Genetic screens for genes controlling motor nerve–muscle development and interactions

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Abstract

Motor growth cones navigate long and complex trajectories to connect with their muscle targets. Experimental studies have shown that this guidance process critically depends on extrinsic cues. In the zebrafish embryo, a subset of mesodermal cells, the adaxial cells, delineates the prospective path of pioneering motor growth cones. Genetic ablation of adaxial cells causes profound pathfinding defects, suggesting the existence of adaxial cell derived guidance factors. Intriguingly, adaxial cells are themselves migratory, and as growth cones approach they migrate away from the prospective axonal path to the lateral surface of the myotome, where they develop into slow-twitching muscle fibers. Genetic screens in embryos stained with an antibody cocktail identified mutants with specific defects in differentiation and migration of adaxial cells/slow muscle fibers, as well as mutants with specific defects in axonal pathfinding, including exit from the spinal cord and pathway selection. Together, the genes underlying these mutant phenotypes define pathways essential for nerve and muscle development and interactions between these two cell types.
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Keywords: Zebrafish; Mutagenesis; Genetic screen; Motor neuron; Adaxial cells; Slow-twitch muscle fibers; Axonal pathfinding; Axonal guidance; Motoneurons

Introduction

Genetic screens to uncover genes critical for cell migration and axonal guidance have been extremely successful in flies and worms. There, antibody-based or reporter-based screens have identified key genes for axonal guidance towards and across the midline (Seeger et al., 1993; Zallen et al., 1999), guidance along the anterior–posterior axis (Wightman et al., 1997), motor axon guidance (Kraut et al., 2001; Van Vactor et al., 1993) and guidance of specialized cell types, including canal associated neurons (Forrester and Garriga, 1997) or pharyngeal axons (Morck

et al., 2003). In vertebrates, comparable genetic screens using antibodies or reporter lines to identify essential genes are limited by the greater complexity of the vertebrate nervous system and the logistic requirements to perform such screens. Gene trapping strategies in mice and genetic screens in zebrafish have been successfully used to identify genes with critical roles in cell migration and axonal guidance (Beattie et al., 1999; Granato et al., 1996; Karlstrom et al., 1996; Leighton et al., 2001). However, of these screens, only two were designed to identify genes critical for motor axonal guidance. In the first, Beattie et al. performed a small scale antibody-based parthenogenetic screen using diploid embryos (Beattie et al., 1999), while in the second, large-scale screen, pre-selected mutants with defects in locomotion were re-screened using antibodies (Granato et al., 1996). Together, these screens identified only five genes essential for motor axon guidance (reviewed in: Beattie, 2000).

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Recently, a large-scale insertional mutagenesis screen in the zebrafish has identified 315 embryonic essential genes (Amsterdam et al., 2004). Surprisingly, mutations in well-studied axonal guidance genes such as Robos, Slits and Semaphorins are absent from this collection. Moreover, mutations in the zebrafish *astray* (*robo2*) and *robo3* genes, previously identified in the Tuebingen large-scale screen, result in partially viable animals (M. G. unpublished results and Fricke et al., 2001). These results, combined with the low number of motor axon genes recovered in the previous locomotion screen, suggest that mutations in genes critical for motor axonal guidance may not result in embryonic lethality or easily detectable locomotion defects. Consistent with this notion, we find that null mutant embryos for the *unplugged* gene, which is essential for motor axonal pathway selection, initially display a locomotion defect, but recover from this within 1 day and develop into viable adults (Zhang et al., 2001). Similarly, *stumpy* and *topped* mutants, in which motor axon guidance is severely compromised, display no other discernible phenotype, and mutant alleles for each of these genes are homozygous viable (Beattie et al., 2000; Rodino-Klapac and Beattie, 2004).

To identify additional genes essential for motor axon guidance, we performed an antibody-based screen using diploid embryos. We focused on the primary spinal motoneurons, because there are only three per hemisegment, and because they are the first to pioneer into the periphery (reviewed in: Beattie, 2000). Growth cones of the three primary motoneurons initially share a path into the periphery along the medial surface of the somites (Bernhardt et al., 1998; Eisen et al., 1986). At the distal end of this shared or common path, all pioneering growth cones contact a group of specialized cells called the muscle pioneers (Felsenfeld et al., 1991; Melançon et al., 1997). After reaching this choice point, they pause before selecting cell-type specific paths to ventral, dorsal and medial myotomal regions (Eisen et al., 1986; Myers et al., 1986; Westerfield et al., 1986). We have previously shown that growth cone migration along the common path, as well as pathway selection at the choice point, critically depends on signals provided by dorsal adaxial cells (Zeller and Granato, 1999; Zeller et al., 2002; Zhang and Granato, 2000). Adaxial cells form in response to Hedgehog (Hh) signals and represent a small population of myotomal cells that develop into slow-twitching muscle fibers (Currie and Ingham, 1996; Devoto et al., 1996). Intriguingly, dorsal adaxial cell delineate the prospective common path on the medial somite surface and migrate to the lateral somite surface as the first motor growth cones enter the common path (Devoto et al., 1996; Zeller and Granato, 1999). While there is clear genetic evidence that adaxial cells play a critical role in providing guidance signals to motor growth cones, little is known about the differentiation and migration of these cells, or the nature of guidance signals they provide. Thus, we used antibodies to visualize motor axonal trajectories and adaxial

cells/slow muscle fibers in a genetic pilot screen to identify genes that govern the development of motor axons and adaxial cells/slow muscle fibers, as well as for genes critical for interactions between these cell types.

Here, we report on the isolation of 15 mutants which cover a broad spectrum of phenotypes but can be divided into three categories. Through phenotypic analyses, chimera analyses and molecular cloning of some of these mutants, we conclude that (1) adaxial cells play a pivotal role in motor axonal guidance; (2) antibody-based screens can identify mutations in presumptive guidance genes, without associated defects in morphology or locomotion; (3) some of the mutants provide key entry points into biological processes not well understood, such as differentiation of muscle cell types towards their unique fiber type profile or axonal guidance towards and through segmental central nervous system exit points.

Materials and methods

Mutagenesis, fish maintenance and breeding

Zebrafish were raised and maintained as previously described by Mullins et al. (1994). Embryos were staged as reported in Kimmel et al. (1995). ENU mutagenesis was performed as described in Mullins et al. (1994) and in Dosch et al. (2004).

Screening procedure

26–28 hpf (hours post fertilization) F3 embryos were anesthetized (0.01% Tricaine), fixed overnight in 4% PFA in 0.1 M phosphate buffer, pH 7.4 (81 mM Na_2HPO_4, 19 mM NaH_2PO_4, pH 7.4) plus 1% DMSO, and then washed several times in 0.1 M phosphate buffer pH 7.4. Fixed embryos were dehydrated through a MeOH series and stored in 100% MeOH at $-20°C$. Stored embryos were then transferred into prechilled 100% acetone and incubated for 30 min at $-20°C$, and then washed several times with incubation buffer (0.2% BSA, 0.5% triton-X in 0.1 M phosphate buffer, pH 7.4). All subsequent antibody stainings with primary and secondary antibodies were performed in 24 well plates. Stained embryos were transferred into Vectashield mounting medium (Vector Laboratories). From each clutch, 12 embryos were screened for defects using a Leica MZFLIII stereomicroscope equipped with epifluorescence.

Antibody stainings and α-bungarotoxin labeling

Antibody stainings and cross sections were performed as previously described in Zeller et al. (2002). The following primary antibodies were used: znp-1; (1:200, Antibody Facility, University of Oregon, Trevarrow et al., 1990); F59 (1:10, kindly provided by F. Stockdale, Crow and