RESEARCH ARTICLE

Zebrafish foxc1a drives appendage-specific neural circuit development

Santanu Banerjee¹, Katharina Hayer², John B. Hogenesch² and Michael Granato¹,*

ABSTRACT

Neural connectivity between the spinal cord and paired appendages is key to the superior locomotion of tetrapods and aquatic vertebrates. In contrast to nerves that innervate axial muscles, those innervating appendages converge at a specialized structure, the plexus, where they topographically reorganize before navigating towards their muscle targets. Despite its importance for providing appendage mobility, the genetic program that drives nerve convergence at the plexus, as well as the functional role of this convergence, are not well understood. Here, we show that in zebrafish the transcription factor foxc1a is dispensable for trunk motor nerve guidance but is required to guide spinal nerves innervating the pectoral fins, equivalent to the tetrapod forelimbs. In foxc1a null mutants, instead of converging with other nerves at the plexus, pectoral fin nerves frequently bypass the plexus. We demonstrate that foxc1a expression in muscle cells delineating the nerve path between the spinal cord and the plexus region restores convergence at the plexus. By labeling individual fin nerves, we show that mutant nerves bypassing the plexus enter the fin at ectopic positions, yet innervate their designated target areas, suggesting that motor axons can select their appropriate fin target area independently of their migration through the plexus. Although foxc1a mutants display topographically correct fin innervation, mutant fin muscles exhibit a reduction in the levels of pre- and postsynaptic projections of fin-innervating motor nerves, and we demonstrate that this mutant phenotype is caused by a presumptive null mutation in the foxc1a gene. The expression of foxc1a in muscle cells is crucial for appendage mobility.

KEY WORDS: Zebrafish, Limb motor neuron, Motor axon, Plexus, Fin nerves, Foxc1a, Pectoral fin, Axon guidance

INTRODUCTION

During development, the fidelity with which connections between spinal motor neurons and their appropriate limb muscle targets form is paramount for precise and coordinated motor behaviors. Motor neurons express specific set of guidance receptors that ensure correct navigation at intermediate targets where growth cones select a path towards their appropriate final synaptic targets. For example, after exit from the spinal cord, limb-innervating motor axons avoid a path towards dorsal axial muscles, and instead extend ventrally towards an intermediary target at the base of the limb. Here, motor axons from adjacent spinal cord segments converge and re-organize to innervate their final limb muscle targets. This intermediate target, the plexus, is considered crucial for proper target innervation, in part because here axons make their final navigational decisions, and are able to compensate for previous migration errors (Ferguson, 1983; Ferns and Hollyday, 1993; Lance-Jones and Landmesser, 1981; Tosney and Landmesser, 1984). For example, rotating spinal cord segments along the anterior posterior axis causes limb nerves to approach the plexus from inappropriate directions, yet compensatory changes within the plexus are thought to correct target innervation (Lance-Jones and Landmesser, 1981). Similarly, following dorsoventral rotation of the limb bud, innervating motor axons also adjust their trajectories in the plexus accordingly, suggesting involvement of local guidance cues within the plexus and in the developing limb (Ferguson, 1983; Tosney and Landmesser, 1984). Combined, these experiments suggest that axonal migration through the plexus area contributes to proper target innervation; however, this has not been tested directly by examining synaptic target selection of axons that bypass the plexus and enter the limb at ectopic locations.

Studies in chick and mouse have revealed that motor neurons express a specific set of cell-adhesion molecules and guidance receptors that are crucial to steer limb motor axons from the limb plexus to target muscles (Bonanomi and Pfaff, 2010; Tang et al., 1992, 1994). For example, motor neurons expressing EphA4 are repelled by ephrin A proteins expressed in the ventral limb mesenchyme and innervate the dorsal limb (Eberhart et al., 2000, 2002; Kania and Jessell, 2003; Luria et al., 2008). By contrast, neuropilin 2 receptor-expressing motor neurons are repelled by the semaphorin 3F ligand expressed in the dorsal limb, and innervate the ventral limb. Similarly, neuropilin 1/sema- phosphorin 3A signaling regulates multiple aspects of limb nerve guidance, including axon fasciculation, timing of entry into the plexus and bifurcation of nerves into dorsal and ventral limb (Huber et al., 2005). Despite this, the genetic program that ensures the convergence of multiple appendage innervating spinal nerves at the plexus, and the significance of this nerve convergence for the innervation pattern and function of vertebrate appendages, is not well understood.

Similar to tetrapods, zebrafish spinal nerves that innervate the pectoral fins, equivalent to the tetrapod forelimbs, converge at base of the pectoral fins to form a plexus before entering their distinct target areas in the fin. Importantly, fin muscle innervation is highly stereotyped (Ma et al., 2010; Thorsen and Hale, 2007), providing an excellent model system with which to determine whether the convergence of spinal motor nerves at the plexus is a prerequisite for proper fin innervation and function, and to identify key players selectively required for the development of the neural circuitry critical for the mobility of vertebrate appendages. Using a combination of forward genetics and whole-genome sequence analysis, we first report on a mutant we isolated in a forward genetic screen based on aberrant projections of fin-innervating motor nerves, and we demonstrate that this mutant phenotype is caused by a presumptive null mutation in the foxc1a transcription factor. We show that foxc1a is required selectively for pectoral fin-innervating motor axons to converge at the plexus, and...
that nerve convergence requires *foxc1a* function in somitic muscle cells located along the nerve path. Moreover, we find that *foxc1a* mutant nerves that bypass the plexus still target their original target area within the fin, providing compelling evidence that convergence of fin nerves at the plexus is not a prerequisite for fin muscle target selection. Finally, we show that despite proper target selection, *foxc1a* mutants exhibit reduced fin innervation, concomitant with a reduction in the amplitude and frequency of fin movement (Fig. 1C, n = 160 hemisegments in 30 embryos). In *p162* mutants the muscle intrinsic mechanisms required for motor axon guidance through anterior somite segments. To exclude the possibility that the observed axonal defects were secondary, i.e. due to defective somite development, we examined adaxial muscle cell specification, somite polarity and muscle fiber differentiation, as defects in these processes are known to impair motor axon guidance (Birely et al., 2005; Zeller et al., 2002). Antibody staining revealed that adaxial muscle cell numbers, their specification, as well as somite polarity is unaffected in *p162* mutants (Fig. 1F,G; supplementary material Fig. S2). Finally, we examined postsynaptic differentiation as a hallmark of muscle fiber differentiation. Primary motor axons form stereotypic en passant neuromuscular synapses, characterized by the accumulation of acetylcholine receptor (AChR) clusters, at the center of muscle fibers (Fig. 1H; Westerfield et al., 1986). In *p162* mutants, en passant synapses properly localized in the center of muscle fibers, along the entire length of their shortened motor axons (Fig. 1I), suggesting that in *p162* mutants the muscle intrinsic mechanisms crucial for postsynaptic specialization are operational. Taken together, we have identified a mutation in a gene specifically required for motor axon guidance through anterior somite segments.

**The *p162* phenotype is caused by a premature stop codon in *foxc1a***

To identify the gene disrupted by the *p162* mutation, we applied a combination of positional cloning and whole-genome sequence analysis. In brief, we first used genetic linkage analysis via microsatellite mapping to position the *p162* mutation within a 2 Mb interval on chromosome 2, and then performed whole-genome sequencing analysis. Sequence data was aligned to the Zv9/danRer7 genome (UCSC), and then processed through the GATK pipeline (DePristo et al., 2011; McKenna et al., 2010; Van der Auwera et al., 2013). Within the 2 Mb interval defined by genetic mapping, this revealed a unique ‘deleterious’ single nucleotide polymorphism (SNP) (Fig. 2A,B). This SNP was considered ‘deleterious’ because it changes a conserved tryptophan to a premature stop codon in the *foxc1a* gene (W118*, Fig. 2A,B). The zebrafish *foxc1a* gene encodes a 476 amino acid protein, which is 63% identical to the human and mouse FoxC1 homologs. FoxC1 proteins belong to the forkhead family of transcription factors characterized by the forkhead domain consisting of two DNA-binding wing helix domains, crucial for FoxC1 function (Murphy et al., 2004; Nishimura et al., 2001; Saleem et al., 2004; Weisschuh et al., 2006). The *p162* mutation W118* truncates the open reading frame in the forkhead domain (amino acids 72-163). Consistent with previously described morpholino knockdown phenotypes, *foxc1a*
mutants eventually develop small eyes, pericardial edema and circulation blockage (data not shown; Skarie and Link, 2009; Veldman and Lin, 2012).

To demonstrate that the mutation in the foxc1a gene is causative of the observed axon guidance defects, we performed mRNA rescue experiments. For this, we injected mRNA encoding wild-type foxc1a and truncated foxc1a<sup>p162</sup> allele (see Materials and Methods) into wild-type and mutant embryos, and examined axonal projection pattern of spinal motor axons at 28 hpf. Injection of wild-type foxc1a mRNA into wild-type or sibling embryos did not affect axonal projection nor did it rescue axon guidance defects in foxc1a mutants, even when injected at 10-fold higher levels than wild-type foxc1a mRNA (sufficient to rescue foxc1a mutants), consistent with the idea that foxc1a<sup>p162</sup> represents a presumptive null allele (n=48 hemisegments in eight embryos for each genotype, data not shown). To our knowledge this is the first evidence for a role for foxc1a in motor axon guidance.

**foxc1a is required for pectoral fin nerve guidance**

Given the role of foxc1a in guiding pioneering primary motor axons, we examined the projection patterns of later developing secondary motor axons, which extend along the same path to reach their peripheral targets, yet largely independent of primary motor axons (Myers, 1985; Pike et al., 1992). In somitic segments 5-36, 60-80 secondary motor axons from each spinal cord segments bundle together to form spinal nerves that innervate trunk muscle (Fig. 3A,B). In foxc1a mutants, motor neurons innervating trunk muscles appear indistinguishable from those in wild type (Fig. 3A-C; n=120 hemisegments in 20 embryos), suggesting that foxc1a is dispensable for the guidance of trunk innervating motor nerves. By contrast, in foxc1a mutants, pectoral fin-innervating nerves displayed severe defects. Wild-type pectoral fins are innervated by a set of four segmental spinal motor nerves, extending through the four anterior-most somites (Fig. 3A,D,F,H,J). By 48 hpf, nerves 1-3 have converged at the plexus located at the base of the fin, and have just entered the dorsal aspect of the fin (Fig. 3D,F, arrowheads). Nerve 4, without entering the plexus, enters the fin at the ventral aspect (Fig. 3D,F, arrows). In foxc1a mutants, the fourth nerve completes migration through the axial myotome, stalls before reaching the ventral extent of the fin but eventually enters the fin (Fig. 3E,G, arrows; Fig. 3I-K). Nerves 1-3 in foxc1a mutants fail to converge at the base of the fin plexus (Fig. 3E,G, arrowheads), and instead made contacts with each other at ectopic locations (cyan asterisks in Fig. 3E,G, arrowheads), and instead made contacts with each other at ectopic locations (cyan asterisks in Fig. 3E,G, n=115/132 hemisegments in 33 embryos). For example, the third nerve frequently contacted the second or the fourth fin nerve at ectopic locations, from where they extended further ventrally, bypassing the plexus (n=115/132 nerves in 27/33 embryos; cyan asterisks in Fig. 3E,G). Furthermore, within individual nerves, motor axons defasciculated and extended along ectopic trajectories (Fig. 3E,G, red asterisks). To determine whether axonal guidance defects result in motor neuron death, we examined motor neuron cell death by TUNEL staining around 48 hpf. Identical to the situation in wild-type embryos, motor neuron death in foxc1a mutants was undetectable (supplementary material Fig. S3).

Finally, to determine whether those nerves bypassing the plexus area were ultimately able to enter the fin, we examined fin innervation at later stages. At 96 hpf, foxc1a mutant nerves still exhibited ectopic fasciculation with other nerves (Fig. 3I-K), yet were able to enter the pectoral fin, albeit mutant fins displayed a significant reduction in the number of terminal axonal arbors (Fig. 3I-K; and below, see Fig. 6). Combined, our data demonstrate that although foxc1a is dispensable for the projection of trunk innervating motor nerves, it is required for the convergence of fin innervating nerves at the fin plexus.

**foxc1a acts cell non-autonomously in somitic muscle**

To determine how foxc1a regulates fin nerve guidance, we examined foxc1a mRNA expression at the time of pectoral fin innervation (see Fig. S4 in the supplementary material). At 40 hpf, foxc1a expression is enriched in the ventral region of those anterior somite segments through which the first three fin nerves migrate (Fig. 4A-F). Thus,
consistent with the pectoral fin-innervating nerve defects, we find that \textit{foxc1a} mRNA is expressed at the time when motor axons navigate towards the plexus region, and along their path. We therefore tested whether \textit{foxc1a} functions in somitic muscles to guide fin nerves. To test this we expressed wild-type \textit{foxc1a} under the control of a muscle-specific promoter (\textit{α}-actin::\textit{foxc1a}-p2a-

Fig. 3. \textit{foxc1a} is required for fin-innervating motor nerves to convert at the plexus. (A) Lateral (composite) view of a 96-hpf-old Tg\textit{(mnx1:GFP)} larvae expressing GFP in all motor nerves. Yellow dashed boxes mark anterior segments (AS) and posterior segments (PS) considered in this study; white dotted area outlines the pectoral fin. (B,C) Compared with wild-type larvae, trunk innervation (posterior segments) is unaffected in \textit{foxc1a} mutants. (D-G) By contrast, three out of the four fin-innervating nerves in \textit{foxc1a} mutants display axonal defects at 48 hpf (D-G) and at 96 hpf (H-K). Dotted areas outline the pectoral fin, arrowheads indicate the position of the fin plexus and arrows indicate the fourth pectoral fin nerve. Cyan asterisks indicate ectopic fasciculation; red asterisks mark defasciculation. Scale bars: 50 \textmu m.

Fig. 4. \textit{foxc1a} is expressed and functions in somitic muscle to guide fin nerves. (A-C) At 40 hpf, \textit{foxc1a} mRNA is highly enriched in anterior (A,B) but not posterior somite segments (A,C). (B,C) Magnified views of anterior and posterior somitic segments shown in A, asterisks indicate the anterior most three segments. (D-F) Cross-sections through anterior and posterior segments (at the level of arrows in A) reveal \textit{foxc1a} mRNA accumulation in muscle fibers located along the fin nerve path. (G-J) Stochastic expression of a \textit{foxc1a}-p2a-mKate transgene in muscle fibers (I,J) restores fin nerve guidance. (K-O) RNA-seq identifies transcripts selectively enriched in anterior segments and downregulated in \textit{foxc1a} mutants, as confirmed by real-time PCR experiments (L,M, asterisks indicate significance; \(P<0.05\), Student’s \(t\)-test) and by in situ hybridization of \textit{mybpha} (N, cross-section through anterior segment; O, cross-section through posterior segment). Data are mean±s.e.m.
function is dispensable for fin target area selection

In wild-type animals, nerves 1-3 stereotypically converge at the plexus before they enter the fin and innervate their synaptic muscle targets. To determine whether this convergence is important for synaptic target selection in the fin, we examined the innervation pattern of individual foxc1a mutant nerves. We focused on the fourth nerve, which is unaffected in foxc1a mutants, and on the third nerve, which in foxc1a mutants bypasses the plexus and enters the fin at an ectopic position (Fig. 3J,K). To visualize the trajectory of individual nerves, we generated a stable transgenic line [Tg(mnx1: Kik-GR)] and photoconverted (green to red) motor neurons expressing the photoconvertible Kik-GR protein. Photoconverting motor neurons contributing to the fourth fin nerve (pseudocolored in magenta) enabled us to visualize the trajectories of many axons within this nerve, as they entered the fin at the ventral aspect and occupied their stereotyped domain restricted to the ventroposterior region of the fin (Fig. 5A,C; n=4/4 in four embryos). Although the branch density as well as the total area covered by these axons was reduced in foxc1a mutants (see below, Fig. 6), there was no significant difference between wild type and foxc1a mutants regarding the territory occupied by fourth-nerve axons (Fig. 5B,D; n=7/7 in seven embryos).

Photoconversion of the third nerve confirmed that in foxc1a mutant axons enter the fin at an ectopic, ventral position (Fig. 5E-H, arrowheads, n=5/7 in seven embryos). Despite their ectopic entry, third-nerve axons mostly innervated their nerve specific fin territory, with only few axons projecting to ectopic sites (Fig. 5E-H). Thus, pectoral fin-innervating axons can select their original fin target areas independently of their prior pathfinding history. This strongly suggests that the mechanisms that control synaptic target innervation in the fin operate independently of those required for appropriate plexus formation and entry into the fin.

mutants display reduced fin nerve branching and synapse formation

Although foxc1a pectoral fin nerves innervate their appropriate territories, we asked whether the levels of axonal branching and synapse formation are affected. foxc1a mutants develop large pericardial edemas and die between 6 and 8 dpf. We therefore performed our analysis on 4-dpf (96 hpf) animals. Immunohistochemistry using the MF20 skeletal muscle myosin-specific antibody (Patterson et al., 2008) did not reveal any significant differences in pectoral fin muscle morphology or total fin muscle area when compared with wild type, suggesting that in foxc1a mutants fin muscles are properly differentiated (Fig. 6A-C; n=7 fins). Image analysis (for details, see Materials and Methods) revealed that in wild-type embryos ∼35% of the total fin muscle territory is occupied by thin axonal branches (Fig. 6D,F, n=7 fins). By contrast, foxc1a mutant nerves are less branched and occupied ∼20% area of the pectoral fin muscles (Fig. 6E,F, n=7 fins in seven embryos; for details on quantification, see Materials and Methods). Finally, we used α-bungarotoxin to quantify postsynaptic AChR clusters in pectoral fin muscles (Thorsen and Hale, 2007). In foxc1a mutants, AChRs were associated with presynaptic axonal branches (Fig. 6G-K), suggesting that mutant fin muscles have retained their ability to clusters AChRs. To quantify synapses between wild-type and mutant fin, we considered individual AChR cluster juxtaposed to an axon branch as individual synaptic contact sites. This revealed that compared with wild-type animals, the total number of synaptic contact sites in foxc1a mutant fins is reduced to about 66% (Fig. 6I). Thus, in foxc1a mutants, fin innervating axons target their proper synaptic territories, albeit in reduced numbers.
To examine whether the overall reduction in pectoral fin synapses observed in foxc1a mutants has functional consequences, we embedded 96-hpf-old zebrafish larvae with their fins able to move freely, and used a high-speed camera (200 frames/second) to record spontaneous fin movements. From these high-speed video recordings we calculated the amplitude of fin movements as the difference between the largest angle during the upward stroke (i.e. away from the body, angle X in Fig. 7C,D) and the smallest angle during the downward stroke (i.e. towards the body, angle Y in Fig. 7C,D). This kinematic analysis revealed that compared with wild-type animals both the mean fin-beating amplitude and the mean fin-beating frequency in foxc1a mutants are significantly reduced (Fig. 7E,F; n=9 fins from five animals for each genotype). Thus, foxc1a plays a crucial role in the development of appendage-specific neural circuits that drive appendage motility.

**DISCUSSION**

foxc1 encodes a transcription factor with well-defined roles in a wide range of developmental processes, including cerebellar, eye, vascular and organ development (Aldinger et al., 2009; Komaki et al., 2013; Kume et al., 2001; Seo and Kume, 2006; Seo et al., 2012; Sowden, 2007). In zebrafish, morpholino knockdown of foxc1a has been reported to cause defects in angioblast development (Veldman and Lin, 2012), and defects in eye vascularization (Skarie and Link, 2009). In a forward genetic screen for axonal guidance mutants, we identify a presumptive null allele in the zebrafish foxc1a gene, and demonstrate a previously unknown role of foxc1a as a key regulator to establish functional connectivity between the spinal cord and pectoral fins. Although transcription factors are known to control neuron intrinsic programs that also regulate growth cone behaviors, we find that foxc1a acts in the environment, in muscle cells along the axonal path towards the plexus region. Furthermore, we demonstrate that loss of foxc1a in muscle cells does not alter their identity. Instead, we propose that foxc1a expression in muscle cells generates a microenvironment selective...
expression of guidance receptors (Bonanomi and Pfaff, 2010). Intrinsic LIM homeodomain genes that then control the selective guidance at the base of the limb to project dorsally or ventrally within the limb is also established by the differential expression of neuron axons ventrally towards the limb (Dasen et al., 2008; Polleux et al., 2004).

Motor behaviors depend on precise connections between spinal motor neurons and their appropriate muscle targets, and one of the most complex sets of neuromuscular connections is the vertebrate limb. This is in part because the limb contains dozens of anatomically and functionally distinct muscle groups, and in part because, compared with axial motor axons, limb-innervating motor axons are confronted with additional guidance decision even before reaching the limb (Dasen and Jessell, 2009). Work over the past decade has identified a sequential motor neuron intrinsic transcriptional program that governs limb innervation. For example, motor neurons at limb levels of the spinal cord express the LIM homeodomain gene *Lhx3*, which directs their axons dorsally toward axial muscle, or express *Foxp1*, which directs their axons ventrally towards the limb (Dasen et al., 2008; Polleux et al., 2007; Roussos et al., 2008; Shirasaki et al., 2006). Similarly, guidance at the base of the limb to project dorsally or ventrally within the limb is also established by the differential expression of neuron intrinsic LIM homeodomain genes that then control the selective expression of guidance receptors (Bonanomi and Pfaff, 2010).

By contrast, the molecular program that controls axonal behaviors between their exit from the spinal cord en route to the limb and their entrance into the limb is not well understood. Between these two points, several segmental nerves converge at a defined area to form a plexus where axons mingle and then emerge to enter the limb. We find that *foxc1a* is dispensable for all aspects of axial motor axon guidance but is selectively required to guide pectoral fin-innervating motor axons, enabling the fin-innervating nerves to convergence onto a common plexus. In fact, their unique projection pattern suggests that fin nerves are dependent on guidance mechanisms that are distinct from those used by axial muscle motor nerves. Consistent with this, zebrafish mutants in which axial motor axon guidance is perturbed, display normal pectoral nerve projections (Palaisa and Granato, 2007; Zeller and Granato, 1999; Zeller et al., 2002; Zhang and Granato, 2000; Zhang et al., 2004).

*foxc1a* expression is strongly enriched in the somitic segments through which fin-innervating axons migrate where it functions cell non-autonomously in somitic muscle cells that delineate the axonal path towards the plexus. So how does *foxc1a* control axon guidance? One possible mechanism is that *foxc1a* regulates the expression of extracellular matrix (ECM) components or guidance cues in anterior somitic segments, thereby enabling axons to converge properly towards a plexus. For example, the secreted semaphorin Sema 3A, through its neuropilin 1 receptor, is thought to act through a ‘surround repulsion’ mechanism to maintain fasciculation as limb-innervating axons grow towards the plexus (Huber et al., 2005). Our RNA-seq analysis of anterior somitic segments did not identify ECM or ‘classical’ axonal guidance cues to be regulated by *foxc1a*, and in fact only identified 12 genes downregulated in *foxc1a* mutants. Among these downregulated genes, several (*foxd5*, *wm8*-2, *sgcg*, *mybpha*) have been implicated in muscle patterning and structure (Klinge et al., 2008; Lee et al., 2009; Lekven et al., 2001). However, we did not find any gross patterning or structural muscles defects (Fig. 1 and supplementary material Fig. S2) in *foxc1a* mutants, confirming that the axonal defects in *foxc1a* mutants are not secondary, e.g. to the loss of muscle cell identity or integrity. Instead they suggest that *foxc1a* regulates the localization and/or stability of ECM molecules or guidance cues in the local nerve environment in a manner too subtle for detection at the transcriptional level. Importantly, many neuron-intrinsic transcription factors that establish wiring specificity have been identified over the past decade, yet the identification of relevant target genes still remains a challenge (Santiago et al., 2014). The application of recent technological advancements that allows transcriptional and translational profiling of individual muscle cells will be required to identify *foxc1a*-dependent genes relevant for motor axon guidance.

**foxc1a defines a novel neuron extrinsic program selective for pectoral fin innervation**

Motor behaviors depend on precise connections between spinal motor neurons and their appropriate muscle targets, and one of the most complex sets of neuromuscular connections is the vertebrate limb. This is in part because the limb contains dozens of anatomically and functionally distinct muscle groups, and in part because, compared with axial motor axons, limb-innervating motor axons are confronted with additional guidance decision even before reaching the limb (Dasen and Jessell, 2009). Work over the past decade has identified a sequential motor neuron intrinsic transcriptional program that governs limb innervation. For example, motor neurons at limb levels of the spinal cord express the LIM homeodomain gene *Lhx3*, which directs their axons dorsally toward axial muscle, or express *Foxp1*, which directs their axons ventrally towards the limb (Dasen et al., 2008; Polleux et al., 2007; Roussos et al., 2008; Shirasaki et al., 2006). Similarly, guidance at the base of the limb to project dorsally or ventrally within the limb is also established by the differential expression of neuron intrinsic LIM homeodomain genes that then control the selective expression of guidance receptors (Bonanomi and Pfaff, 2010).

For fin innervating nerves to promote their convergence to the plexus region at the base of the fin.

**The role of the fin plexus for axonal targeting**

Before entering the developing limb, limb innervating spinal nerves converge from adjacent spinal cord segments at the plexus. Previous studies revealed important roles for the plexus region in guiding axons to their designated muscle domains (Huber et al., 2005; Landmesser et al., 1990; Tang et al., 1992, 1994), yet its functional requirement in target selection is not clear. Like the limb nerves of birds and mammals, in zebrafish the first three fin nerves converge into the plexus at the base of the pectoral fin. In this study, we tested whether fin nerves that migrate through the plexus are dependent on plexus-derived signals to innervate their appropriate fin muscle targets. We find that, in *foxc1a* mutants, pectoral fin nerves bypassing the plexus enter the fin in an ectopic position, yet
inervate the designated muscle target within the fin, suggesting that plexus-independent cues within the fin contribute to the selection of final muscle target. The fact that, despite taking an aberrant route, the nerve still finds its designated muscle domain suggests signaling pathways implicated in nerve-muscle matching such as Ephrin signaling (Feng et al., 2000; Lampa et al., 2004) are still sufficiently operational in foxc1a mutants to direct fin nerves to their designated target muscles. This is consistent with the fact that, even in wild-type zebrafish, the fourth pectoral fin-innervating nerve selects its target domain without entering the plexus (Thorsen and Hale, 2007).

Why, then, do three of the four fin-innervating nerves converge at the plexus before entering the fin? Although migration through the plexus is not a prerequisite for target selection, it might enhance the efficacy of functional innervation by ensuring high axonal density in the fin. Consistent with this, we find that in foxc1a mutant fins, axonal branching and synaptic contact sites are reduced (Fig. 6). Although foxc1a expression is not detectable in the fin, it is possible that foxc1a promotes axonal arborization once axons enter the fin. Nonetheless, it is tempting to speculate that migration through the plexus promotes axonal entry into the fin and/or enhances innervation. Finally, although the precise mechanism by which foxc1a controls pectoral fin nerve guidance still remain to be defined, we propose that by generating a microenvironment that promotes the convergence of fin-innervating nerves into a plexus, foxc1a plays a specific and crucial role for the development of appendage-specific neural circuits that drive appendage motility.

MATERIALS AND METHODS
Ethics statement
All experiments were performed according to an Animal Protocol approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) on 1/24/2014, protocol number 803446. Veterinary care is under the supervision of the University Laboratory Animal Resources (ULAR) of the University of Pennsylvania.

Zebrafish genetics
All embryos and larvae used in this study were raised at 28°C for the required amount of time (see Mullins et al., 1994). For all experiments, we used the foxc1a<sup>−/−</sup> mutant allele (referred as foxc1a mutant throughout the text) and as foxc1a<sup>118-476</sup> in figures) in the TL3 background. Tg(mnx1:GFP) m2 and Tg(mnx1:Kik-GR<sup>162</sup>) transgenic fish were either used alone, in combination with each other, or in combination with foxc1a mutants.

Molecular biology
foxc1a full-length cDNA was isolated from 28 hpf zebrafish embryos and cloned into pTOPO vector. The foxc1a cDNA was then cloned into BamHI and XhoI sites of pCS2+ using the following primers (forward, 5′-GGGATCCGAATTTGCTGCAGTCATACA; reverse, 5′-AAGAATTCTTCTTGACGACTGTTC-)

DNA extraction and library preparation for whole-genome sequencing
For whole-genome sequencing library preparation, 1-1.5 µg genomic DNA was sheared to 400 bp fragments using a Covaris sonicator. Sequencing library for the Illumina platform were prepared according to manufacturer’s protocol using the Illumina low-throughput library preparation kit.

Whole-genome sequencing and SNP calling
Paired-end 100 bp high-throughput sequencing was performed on the Illumina HiSeq 2000 platform. The variants were called by applying best practices described in the GATK recommendations (DePristo et al., 2011; McKenna et al., 2010; Van der Auwera et al., 2013), which involved aligning the reads to the Zv9/danRer7 genome, and using the Burrows-Wheeler Aligner (BWA) (Li and Durbin, 2009) to map sequences. Variants not present in at least five read pairs were rejected from further analysis. Variants were characterized for their potential to damage protein function with SnpEff (Cingolani et al., 2012). Gene annotations were obtained from RefSeq (http://www.ncbi.nlm.nih.gov/refseq/) and Ensemble (http://www.ensembl.org/index.html).

RNA extraction and RNA sequencing
For RNA-seq, mRNA was extracted from 38-40-hpf embryos. We isolated wild-type and foxc1a mutant samples by dissecting the entire first six anterior somitic segments (AS) through which the fin nerves migrate, and the adjacent posterior segments (PS; segments 7 to ∼12) devoid of fin-innervating nerves. Heads and yolks were excluded from all samples. Tissues were stored in RNALater solution (Life Technologies) for up to 2 days at 4°C before RNA was extracted using the RNeasy kit (Qiagen) according to the manufacturer’s protocol. RNA was tested for integrity using a Bioanalyzer (Agilent Technologies). RNA samples showing RIN value of eight or higher were used for generating cDNA libraries as described in the TruSeq Stranded mRNA sample preparation guide. At the final stage, 15 cycles of PCR amplifications were performed. Barcoded libraries representing duplicates of AS and PS samples of wild type and mutants were validated using Bioanalyzer (Agilent Technologies) and finally sequenced in Illumina HiSeq 2500 analysis. Variants were reannotated using their potential to damage protein function with SnpEff (Cingolani et al., 2012). Gene annotations were obtained from the gene ontology database (http://www.geneontology.org/). Next, we filtered this list for the ‘Danio rerio’ taxon (resulting in 116 unique genes) and used them to annotate our RNA-seq results. A full description of these data can be found at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE64125.

Quantitative RT PCR
RNA samples were prepared in the same manner as described above for RNA-seq experiments using RNeasy kit (Qiagen), and were converted into cDNA using Superscript II kit (Life Technologies). qRT-PCRs were performed using the SYBR Green JumpStart qPCR mix (Sigma-Aldrich). The Ct (cycle threshold) values of the target genes (mybpha, grapb) in AS and PS samples were normalized to Ct values of eef1a1l gene (housekeeping gene) in the AS and PS samples, respectively.

Immunohistochemistry
Antibody staining was performed as described previously (Zeller et al., 2002). The following primary antibodies were used: znpl-1 (1:200) (Trevorrow et al., 1990), Antibody Facility, University of Oregon; SV2 (1:50), Developmental Studies Hybridoma Bank (DSHB), University of Iowa; MF20 (1:20, DSHB); F59 (1:20, DSHB); Engrailed/4D9 (1:5, 1:20, DSHB).
mRNA injections

mRNA was transcribed from linearized expression constructs using the SP6 mMessage mMachine kit (Ambion). mRNA was diluted in DEPC-water plus 0.5% Phenol Red. All embryos were injected at the one-cell stage with ~20 pg of wild-type foxc1a mRNA and 200 pg of foxc1a \textsuperscript{35S}-mRNA.

Imaging

Embryos/larvae of desired age were mounted in Vectashield (Vector Labs) and imaged using 10×, 20× and 40× lenses using either a Spinning disk (Olympus) or a laser scanning confocal (Zeiss, LSM10) microscope. Appropriate numbers of z-sections were used to create maximum intensity projection image using Slidebook (3i) or NIH Image/Fiji. Images were further processed using ImageJ and/or Photoshop. Images in Fig. 1 and Fig. 3A were created by stitching multiple xy-plane images spanning the whole embryo. Final versions of the figures for the manuscript were prepared using Adobe Illustrator. Live imaging was performed as previously described (Banerjee et al., 2012).

Photoconversion of Kik-GR protein

Transgenic larvae expressing the Kik-GR protein in motor neurons [Tg (mnx1:Kik-GR)] were anesthetized using tricane and mounted in 1.2% low melt agarose in Ringer solution. Motor neuron cells bodies of a given fin nerve expressing the Kik-GR protein were then converted from green to red using the UV line of standard Zeiss Axioscope microscope. Larvae were subsequently released from the agar. At least 6 h were given to allow the photomodified Kik-GR protein to spread through the entire axon of the converted neurons. Larvae were then mounted again and imaged for fin nerve targeting.

Quantification of nerve branching and AChR clusters

All imaging for the fin nerve branching analysis was performed using an identical acquisition parameter in LSM10 (Zeiss). Images were analyzed by Fiji (ImageJ). Briefly, we converted maximum projection images into binary images, and muscle area was outlined and calculated. In the same stack the total axonal branching area was calculated in the axonal channel of the binary images by dividing the total Integrated Density with 255. For Synaptic AChR measurement, we first outlined the muscle area and performed background subtraction and mean noise reduction. Then we used the ‘find maxima’ command to count the number of AChR cluster and considered those as synaptic AChR clusters where we had axonal signal in the same spot. We found more than 95% of the total AChRs are synaptic in both wild type and mutants. Statistical analysis was performed using the Student’s t-test.

Fin movement analysis

Five foxc1a mutant and five wild-type zebrafish larvae (96 hpf) were randomly selected, briefly anesthetized with tricane and mounted in 1.2% low melt agar, keeping the pectoral fin exposed so that they could move freely. Larvae were allowed to recover for 15 min before recording spontaneous fin movements using a high-speed video camera (200 frames/second) mounted on a stereomicroscope. Each frame was analyzed manually, and a minimum intensity projection image was created for a given fin beating cycles (as shown in Fig. 7A,B). X and Y angles were calculated in Adobe illustrators. All statistical analysis was carried out using the Student’s t-test.


