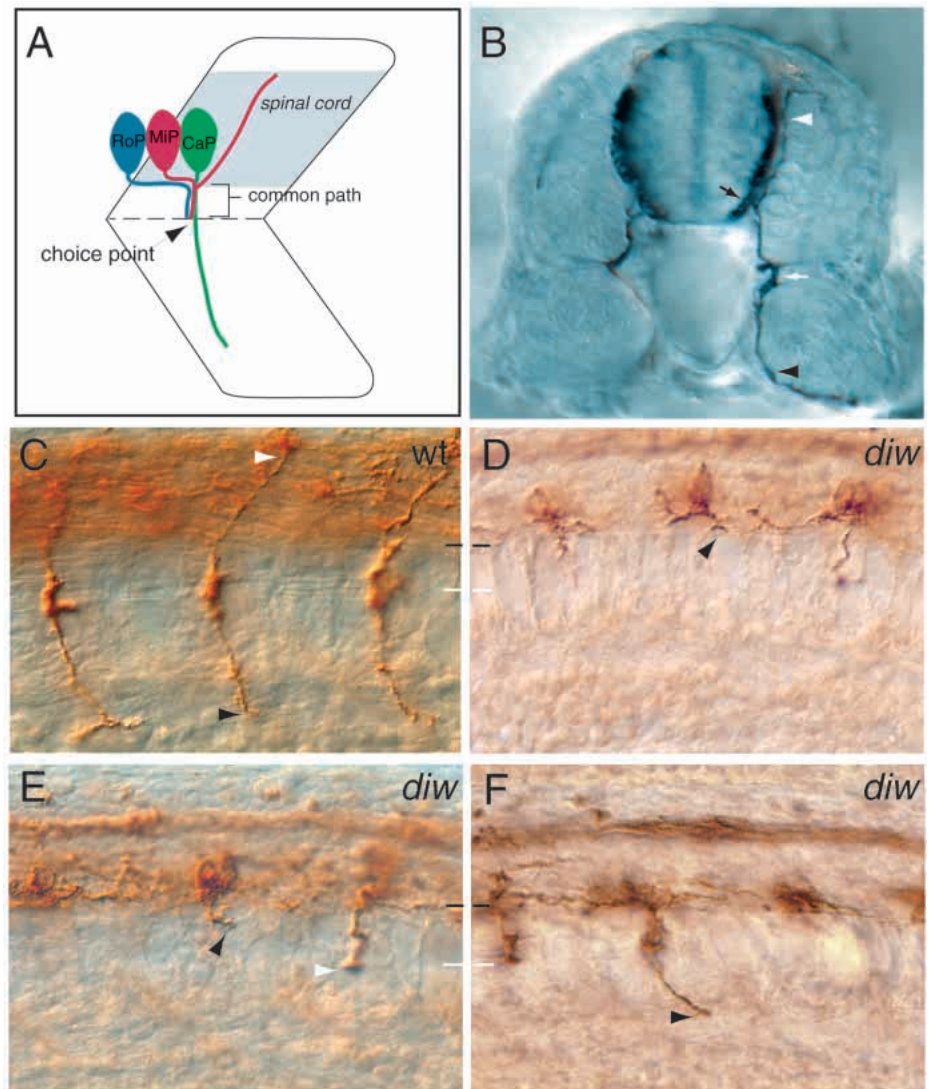


Fig. 1. *diwanka* mutant embryos display severe motor axon defects. (A) Schematic drawing of a spinal hemisegment showing CaP, MiP and RoP neurons and their axonal projection (lateral view). (B) Cross-section through a 28 hpf embryo stained with the *znp-1* antibody. Motor growth cones exit the spinal cord (black arrow) and migrate on the medial surface of the somites to the ventrally located choice point (white arrow) at the horizontal myoseptum. CaP (black arrowhead) axons continue further ventrally (white arrowhead), whereas MiP neurons form a branch to the dorsal somite (white arrowhead). Wild-type (C) and *diwanka* mutant (D-F) embryos at 23 hpf stained with *znp-1* antibody. The common path extends between the lower end of the spinal cord (black line) and the horizontal myoseptum (white line). (C) Wild-type CaP (black arrowhead) and MiP (white arrowhead) axons have completed the common path and extended on their cell-type-specific paths. (D-F) Arrowheads point to *diwanka* mutant motor axons which failed to exit the spinal cord (D), stalled along the common path (black arrowhead in E), stalled at the distal end of the common path (white arrowhead), or have extended on the cell-type-specific path (F).

II collagen (Yan et al., 1995) showed that development of these tissues appeared unaffected in *diwanka* mutant embryos (Fig. 2C,D). Thus, analysis of markers specific for the notochord, floorplate and spinal motoneurons indicates that *diwanka* functions in axonal migration rather than in specification or maintenance of the motoneuronal fate.

***diwanka* exhibits functions specific for spinal motor axons**

To investigate if mutations in the *diwanka* gene affect axonal projections throughout the nervous system or specifically affect motor axons, axonal trajectories of various neuronal cell types ($n=5$) were examined. Wild-type and mutant embryos ($n=10$) were stained at 28 hpf with an anti-acetylated tubulin antibody (Piperno and Fuller, 1985), which recognizes most if not all axonal projections in the zebrafish embryo (Bernhardt et al., 1990), and an anti-neurofilament antibody (3A10, a gift from Dr T. M. Jessell), which recognizes a subset of hindbrain neurons and commissural interneurons in the spinal cord (Hatta, 1992). Immunohistochemical analysis of spinal CoPA, DoLA, RB and CoSA axons (Bernhardt et al., 1990; Kuwada and Bernhardt, 1990) and hindbrain Mauthner axons (Metcalfe et al., 1986) revealed that all axonal projections examined in *diwanka* embryos appear indistinguishable from those in wild-type embryos (Fig. 2 and data not shown). For example, 3A10-positive commissural interneurons in the spinal cord extend to the ventral midline, cross the midline and ascend on the contralateral side of the spinal cord in *diwanka* mutant embryos, indistinguishable from those in wild-type embryos (Fig. 2E,F). We conclude that the *diwanka* gene, rather than participating in general axonogenesis, exhibits a function specific for motor axons.

***diwanka* is required for CaP, MiP and RoP axons to migrate from the spinal cord towards the somites**

In *diwanka* mutant embryos, a significant number of *znp-1*-positive motor axon trajectories were completely absent (Fig. 1D). We reasoned that if longitudinal migration of MiP and RoP growth cones within the spinal cord are affected, then *diwanka* is likely required for motor axon migration both within and outside the spinal cord. Alternatively, if longitudinal migration within the spinal cord is unaffected, then this would suggest that *diwanka* is required specifically for the migration of motor axons along their common path, from the spinal cord to the choice point. To distinguish between these two possibilities, we examined the entire

axonal trajectories of CaP, MiP and RoP motoneurons in wild-type and mutant embryos.

Individual blastomeres at the 32- to 128-cell stage were injected with fixable dyes, and the resultant clones of labeled cells were assayed at 26 hpf for the presence of primary motoneurons (see Materials and Methods). Labeling of CaP, MiP and RoP neurons in wild-type embryos revealed their well-described stereotypic axonal trajectories, in particular the distinct longitudinal path of RoP growth cones within the spinal cord (Fig. 3A-C; Eisen et al., 1986; Myers et al., 1986; Westerfield et al., 1986). Labeling of RoP motor axons ($n=19$) in *diwanka* mutant embryos demonstrated that longitudinal migration of RoP axons inside the spinal cord was unaffected, whereas migration on the common path was strongly impaired ($n=4$) or, completely abolished ($n=15$; see Table 1 and Fig. 3G). Moreover, mutant RoP growth cones often missed the exit point and extended further caudally within the spinal cord, which we never observed in wild-type embryos (compare Fig. 3C to G). Labeling of CaP and MiP motor axons in *diwanka* mutant embryos showed that the longitudinal path of MiP axons within the spinal cord was unaffected, while migration

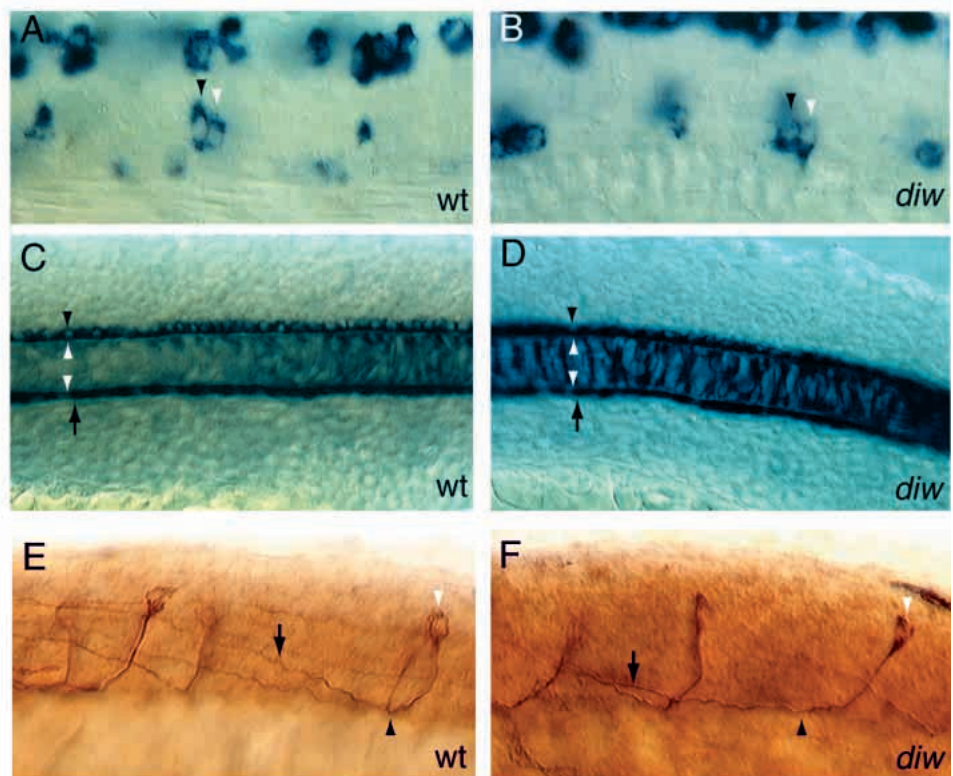


Fig. 2. Motoneuron specification, notochord and floorplate development and spinal axonal projections are not affected in *diwanka* mutant embryos. (A,B) *islet-1*-positive cells in the ventral spinal cord consistent with the position of RoP neurons (black arrowhead) and MiP neurons (white arrowhead) are present in wild-type and mutant embryos. CaP, MiP and RoP motoneurons were identified by their dorsoventral and rostrocaudal position within the spinal cord and their soma size (see Table 1 for details). (C,D) *type II collagen* expression in the notochord (delineated by white arrowheads), in the floorplate (between black arrowhead and upper white arrowhead) and hypochord (between arrow and lower white arrowhead) is indistinguishable between wild-type and mutant embryos, suggesting that these cell types are not affected by mutations in the *diwanka* gene. (E,F) In wild-type and mutant embryos 3A10-positive commissural spinal interneurons (white arrowhead) extend their axons ventrally to the midline (black arrowhead), cross the midline and ascend on the contralateral side (arrow). Multiple focal planes were compiled to follow the entire axonal projections.

Table 1. Motor axon defects in *diwanka* mutants

	Wild type				<i>diwanka</i>			
	CaP‡	MiP‡	RoP‡	Σ	CaP‡	MiP‡	RoP‡	Σ
Total number of labeled primary motoneurons*	21 100%	10 100%	21 100%	52 100%	34 100%	12 100%	19 100%	65 100%
Axons extending on their cell-type-specific path	20 95%	10 100%	21 100%	51 98%	4 12%	0 0%	0 0%	4 6%
Axons stalled before or at the choice point	1 5%	0 0%	0 0%	1 2%	22 65%	8 67%	4 21%	34 52%
Axons failed to exit the spinal cord	0 0%	0 0%	0 0%	0 0%	8 23%	4 33%	15 79%	27 42%

*Blastula-staged embryos were injected into a single blastomere with lineage tracer before the 128-cell stage. Labeled motoneurons in 216 mutant and 51 sibling embryos were examined.

‡CaP, MiP and RoP motoneurons 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, if present, (d) by their axonal trajectories. The only other ventral neurons present at 26 hpf are secondary motoneurons and VeLD neurons (Kuwada et al., 1990; 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 motoneurons can be distinguished by their smaller soma size (~1/2 the size of primary motoneurons, Myers et al., 1986) and the more ventral position of their somata in the cord (Myers et al., 1986). Although the VeLD neuron are located in the same region as the primary motoneurons, at 26 hpf, VeLD growth cone have extended up to 10 segments caudally (Kuwada et al., 1990).

of CaP and MiP along the common path was completely abolished or strongly affected (Table 1; compare Fig. 3A to E and B to F). Overall, more than 40% of the primary motoneurons in *diwanka* mutant embryos failed to exit the spinal cord and did not enter the common path. Together, these results strongly suggest that *diwanka* is not required for general motor axon outgrowth, but is essential for primary motor growth cones as they migrate from the spinal cord to the somites (Fig. 3D,H).

The medial surface of the somites on which the common motor axon path extends is also navigated by migrating neural crest cells (Jesuthasan, 1996; Raible and Eisen, 1994; Raible et al., 1992) and sclerotomal cells (Morin-Kensicki and Eisen, 1997). This raises the possibility that *diwanka* might function as a migration cue for such motile cells. To determine if mutations in the *diwanka* gene affect cell as well as axonal migrations, we analyzed neural crest cell migration in *diwanka* mutant embryos. In the zebrafish embryo, neural-crest-derived melanophores migrate on the medial surface of the somites and can readily be identified by their melanin expression (see Fig. 5 in Raible and Eisen, 1994). When compared to wild-type embryos ($n=10$, data not shown), the number and migration of melanophores appeared unaffected in *diwanka* mutant embryos ($n=10$, data not shown). This indicates that *diwanka* does not simply provide a permissive cue on the medial surface of the somites for the migration of axons and neural crest cells.

The *diwanka* gene acts cell non-autonomously in somitic cells

In order to define the biological mechanism of motor growth cone migration mediated by *diwanka* gene activity, it was of great interest to identify the cell type(s) providing *diwanka* gene activity. Axonal pathfinding requires the interaction of the migrating growth cone with its environment (Goodman and Tessier-Lavigne, 1996). Accordingly, molecules involved in this process can be subdivided into two large groups, depending on where they function: in/on migrating axons or in/on surrounding tissues. The question of where gene activity is required can be addressed in the zebrafish embryo through

the analysis of chimeric embryos (Ho and Kane, 1990; Materials and Methods). To generate chimeric embryos, we transplanted genotypically wild-type, dye-labeled cells at the blastomere stage into embryos derived from two heterozygous *diwanka* fish. At 26 hpf, *diwanka* mutant embryos were identified by their motility phenotype and scored for the presence of labeled, genotypically wild-type primary motoneurons. When genotypically wild-type primary motoneurons developed in mutant host embryos they formed mutant motor axonal trajectories ($n=27$, Table 2; Fig. 4A). In the converse experiment, when genotypically mutant motoneurons developed in wild-type host embryos, they formed wild-type-like axonal trajectories ($n=8$, Fig. 4B). These results demonstrate that *diwanka* functions cell non-autonomously.

To identify which cell type(s) provides *diwanka* activity, we assessed the potential of various genotypically wild-type cell types, by themselves or in combinations, to restore motor axonal trajectories in *diwanka* mutant embryos. To perform this analysis, it was necessary to generate about 300 chimeric *diwanka* mutant embryos containing transplanted, genotypically wild-type cells, as described above. At 26 hpf, *diwanka* mutant embryos were identified by their motility phenotype and stained with the motor-axon-specific antibody znp-1. We then correlated the distribution of genotypically wild-type cells with their ability to rescue mutant motor trajectories. We chose to focus our analysis on the CaP trajectories, because the znp-1 antibody stains CaP axons more consistently than MiP and RoP axons. As summarized in Table 2, the presence of genotypically wild-type cells in most tissues located in the vicinity of motoneurons, including floorplate (Fig. 4C), notochord (Fig. 4D) and neural crest cells was not sufficient to rescue the axonal defect. Even when genotypically wild-type cells populated combinations of tissues, such as the notochord and floorplate ($n=6$, Table 2), we did not observe rescue of motor axon projections.

In contrast, in 62% of the cases ($n=24$, Table 2) in which wild-type cells were present in medial and lateral regions of the somites, CaP trajectories were indistinguishable from wild-

type CaP axons. When genotypically wild-type cells were confined to mostly medial regions of the somites, we observed a mutant axonal phenotype in the majority of the hemisegments (Fig. 4F; Table 2). Moreover, when wild-type cells were restricted to only the lateralmost region of the somites, CaP axons were rescued in 80% of the cases ($n=10$; Table 2; Fig. 4E). In 30% of the hemisegments in which we observed rescue of the CaP axon, we also observed completely rescued MiP axons, indicating that the ability of lateral somitic cells to restore migration of primary motor axons was not restricted to CaP neurons. Together, this shows that motor growth cones require *diwanka* activity in cells predominantly located in the lateral portion of the somite.

The *diwanka* gene is required in a subset of adaxial cells for motor axon migration

The position of cells able to rescue the axonal phenotype in *diwanka* mutant embryos suggested that they might be adaxial cells. These cells constitute one of the two populations of muscle cells present in the segmental plate (Devoto et al., 1996; Thisse et al., 1993; Weinberg et al., 1996). Initially, adaxial cells are the most medial cells in the segmental plate, arranged in columns along both sides of the notochord (Devoto et al., 1996; Thisse et al., 1993; Weinberg et al., 1996). After somite formation, individual adaxial cells move either dorsally or ventrally to form a single layer along the medial surface of the somite (Devoto et al., 1996). Most adaxial cells migrate radially through the myotome and form a lateral, superficial layer of slow muscle fiber cells (Devoto et al., 1996; Van Raamsdonk et al., 1978). Only a subset of adaxial cells, the muscle pioneers, does not migrate radially, but extends from the notochord to the surface of the myotome, at the level of the future horizontal myoseptum (Devoto et al., 1996).

We generated a second set of chimeric mutant embryos to determine if the lateral somitic cells that rescued the *diwanka* motor axonal phenotype were differentiated adaxial cells. In these chimeras, we examined simultaneously the distribution of genotypically wild-type cells (using a fluorescent lineage marker), motor axon projections (using the *znp-1* antibody) and adaxial cells (using the adaxial-specific marker F59; Devoto et al., 1996). In hemisegments in which motor trajectories were restored, we detected colocalization between the lineage marker (in red) and the adaxial-cell-specific marker F59 (in green; $n=11$; Fig. 4G,I). In contrast, in hemisegments in which all adaxial cells were genotypically mutant for *diwanka* but the majority of the remaining somitic cells were genotypically wild type, the axonal phenotype was not rescued ($n=40$; Fig. 4H,J). These results demonstrate that those cells providing *diwanka* gene activity to migrating motor axons are adaxial cells.

We analyzed the number and spatial distribution

of double-labeled cells to investigate whether the rescue of the motor axon defect was mediated by adaxial cells with a particular distribution. In wild-type embryos, about 20 adaxial cells form a superficial layer along the dorsoventral axis of each myotome (Devoto et al., 1996). About five of these adaxial cells are located between the ventral part of the spinal cord and the horizontal myoseptum, which delimits the

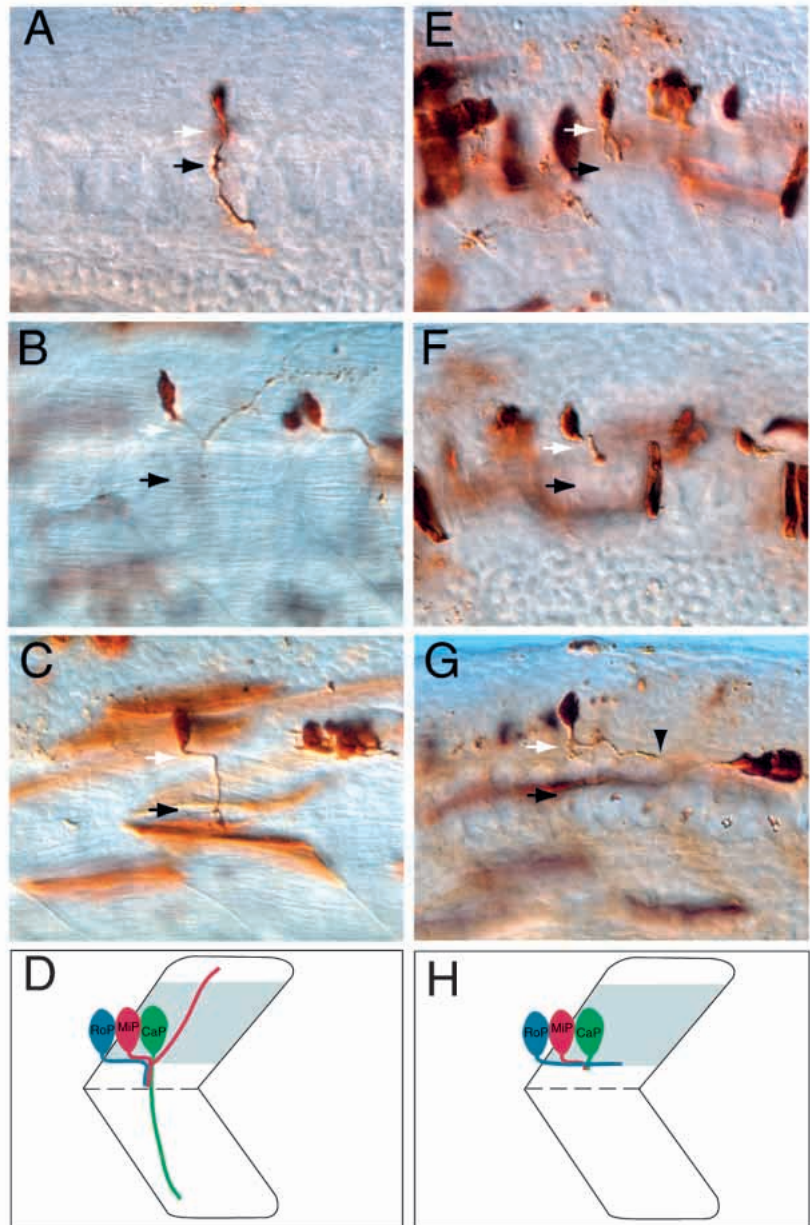


Fig. 3. *diwanka* is required for motor axon migration from the spinal cord to the somites. Stereotypic axonal projections of CaP (A), MiP (B) and RoP (C) neurons in wild-type embryos. White arrows point to the lower end of the spinal cord (out of focal plane) and black arrows point to the choice point. In *diwanka* mutants, all CaP axons (E) and MiP axons (F) extend normally within the spinal cord, but are severely affected as they migrate on the common path (black arrow). (G) All RoP axons in *diwanka* mutants complete the longitudinal path within the spinal cord, but most fail to exit the spinal cord and instead project further caudally within the spinal cord (arrowhead points to the growth cone). Note that the longitudinal projection of the mutant RoP axon is about twice the length of the corresponding projection of wild-type RoP in Fig. 4C.

Table 2. Analysis of chimeric embryos

Motoneurons			noto	flp	not & flp	epi.	neu. crest	somite m*	somite m&l*	somite l*	musc. pion.
CaP	MiP	RoP									
0/11	0/9	0/7	0/23	1/105	6	1/42	0/45	6/41	15/24	8/10	2/33
0%	0%	0%	0%	1%	0%	2.3%	0%	14.6%	62%	80%	6%

A total of 301 chimeric embryos were analyzed at 26 hpf, whereby *n* indicates the number of rescued CaP motor axon trajectories per number of events in which genotypically wild-type cells contributed to the indicated tissue/cell type. Only cell types that could be identified unambiguously by their morphology and/or position are considered here and are abbreviated as follows: CaP, caudal primary; MiP, middle primary; RoP, rostral primary; noto, notochord; flp, floorplate; epi, epidermal; neu.crest, migrating trunk neural crest; somite m, medial somite; somite m & l, medial and lateral somites; somite l, lateral somites; musc. pion., muscle pioneers.

*A total of 229 somitic wild-type clones were identified, of which 75 were sectioned in order to analyze the spatial distribution of the clones within the somites. Only cases in which *znp-1*-positive CaP and/or MiP motor axons had extended on their cell-type-specific pathways similarly to wild-type motor axons were considered as rescued. The *znp-1* antibody has been shown to also stain secondary motoneurons (Melançon, 1994), which project on the paths pioneered by primary motor axons. However, at 26 hpf secondary motor axon in somitic segments 5-16 of wild-type embryos had not extended along CaP or MiP cell-type-specific pathways. We observed wild-type-like CaP and/or MiP motor axon projections without observing any wild-type cell within the same segment in 2% of the somitic segments examined. This is comparable to the frequency with which wild-type-like projections are observed in homozygous mutant *diwanka* embryos.

proximal and distal ends of the common motor path. During radial migration, adaxial cells maintain their dorsoventral positions (Devoto et al., 1996) and, at the time point of our chimeric analyses (26 hpf), they have formed a superficial layer of slow muscle fiber cells (Fig. 4). By confocal microscopy, we scored the number and positions of cells that were positive for the lineage tracer (transplanted, genotypically wild-type cells) and for the adaxial-specific marker F59. In all except one case (*n*=10), motor axonal trajectories were restored when one to three F59-positive adaxial cells of wild-type origin were located between the ventral part of the spinal cord and the horizontal myoseptum (Fig. 4G). Because the dorsoventral positions of adaxial cells are maintained during radial migration (Devoto et al., 1996), we conclude that, prior to their radial migration, adaxial cells providing *diwanka* activity are likely to delineate the common motor path.

Adaxial cell are in close proximity to migrating motor growth cones

Our experiments show that adaxial cells provide *diwanka* activity, enabling motor axons to establish a common axonal path from the spinal cord to the somites. We therefore reasoned that adaxial cells should be in contact with or in proximity to motor growth cones, shortly before or during their migration. Based on the timing of primary motor axon migration (Eisen et al., 1986; Myers et al., 1986) and on the position and the timing of adaxial cell migration (Devoto et al., 1996), it has been suggested that adaxial cells might be involved in motor axon pathfinding (Melançon, 1994); however, simultaneous observations of motor growth cone and adaxial cell migration have not been reported yet. To determine the spatial relationship between motor axons and adaxial cells, both cell types were labeled with antibodies at the time when motor axons migrate to the somites (Fig. 5). In somitic segments in which adaxial cells have not yet migrated radially, *znp-1*-positive motor axons were not yet detectable outside the spinal cord (Fig. 5A). In contrast, in segments in which adaxial cells had just begun to migrate, the first *znp-1*-positive growth cones pioneered the common path (Fig. 5B). These are likely to be CaP growth cones, as they pioneer the common path before the MiP and RoP growth cones (Eisen et al., 1986, 1989). Interestingly, adaxial cells at the level of the *znp-1*-positive growth cone have migrated one to several cell diameters

radially, whereas adaxial cells located one to a few cells further ventrally have not begun to migrate radially. As the motor growth cone projects further ventrally, adaxial cells at the level of the growth cone have again migrated radially, while adaxial cells located one to a few cells further ventrally have not migrated yet (Fig. 5C). At the distal end of the common path, motor growth cones pause at a group of non-migrating adaxial cells, the muscle pioneers (Fig. 5D; Devoto et al., 1996; Liu and Westerfield, 1990; Melançon et al., 1997; Westerfield et al., 1986). When CaP and MiP axons resume migration along their cell-type-specific path to the ventral and dorsal myotomes, respectively, adaxial cells have almost completed their radial migration to the surface of the myotome (Fig. 5E). Thus, adaxial cells delineate the common path before the first motor growth cone arrives and they begin to migrate radially as the growth cone approaches. This suggests that adaxial cells do not provide a substratum on which motor axons migrate, but serve as a source of information, enabling motor axons to enter and complete the common path.

Our experiments show that adaxial cells provide wild-type *diwanka* gene activity, raising the possibility that mutations in the *diwanka* gene interfere with specification or migration of adaxial cells, thereby affecting migration of motor axons. To test this hypothesis, we used two adaxial-specific markers, F59 (Devoto et al., 1996) and prox-1 (Glasgow and Tomarev, 1998; data not shown) to examine adaxial cells at stages before and after radial migration. We observed no difference in the total number of adaxial cells or in their radial migration pattern (*n*=10, Fig. 5F).

DISCUSSION

We used the well-characterized neuromuscular system of the zebrafish embryo to study pathfinding of motor growth cones. Our phenotypic analyses implicate the *diwanka* gene in a local, rather than a global mechanism specialized in motor axon pathfinding. We demonstrate that a distinct set of adaxial cells in the zebrafish embryo generates a *diwanka*-dependent cellular environment essential for different subtypes of motor growth cones to migrate into the periphery. Furthermore, the coherent migration of adaxial cells and motor growth cones suggests a functional connection between these two events.

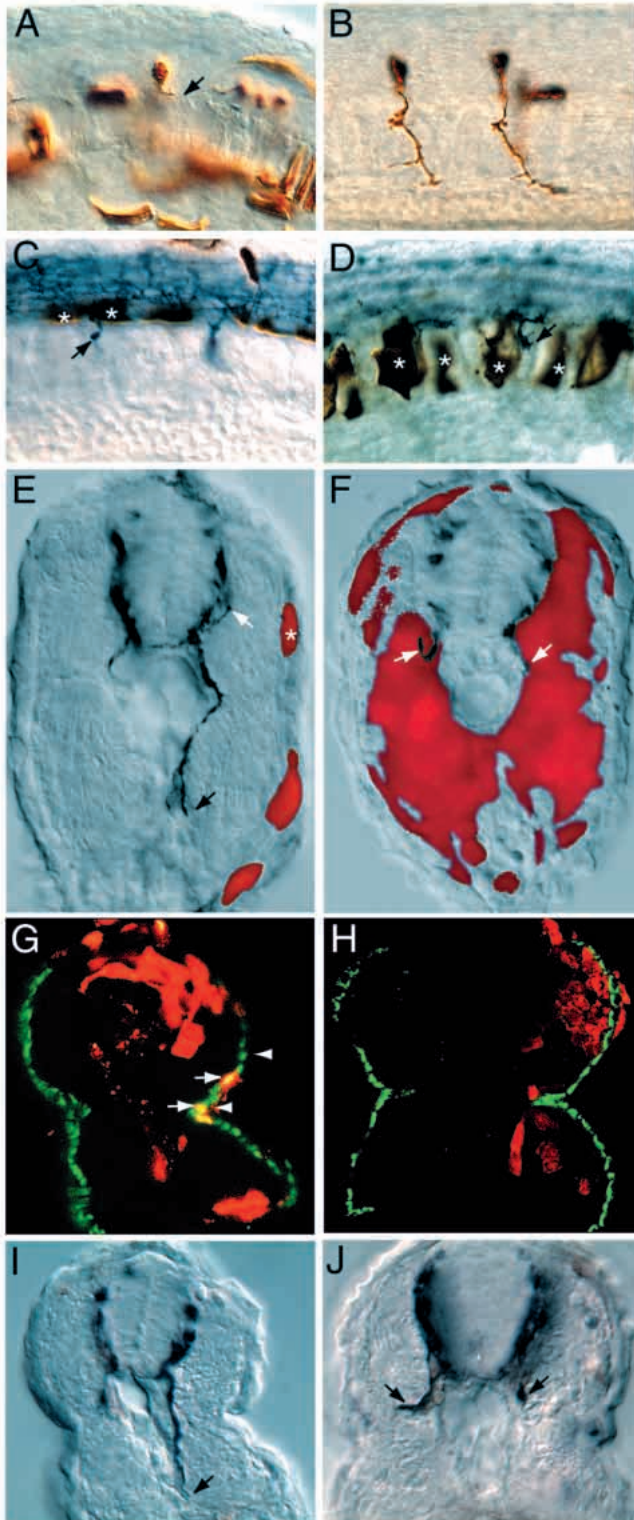


Fig. 4. Analysis of chimeric mosaic embryos reveals that *diwanka* activity is required in adaxial cells. (A) A genotypically wild-type motoneuron (brown) which developed in *diwanka* mutant embryos and failed to exit the spinal cord (arrow). (B) Two genotypically mutant CaP motoneurons (brown) which developed in wild-type embryos and displayed normal axonal trajectories. (C,D) Wild-type-derived floorplate (asterisks in C) or notochord cells (asterisks in D) failed to restore motor axonal trajectories. Arrows point to *znp-1*-positive motor growth cones, which failed to complete the common path. (E,F) Cross-section of mosaic *diwanka* embryos showing wild-type-derived cells (in red) and *znp-1*-positive motor axons in dark blue. (E) Small clones of genotypically wild-type cells in the lateral portion of the somite (asterisk) restored the motor axon projections of CaP (black arrow) and MiP (white arrow, only partially in focal plane). (F) In contrast, large clones of genotypically wild-type cells in the medial part of the somites failed to restore motor axon projections. (G) Cross-section showing colocalization between wild-type-derived cells (red) able to rescue the axonal phenotype in the right hemisegment and F59-positive adaxial cells (green). Arrows point to double-labeled cells (yellow), located between the lower end of the spinal cord (upper arrowhead) and the horizontal myoseptum (lower arrowhead). The rescued motor axon is shown in Fig. 4I. (H) Cross-section through a segment in which wild-type-derived cells (red) occupied most of the segment, but F59-positive adaxial cells (green) are of *diwanka* mutant origin. There is no colocalization between wild-type-derived cells (red) and F59-positive adaxial cells (green) and the motor trajectories were not rescued (see Fig. 4J). (I) DIC image of the cross-section shown in G. The arrow points to the rescued, *znp-1*-positive motor axon. (J) DIC image of the cross-section shown in H. Arrows point to mutant, *znp-1*-positive motor axons.

revealed that the expressivity of the motor axon defect is variable (data not shown, Fig. 1; Table 1), suggesting that residual gene activity might be present in *diwanka* mutant embryos and partially attenuates the full expressivity of the phenotype. However, embryos carrying one *diw^{tv205a}* allele and one allele in which the *diwanka* locus is entirely deleted display a similar range of motor axonal phenotypes as observed in *diw^{tv205a}/diw^{tv205a}* mutant embryos, indicating that the *diw^{tv205a}* allele is a strong hypomorphic or amorphic allele (J. Z. and M. G., unpublished results). Thus, it is conceivable that other, yet unknown genes partially substitute for *diwanka* during motor axon migration. This conclusion is consistent with a number of previous studies in which even amorphic alleles do not completely eliminate axonal guidance (e.g. Desai et al., 1996; Hedgecock et al., 1990; Winberg et al., 1998).

We noticed that RoP growth cones were more strongly affected than MiP growth cones and that MiP growth cones were more strongly affected than CaP growth cones in their ability to enter and extend along the common path. The phenotypic strength appears to increase with the distance between the somata of the individual motor neurons and the common path (Fig. 1A; Table 1). This could suggest that all mutant growth cones migrate the same distance, due to a general migration defect. However, we observe that mutant RoP growth cones, of which 79% failed to exit the spinal cord, always extended caudally past the exit point towards the somitic boundary, about twice the distance wild-type RoP growth cones migrate inside the spinal cord. This strongly suggests that motility of *diwanka* motor growth cones is unaffected. It is possible that RoP and MiP motor growth cones are more strongly affected because they enter the common path

The *diwanka* gene controls motor growth cone migration

Three different lines of fish carrying mutations in the *diwanka* locus were previously isolated in a large-scale genetic screen for genes affecting locomotion behavior in the zebrafish (Granato et al., 1996). Analysis of the motor axonal phenotype of *diwanka* mutant embryos from three different alleles

after CaP growth cones and are therefore less likely to be exposed to residual *diwanka* activity or are less responsive to another, yet unidentified activity which partially substitutes for *diwanka*.

Our analysis of the *diwanka* motor axon phenotype shows that the *diwanka* gene plays a central role in establishing the segmental nerve path on which spinal motor growth cones migrate from the spinal cord into the periphery. Is this the only function of the *diwanka* gene during primary motor axon migration? We noticed that some mutant growth cones reach the distal end of the common path, but fail to extend on their cell-type-specific path (Fig. 1E; Table 2). This could reflect an additional role of the *diwanka* gene in the migration of motor growth cones along their cell-type-specific path. Because the entire axonal path of CaP and MiP axons was rescued by wild-type cells located in the proximity of the common, but not the cell-type-specific path, the *diwanka* gene would have to exert its additional function on motor growth cones before they enter their cell-type-specific path. Alternatively, the failure of CaP and MiP growth cones to migrate on their cell-type-specific path may be a consequence of their impaired migration on the common path. For example, it is conceivable that mutant motor growth cones reach the choice point belatedly, thereby missing signals from the embryo that guide motor growth cones at or beyond the choice point. Evidence for such temporally restricted signals comes from elegant heterochronic somite transplantation experiments, which demonstrate that notochord signalling is essential for cell-type-specific pathfinding of CaP motor axons (Beattie and Eisen, 1997).

The role of mesodermal cells for zebrafish motor growth cone migration

In vitro and in vivo experiments suggest that somitic tissues may play a role during spinal motor axon pathfinding (for review see Eisen, 1994). For example, mouse sclerotome explants and sclerotome-conditioned medium promote the invasion of motor axons into a collagen matrix (Ebens et al., 1996). Agar slabs conditioned by somitic myoblast from *Xenopus* (McCaig, 1986) and chick (Henderson et al., 1981, 1984) are able to attract presumptive growth cones of axial motoneurons, suggesting that the signal(s) attracting motor growth cones might be diffusible. In vivo, surgical ablation of the dorsal portion of the chick somite, the dermamyotome, affects pathfinding of epaxial motor axons (Tosney, 1987). In the zebrafish, embryos homozygous for the *spadetail* mutation lack most axial trunk myotomes and display severe motor axon pathfinding defects (Eisen and Pike, 1991). These and other studies suggest that somitic tissues can provide signals important for motor axon pathfinding (for reviews see Eisen, 1994; Tannahill et al., 1997; Tosney, 1991); however, the identity and in vivo function of such somitic signals have remained unknown.

Our data show that the major source of *diwanka* gene activity for motor growth cones is provided by a small group of migratory mesodermal cells, those adaxial cells that delineate the future common path. Adaxial cells constitute one of the two *myoD*-expressing myotomal cell types in the segmental plate and are initially arranged in columns along both sides of the notochord (Devoto et al., 1996; Weinberg et al., 1996). Previous studies show that *hedgehog* signals emanating from the notochord induce a muscle population distinct from other somitic cells, the adaxial cells (Blagden et al., 1997; Currie and Ingham, 1996; Hammerschmidt et al., 1996; van Eeden et al., 1996). Adaxial cells first elongate to span the entire length of the somite, then move dorsally and ventrally and finally migrate radially through the somites to form a superficial myotomal cell layer (Devoto et al., 1996). Although in most cases a small number of wild-type-derived adaxial cells was sufficient to restore motor growth cone migration (Fig. 4G), we noticed that wild-type non-adaxial muscle cells located in the medial portion of the myotome

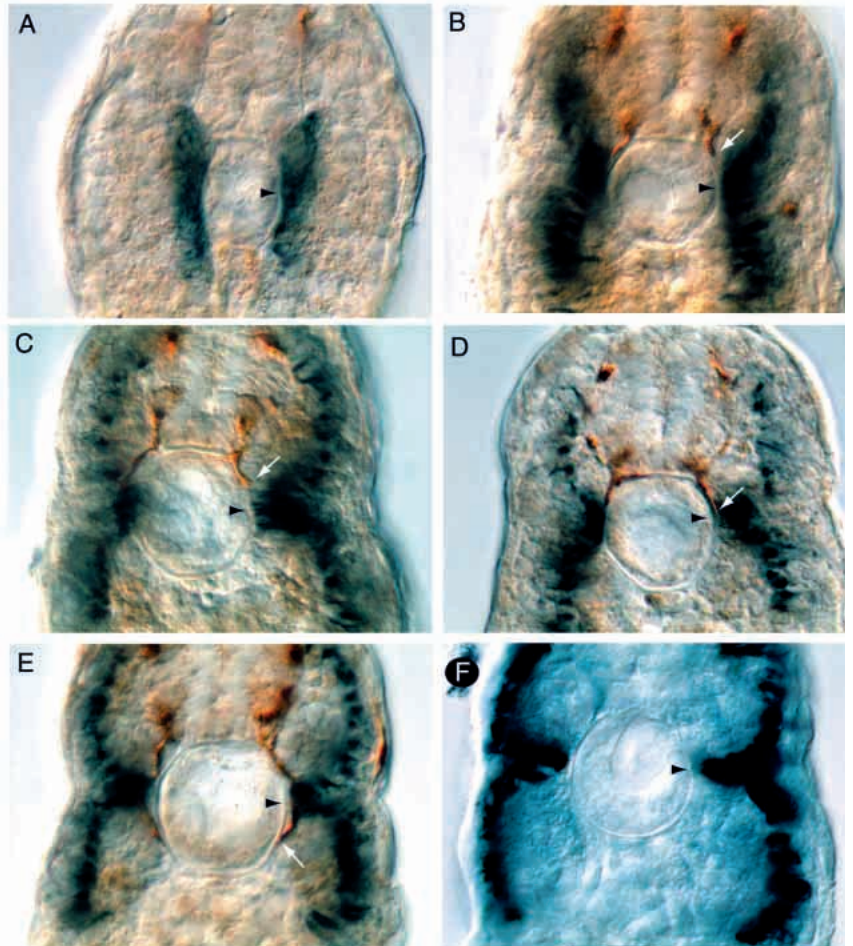


Fig. 5. Motor growth cone and adaxial cell migration coincide. (A-E) Cross-section of somitic segments (18-12) of wild-type embryos at 20 hpf stained with znP-1 antibody (brown) and F59 antibody (blue). White arrows point to the progressing growth cone and arrowheads point to distal end of the common path in the region of the future horizontal myoseptum. (F) Cross-section of somitic segment 14 of a *diwanka* mutant embryo at 24 hpf stained with F59 (blue), demonstrating that, in *diwanka* mutant embryos, the migrated adaxial cells are indistinguishable from those in wild-type siblings.

displayed a restricted potential to restore motor trajectories (14%, Table 2). This might reflect a combinatorial role of somitic tissues to ensure proper formation of the common path: a group of myotomal cells other than adaxial cells might also provide *diwanka* activity to motor axons, although with much lower potency. Together these results demonstrate that a specialized subpopulation of mesodermal cells play a pivotal role in motor growth cone migration.

One prediction from our results is that in embryos lacking adaxial cells or adaxial cell differentiation, primary motor axons should display phenotypes similar to those observed in *diwanka* embryos. This prediction can be tested in *no tail* embryos, which lack a differentiated notochord (Halpern et al., 1993). In *no tail* mutant embryos, the posterior somitic segments have reduced levels or lack completely a marker specific for adaxial cell differentiation (Blagden et al., 1997). The *no tail* gene is expressed in notochord but not in adaxial and neural cells (Schulte-Merker et al., 1994), and nervous system differentiation in *no tail* mutants appears largely unaltered (Halpern et al., 1995). Our analysis of *no tail* (*ntl*^{tc41}) embryos (Odenthal et al., 1996) indicates that the presence of F59-positive, adaxial-cell-derived slow muscle fibers in rostral somites coincides with the presence of znp-1-positive motor axons, whereas the absence of F59-positive slow muscle fibers in caudal somites correlates with severe motor axon defects (J. Z. and M. G., unpublished observations).

The role of *diwanka* in motor axon migration

In wild-type embryos, CaP growth cones are the first to pioneer the common path, followed within 2 hours by MiP, and eventually by RoP growth cones (Eisen et al., 1989). Before motor growth cones enter the common path, a small set of about five adaxial cells delineate their future common path. Radial migration of these adaxial cells and migration of the first motor growth cones to the somites coincide in an intriguing manner. Adaxial cells at the level of the growth cone have just begun their radial migration, while adaxial cells distal to the growth cone have not yet begun to migrate (Fig. 5). Moreover, of the five adaxial cells delineating the common path, the presence of a few wild-type cells in an otherwise mutant *diwanka* embryo is sufficient to restore motor axon trajectories. From these data, we conclude that adaxial cells themselves do not provide a substratum on which motor axons migrate, but might provide a source of information, enabling motor axons to establish an axonal path to the somites. What information could this be? It is unlikely that adaxial cell-derived *diwanka* activity directly influences all three motor growth cones, as MiP and RoP growth cones enter the common path after CaP growth cones, at a time when adaxial cells have migrated away from the common path. It is also unlikely that CaP growth cones pioneer in a *diwanka*-dependent manner a path on which MiP and RoP simply follow, because each motor neuron growth cone has the potential to independently pioneer the common path (Eisen et al., 1989; Pike and Eisen, 1990).

How, then, does *diwanka* influence the ability of all primary motor growth cones to migrate from the spinal cord to the somites? One possible mechanism is that, during normal development, a defined set of adaxial cells, located between the ventral part of the spinal cord and the future myoseptum, modifies the overlying medial surface of the somites in a *diwanka*-dependent manner, thereby enabling CaP growth

cones to pioneer the common path. This modification may be triggered by the onset of radial migration of adaxial cells, and the modification may persist for several hours to allow the later migrating MiP and RoP axons to extend on the somites. In the absence of *diwanka* activity, adaxial cells fail to modify the medial surface of the somites thereby preventing motor axon growth cones from projecting into the periphery. It is possible that the *diwanka* gene, acting in a small subset of adaxial cells, mediates this somite surface modification. This could occur directly, or indirectly by inducing neighboring cells. Such an indirect mechanism has been proposed for the *C. elegans unc-129* gene, which encodes a secreted TGF- β molecule required for motor axon guidance to dorsal muscles (Colavita et al., 1998).

Alternatively, *diwanka* might inactivate an inhibitory cue present on the medial surface of the somites, preventing motor growth cones from entering the common path precociously. Adaxial cells might release this blockage through the function of *diwanka*, thereby enabling primary motor axons to navigate the common path. Again, this modification may be triggered as adaxial cells start migrating radially. In the absence of *diwanka* gene activity the inhibitory force persists, thus precluding motor growth cones to pioneer the common path. Such a double inhibitory mechanism controls the establishment of the dorsoventral body axis in vertebrate embryos (for review see Mullins, 1998). There, the metalloprotease Tolloid cleaves the Chordin gene product, thereby releasing Bone Morphogenetic Protein, a key player in dorsoventral axis formation.

Our detailed analysis of the *diwanka* mutant phenotype demonstrates an essential and cell non-autonomous role for *diwanka* in establishing the segmental motor path. We have started cloning the *diwanka* locus and expect that the molecular identification of the *diwanka* gene will reveal the molecular mechanism by which this gene controls motor axon pathfinding. Our analysis also shows that adaxial cells are important to generate pathfinding information for primary motor axons. The well-orchestrated migration of adaxial cells and motor growth cones raises the possibility that these two events might be functionally connected. Migration of motor growth cones might depend directly on the migration of adaxial cells through modifications of these cells on the extracellular matrix. Examination of mutants in which adaxial cells fail to migrate properly may clarify the role of adaxial cells in motor axon guidance.

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