Development/Plasticity/Repair

Schwann Cells and Deleted in Colorectal Carcinoma Direct Regenerating Motor Axons Towards Their Original Path

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After complete nerve transection, a major challenge for regenerating peripheral axons is to traverse the injury site and navigate toward their original trajectory. Denervated Schwann cells distal to the lesion site secrete factors promoting axonal growth and serve as an axonal substrate, yet whether Schwann cells also actively direct axons toward their original trajectory is unclear. Using live-cell imaging in zebrafish, we visualize for the first time how in response to nerve transection distal Schwann cells change morphology as axons fragment, and how Schwann cell morphology reverses once regenerating growth cones have crossed the injury site and have grown along distal Schwann cells outlining the original nerve path. In mutants lacking Schwann cells, regenerating growth cones extend at rates comparable with wild type yet frequently fail to cross the injury site and instead stray along aberrant trajectories. Providing growth-permissive yet Schwann cell-less scaffolds across the injury site was insufficient to direct regenerating growth cones toward the original path, providing compelling evidence that denervated Schwann cells actively direct regenerating axons across the injury site toward their original trajectory. To identify signals that guide regenerating axons in vivo, we examined mutants lacking the deleted in colorectal carcinoma (DCC) guidance receptor. In these dcc mutants, a significant fraction of regenerating motor axons extended along aberrant trajectories, similar to what we observe in mutants lacking Schwann cells. Thus, Schwann cell and dcc-mediated guidance are critical early during regeneration to direct growth cones across the transection gap and onto their original axonal trajectory.

Key words: DCC; peripheral nerve; regeneration; Schwann cell; zebrafish

Introduction
In the peripheral nervous system, injured axons regenerate, although many aspects of this process, including how regenerating axons navigate across the lesion site toward their original synaptic targets, are not well understood. The regenerative capacity of peripheral axons varies with the severity and the type of injury (Zochodne, 2008; Brushart, 2011; Zochodne, 2012). For example, after a crush injury, glial-derived basal lamina remains intact, providing regenerating axons with a permissive substrate across the injury site (Cajal, 1928; Scherer and Easter, 1984; Nguyen et al., 2002). In contrast, complete nerve transection results in a Schwann cell and basal lamina free gap, presenting a major hurdle for regenerating axons. In rodents, removing the distal nerve stump or increasing the injury gap beyond 12 mm dramatically reduces regeneration, consistent with the idea that the distal nerve provides axons with critical signals for navigation across the gap, toward the distal nerve stump, and onto their original trajectory (Lundborg et al., 1981, 1982, 1986).

After injury, regenerating axons interact with a variety of non-neuronal cells, including macrophages, fibroblasts, perineurial ensheathing glia, and Schwann cells (Beuche and Friede, 1984; Perry et al., 1987; Stoll et al., 1989; Martini et al., 1990; Perry and Brown, 1992; Schröder et al., 1993; Abernethy et al., 1994; Weis et al., 1994; Parrinello et al., 2010; for review, see Brosius Lutz and Barres, 2014), yet if and to what extent these cell types direct regenerating axons toward their original developmental path has not been determined in vivo. Schwann cells in particular are thought to be essential for regeneration. Denervated Schwann cells in the distal nerve stump, known as the bands of Bungner (Bungner, 1891), serve as a substrate for regenerating axons and provide diffusible factors, including NGF, BDNF, and FGF, to promote axonal outgrowth (Lundborg et al., 1986; Ide, 1996; Chen et al., 2007). However, whether Schwann cells also provide directional information to regenerating axons pioneering the injury gap is unclear.

Here we take advantage of the genetic tractability and transparency of larval zebrafish to investigate how regenerating motor axons cross an injury-induced transection gap and select their original trajectory in live, intact animals. Using in vivo microscopy, we compared the dynamic behavior of regenerating axons and Schwann cells in wild-type larvae to mutants lacking all Schwann cells. Remarkably, the absence of Schwann cells did not impede growth cone sprouting or axonal growth, as regenerating axons extended over considerable distances. However, axons...
lacked directionality and traveled along ectopic trajectories. Providing Schwann cell-less axonal scaffolds across the injury site and along the original trajectory was insufficient to fully restore directionality to regenerating axons, suggesting that Schwann cells produce factors that directly regenerating axons to their appropriate trajectory. Finally, in mutants lacking the axonal guidance receptor deleted in colorectal carcinoma (Dcc), regenerating axons strayed from their original path onto ectopic trajectories, reminiscent of the phenotype observed in mutants lacking Schwann cells. We conclude that Schwann cells and Dcc-dependent signaling direct regenerating axons toward their original developmental trajectories in vivo.

Materials and Methods

Zebrafish genetics and transgenes. All transgenic lines were maintained in the Tuebingen or TLf genetic background and raised as described previously (Mullins et al., 2005) and the Tg(Xla.Tubb: DsRed) (Peri and Nüsslein-Volhard, 2008) lines were used to label spinal motor nerves. The Tg(sox10(7.2): mrfp)y234 (Kucenas et al., 2008), Tg(sox10:leoes)y16 (Prendergast et al., 2012), Tg(-7.2ox10:Gal4–VP16)y305Tg (gift from M. Lush and T. Pirotrowski, University of Utah, Salt Lake City, UT), and Tg(UAS:GFP) (Asakawa et al., 2008) lines were used to label Schwann cells, and the Tg(T2KUAS–E1b:nfsB–mCherry) [Parsons et al., 2009] line was used to conditionally ablate Schwann cells. The Tg(x1f:Wld–GFP)y152 line (Rosenberg et al., 2012) expresses the Wld–GFP protein in motor neurons. The sox10/z57 (Kishel et al., 1996; Dutton et al., 2001; Lyons et al., 2005; Jao et al., 2008; Perlin et al., 2011) mutants were used. Male and female zebrafish were used, and all zebrafish work was conducted in accordance with Institutional Animal Care and Use Committee regulatory standards.

Stochastic cell labeling. Axons were stochastically labeled by microinjection of 33 pg mnx1: DsRed DNA at the one-cell stage as described previously (Thermes et al., 2002). The Discomosa red (DsRed) fluorophore is strongly expressed by 24 h after fertilization, concomitantly with the expression of GFP in the transgenic line Tg(x1f:GFP)y322 (Rosenberg et al., 2012). Genotyping. Genotyping protocols for the following mutants were performed as described previously: dcrm130198 (Jain et al., 2014); erbb2b250 and erbb3b458 (Lyons et al., 2005); and nrg1a26 (Perlin et al., 2011).

Whole-mount fluorescent in situ hybridization and immunohistochemistry. Antisense digoxigenin-labeled dcr, net1a (Net4.6 probe), and net1b RNA probes were used for in situ hybridization performed as described previously (Lakhina et al., 2012). In situ signals were amplified using a cyanine 5-coupled tyramide system (TSA Plus Cyanine 5 System; PerkinElmer Life and Analytical Sciences; product number NEL74001KT). In situ hybridization was followed by immunohistochemistry using rabbit anti-GFP (1:400; Life Technologies) and goat anti-rabbit Alexa Fluor 488-conjugated secondary antibody (1:500; Invitrogen) to visualize motor neurons. Processed larvae were mounted laterally in Vectashield (Vector Laboratories) and imaged in 1 μm sections with a 20X water lens and a 40X water-immersion lens on a Zeiss 710 confocal laser scanning microscope (LSM 710) using ZEN2010 software.

The anti-sox10 antibody was a generous gift from S. Kucenas (University of Virginia, Charlotteville, VA). Five-day-old zebrafish larvae were fixed in 4% PFA with 0.1% Triton X-100 for 3 h and then washed one time for 5 min successively with PBS with 1% Triton X-100 (PBStx), deionized water with 1% Triton X-100, and 100% acetone, followed by 100% cold acetone for 10 min at −20°C. Then larvae were washed three times for 5 min in PBStx, blocked in 5% goat serum/PBStx, and incubated in 5% goat serum/PBStx/1% antibody for 1 h at room temperature and then 4°C overnight. Larvae were washed extensively with PBStx and incubated with goat anti-rabbit Alexa Fluor 594-conjugated secondary antibody (1:500; Invitrogen). Larvae were mounted in Vectashield (Vector Laboratories), and images were acquired with an Olympus IX51 equipped with a Yokogawa CSU 10 scan head combined with a Hamamatsu EMCCD camera (model C9100-13); acquisition and hardware were controlled by Slidebook version 5.0.

Fluorescent mRNA quantification. In FIIJ, fluorescent double-labeled 6 μm stacks for mRNA and motor neuron–GFP were made into a series of maximum projection images and then processed to subtract background, and a mean filter of 1 pixel was applied. A region of interest (ROI) was drawn around the ventral spinal cord in which motor neurons were labeled with GFP. mRNA levels within the ROI were counted in two ways: (1) by counting the number of local maxima; and (2) by measuring overall fluorescence levels. This analysis was repeated in smaller ROIs drawn around individual GFP-positive cell bodies.

Nerve transection and live imaging. Nerve transection and live imaging were performed as described previously (Rosenberg et al., 2012).

Axon growth extent quantification. Axon growth extent was quantified a posterior transection (hpt) using a rubric of three semiquantitative categories, illustrated in Figure 1N–P. Transected nerves in which axons failed to regrow or did not extend through the entire length of the ventral myotome were categorized as “no/weak regeneration.” Nerves with at least one fascicle that extended through the entire length of the ventral myotome were categorized as “moderate regeneration.” Finally, nerves with two or more fascicles extending through the entire length of the ventral myotome were categorized as “strong regeneration.”

Axon guidance quantification. Misguided axons were identified at 48 hpt as axons above the lateral line that regrew in areas that were not populated by axons in pre-lesion images.

Axon growth rate, Schwann cell migration, and proliferation quantification. Genotyping protocols for the following mutants were performed as described previously in 5 d post-fertilization (dpf) larvae, and then larvae were removed from agarose and incubated in E3 at 28°C until fixation at 6 or 48 hpt, following previous methods (Rosenberg et al., 2012). Longitudinal coronal sections through the tail segment extending for several myotomes on either side of the transected regions were cut starting from the ventral side and progressing dorsally. When the notochord was reached, a group of thin sections was cut at intervals of ~12–15 μm to view cross-sections of the nerve distal and up to the damaged sites. Cross-sectional profiles of nerves were present immediately adjacent to the notochord midway along each sarcomere. Profiles on the unjured left side of the same animals were used as control undamaged nerves.

Electron microscopy. Nerves were transected as described previously in 5 d post-fertilization (dpf) larvae, and then larvae were removed from agarose and incubated in E3 at 28°C until fixation at 6 or 48 hpt, following previous methods (Rosenberg et al., 2012). Nerves were cut, fixed in 4% formaldehyde, dehydrated, embedded in Epon 812, and sectioned at 600 nm. Sections were stained with uranyl acetate and lead citrate and imaged at 61,000x using a Zeiss 1093 transmission electron microscope.

Image processing. Image stacks were compressed into maximum intensity projections (MIPs) in Slidebook version 5.0. MIPs were exported and gamma adjusted to 0.5 in NIH ImageJ for increased visibility using a macro called “Batch RGB Merge,” modified from a macro called “Batch RGB Merge.” Both datasets were performed as described previously (Mullins et al., 2005) and the Tg(-7.2ox10:Gal4–VP16)y152 line (Rosenberg et al., 2012). Nerves with at least one fascicle that extended through the entire length of the ventral myotome were categorized as “no/weak regeneration.” Nerves with at least one fascicle that extended through the entire length of the ventral myotome were categorized as “moderate regeneration.” Finally, nerves with two or more fascicles extending through the entire length of the ventral myotome were categorized as “strong regeneration.”

Axon growth rate, Schwann cell migration, and proliferation quantification. Regenerating nerves and Schwann cells were imaged approximately every 10 min from ~6 to ~13 hpt, as described previously (Rosenberg et al., 2012). In NIH Image, the scale was set on these movies to 0.192 μm/pixel and pixel distance and aspect ratio to 1.0. Growth was measured at each hour by drawing lines over newly grown axon segments and recording the length in micrometers per hour. Schwann cell distance was measured using the same method, measuring from the top of the distal stump to the leading edge of the Schwann cell nucleus at each time point. Numbers of migrating and proliferating Schwann cells were counted.

Electron microscopy. Nerves were transected as described previously in zebrafish larvae by bath applying 8 μM mCherry to visualize motor neurons. Processed larvae were mounted laterally in Vectashield (Vector Laboratories) and imaged in 1 μm sections with a 20X water lens and a 40X water-immersion lens on a Zeiss 710 confocal laser scanning microscope (LSM 710) using ZEN2010 software.

The anti-sox10 antibody was a generous gift from S. Kucenas (University of Virginia, Charlotteville, VA). Five-day-old zebrafish larvae were fixed in 4% PFA with 0.1% Triton X-100 for 3 h and then washed one time for 5 min successively with PBS with 1% Triton X-100 (PBStx), deionized water with 1% Triton X-100, and 100% acetone, followed by 100% cold acetone for 10 min at −20°C. Then larvae were washed three times for 5 min in PBStx, blocked in 5% goat serum/PBStx, and incubated in 5% goat serum/PBStx/1% antibody for 1 h at room temperature and then 4°C overnight. Larvae were washed extensively with PBStx and incubated with goat anti-rabbit Alexa Fluor 594-conjugated secondary antibody (1:500; Invitrogen). Larvae were mounted in Vectashield (Vector Laboratories), and images were acquired with an Olympus IX51 equipped with a Yokogawa CSU 10 scan head combined with a Hamamatsu EMCCD camera (model C9100-13); acquisition and hardware were controlled by Slidebook version 5.0.
Results

Live-cell imaging after nerve injury reveals orchestrated cellular dynamics of axons and Schwann cells

Changes in Schwann cell morphology are a hallmark of nerve degeneration, yet the in vivo dynamics of this process have not been observed. Therefore, we fully transected individual nerves in larvae aged 5 dpf and then documented changes in Schwann cell morphology using time-lapse microscopy (Fig. 1A). Ventral projecting zebrafish motor nerves consist of ~60–80 motor axons, and individual axons form branches that synapse with muscle fibers and myotendinous junctions along hemisegment boundaries (Fig. 1A, B; Myers, 1985; Myers et al., 1986; Westerfield et al., 1986; Zhang et al., 2004; Rosenberg et al., 2012). At 5 dpf, large-diameter axons are wrapped by approximately three to five layers of myelin, approximately equivalent to that in mouse and rat during the first postnatal week (Peters and Muir, 1959; Schlaepfer and Myers, 1973; Hahn et al., 1987; Garbay et al., 2000). Motor nerves were laser transected as reported previously (Rosenberg et al., 2012) within the first ~20 μm of the peripheral nerve trajectory in larvae expressing GFP in motor neurons and RFP in Schwann cells Tg(mnx1:GFP;sox10:mRFP). Before axon fragmentation, Schwann cell membranes were elongated and closely associated with motor axons (Fig. 1B, C). Once axons started to fragment, Schwann cell membranes distal to the lesion

Figure 1. Schwann cells change morphology as transected motor nerves degenerate and regenerate. A, At 5 dpf, Tg(mnx1:GFP) larva expresses GFP in spinal motor neurons and axons. White box, Single motor nerve. B–H, Schwann cells change morphology as axons fragment. B, I, Pre-lesion nerve in 5 dpf Tg(mnx1:GFP;sox10-mRFP) larva. Red box, Area of laser axotomy. As axons fragment, Schwann cell membranes (magenta) surrounding axons (green) change morphology (C–H) and in 2 h begin degrading axonal debris contained within their membranes (D–H) (arrowheads, E–H). I–L, Schwann cell membrane morphology changes over 48 hpf (insets, 1 μm z-plane). J, At 7 hpt, distal axons have completely fragmented, and remaining denervated Schwann cell membranes appear gnarled. K, At 24 hpt, axons have regrown into the ventral myotome along original trajectory in which Schwann cells remained. L, At 48 hpt after axon regeneration, Schwann cell membranes appear thinner and smoother, like pre-lesion Schwann cells. Scale bar, 10 μm. M–P, Diagram regeneration categories for quantification of nerve regeneration at 48 hpt (Q).
site began to reorganize, changing from a tube-like morphology to a shorter, more rounded morphology containing large blebs of fluorescently labeled axonal debris (Fig. 1D–H). The fluorescent intensity of the encircled debris decreased over the next 2 h (Fig. 1E–H), consistent with the observation that axonal debris is degraded by phagocytic vesicles present in Schwann cells (Holtzman and Novikoff, 1965; Scherer and Easter, 1984). To visualize changes in Schwann cell morphology as axons regenerate, we transected nerves and imaged Schwann cells and regenerating motor axons over a 48 h time period (Fig. 1I–L). By 7 hpt, the nerve segment distal to the transection site had fragmented, leaving behind only axonal debris and a ribbon of denervated distal Schwann cells, known as the band of Bungner (Fig. 1J; Bungner, 1891). Compared with pre-lesion Schwann cell membranes (Fig. 1I), these distal Schwann cell membranes appeared distended and gnarled (Fig. 1E–H). Within 24 hpt, motor neurons reextended axons past the lesion site, in close proximity to the original path delineated by denervated Schwann cells (Fig. 1K). By 48 hpt, regenerating axons had traversed the ventral myotome in which they reestablished complex branches. Concomitantly, Schwann cell membranes reverted to their thinner appearance in proximity to regenerating axons (Fig. 1I–L), and their membranes reacquired a smoother, pre-lesion like morphology (Fig. 1I). Single micrometer z-planes (Fig. 1I–L, insets) demonstrate that axons extended in close proximity to the denervated distal Schwann cells, similar to what has been shown in mammalian systems (Scherer and Easter, 1984; Westerfield et al., 1986; Chen et al., 2007; Zochodne, 2008; Rosenberg et al., 2012). To quantify the extent of axon regeneration at 48 hpt, we applied a three-category rubric illustrated in Figure 1M–Q (for details, see Materials and Methods). In wild-type larvae, ~80% of transected motor nerves (n = 85 nerves from 22 larvae) regrew successfully through the length of the ventral myotome (Fig. 1Q, “strong” and “moderate” categories) and formed functional synapses (Rosenberg et al., 2012). Thus, laser axotomy triggers changes in axonal and Schwann cell morphology, and live imaging reveals that these changes are highly coordinated between the two cell types, during both degeneration and regeneration.

Schwann cell migration and proliferation during the early phase of axon regeneration

We next asked how individual Schwann cells respond to nerve transection. Previous studies have shown that Schwann cell migration and proliferation are critical to axon regeneration because blocking these processes leads to misrouted axons (Cajal, 1928; Politis et al., 1982; Hall, 1986a; Stoll et al., 1989; Chen et al., 2005). To visualize Schwann cell behaviors, we used larvae in which motor axons are labeled with DsRed via the Tg(Xla.Tubb: DsRed) transgene (Peri and Nüsslein-Volhard, 2008) and Schwann cell nuclei with the photoconvertable fluorophore Eos via the Tg(sox10:nlsEos) transgene (Prendergast et al., 2012). Using an anti-sox10 antibody, we first confirmed that the sox10: nlsEos line faithfully labels all Schwann cell nuclei on ventral motor nerves (Fig. 2H–J; n = 348 of 351 nuclei cotubed).

We then transected and imaged motor nerves every 10 min from ~6 to ~13 hpt, a period during which motor axons are actively regrowing (Fig. 2A–F; see Fig. 6A–H). Live-cell imaging revealed that sox10-positive cells migrated in ~42% of transected nerves (n = 27 migrating cells on 19 nerves of 45 nerves analyzed; Fig. 2G). Migration on the distal nerve stump toward the transection site was more frequent (n = 24 migrating cells on 19 nerves) than migration on the proximal nerve stump toward the transection site (n = 3 migrating cells on 19 nerves), suggesting that Schwann cells respond differently to nerve transection based on their relative position to the injury site. Finally, we observed dividing Schwann cells in only 14% of transected nerves (n = 6 Schwann cells on 45 nerves). Given that ~80% of transected nerves regenerate successfully (Fig. 1Q), our data suggest that Schwann cell migration and proliferation are likely less important for the initial stages of axon regeneration when axons cross the lesion site and extend toward their original trajectory.

Motor axon regeneration is impaired in the absence of Schwann cells

To define the in vivo role of Schwann cells for axonal regeneration, we examined four genetic mutant strains lacking differentiated Schwann cells. Specifically, we analyzed mutants lacking the sox10 transcription factor, which is critical for early neural crest development, mutants lacking erbb2 or erbb3, which form a heterodimeric receptor critical for Schwann cell differentiation, and mutants lacking the ErbB2/ErbB3 ligand nrg1typeIII (Kelsh et al., 1996; Dutton et al., 2001; Lyons et al., 2005; Perlin et al., 2011). In all mutants, motor axons develop without delay, and through 5 dpf, their motor axons are morphologically indistinguishable from those in wild-type siblings (Fig. 3A, E; data not shown). Furthermore, the absence of Schwann cells did not affect Wallerian degeneration and debris removal (Rosenberg et al., 2012).

Compared with wild-type siblings, axon regeneration was severely affected in all mutants lacking Schwann cells (Fig. 3C,D compared with G,H), consistent with what has been reported for lateral line nerve regeneration (Villegas et al., 2012). Quantification of axon regeneration at 48 hpt revealed that, in mutants lacking Schwann cells, in 50–80% of transected nerves, the regenerating axons failed to extend along their original trajectory through the ventral myotome compared with <20% in wild-type siblings (Fig. 3I). Instead, regenerating axons grew extensively into lateral territories, in which they formed thick fascicles through the lateral myotome in areas that are sparsely innervated both before lesion and after regeneration in the presence of Schwann cells (Fig. 3G,H), suggesting that Schwann cells direct regenerating axons.

We next wanted to determine the degree of regeneration at the ultrastructural level. For this, we performed electron microscopy at 6 hpt when most axons have fragmented and at 48 hpt when axons have regenerated (Rosenberg et al., 2012). Compared with uninjured nerves, which contain Schwann cells adjacent to axonal profiles separated by myelin sheets (Fig. 4A,B), axotomized nerves at 6 hpt appeared disorganized at the level of the transection site, lacking discernible axonal membranes and instead containing collapsed myelin sheets (Fig. 4C). Similar to intact control nerves (Fig. 4D,E), transected nerves at 48 hpt contained Schwann cells adjacent to both small- and large-diameter axons, which had regrown past the lesion site onto their original path (Fig. 4F). At this time point, myelin levels had not yet returned to pre-lesion levels, with only partially formed myelin sheets detectable (Fig. 4F compared to Fig. 4E). To understand how axons regrow in the absence of Schwann cells, we examined nerves in sox10–/– mutants. Except for the lack of Schwann cells, axonal profiles and the nerve environment appeared normally organized in uninjured nerves (Fig. 4G,H). However, after injury in sox10–/– mutants, axonal profiles at 48 hpt were observed invading aberrantly into muscle territories, deviating far from the normal motor nerve path (Fig. 4I), consistent with the misguided regrowth observed using confocal microscopy (Fig. 3G,H).
Schwann cells direct the growth of regenerating axons toward the original nerve path

One possible mechanism by which Schwann cells might direct regenerating axons is through extracellular marks produced by Schwann cells during development. To exclude this possibility, we ablated Schwann cells acutely, just before nerve transection. Schwann cells were ablated by treating larvae expressing the enzyme nitroreductase under the sox10 promoter in Tg(-7.2sox10:Gal4–VP16; T2KUAS–E1b:nfsB–mCherry (abbreviated as sox10:NTR–mCherry; Curado et al., 2007; Parsons et al., 2009). In control larvae (sox10:NTR–mCherry positive; Mtz negative and sox10:NTR–mCherry negative; Mtz positive), transected motor nerves regenerated indistinguishably from those in wild type (Fig. 5A–D, quantified in G). In contrast, beginning Mtz exposure of sox10:NTR–mCherry-positive larvae at 3.5 dfp efficiently ablated sox10:NTR–mCherry-positive cells by 5 dfp (data not shown). Importantly, by 48 hpt, regenerating axons had extended along the same aberrant trajectories we observed in sox10−/− mutants lacking Schwann cells (Fig. 5F, G). This suggests that the presence and activity of Schwann cells before nerve transection does not influence axon regeneration after injury and strongly suggests that Schwann cells must be present during regeneration to direct regrowing axons.
We next asked when axons begin to depend on Schwann cells. For this, we transected motor nerves in siblings and sox10\(^{+/-}\)/H11002\(^{+/-}\) mutants during the time period when motor axons are still pioneering through the myotome (1 dpf), at 2 dpf when additional axons extend along the pioneering tracts, and at 3 and 4 dpf when axons have fully extended through the myotome. In wild-type animals, nerves were able to fully regenerate, independent of when the nerves were transected (Fig. 5H). In contrast, there was a marked shift in the regenerative capacity of sox10\(^{+/-}\) mutants. Although nerves transected at 1 dpf regenerates equally as well as wild-type nerves (Fig. 5H), nerves transected at 2–4 dpf resulted in the same aberrant growth of regenerating axons observed in sox10\(^{+/-}\) mutant nerves transected at 5 dpf (Fig. 5H). Thus, the capacity for motor axons to regrow along their original path rapidly shifts from a Schwann cell-independent mechanism, present only during the phase of initial outgrowth, to a life-long Schwann cell-dependent mechanism that has already begun during development.

Finally, we asked whether Schwann cells influence growth rates and/or growth direction when regrowing axons cross the injury gap and extend toward their original trajectory. Time-lapse analyses of regenerating wild-type axons revealed that regeneration commences as early as 3–5 hpt, when multiple growth cones sprouted from the proximal stump in all directions (data not shown). Beginning at ~5–7 hpt, growth cones had crossed the injury gap (Fig. 6A–C), and then over the next few hours rapidly pioneered a path along their original trajectory (Fig. 6C–E). Additional regenerating axons joined and formed fascicles with the pioneer axons, and over the next 10 hours, these fascicles extended through the entire length of the ventral myotome (Fig. 6E–H).

Analysis of sox10\(^{-/-}\) mutants revealed that regenerative growth cones sprouted and extended from the proximal stump in...
multiple directions, indistinguishable from those in wild type (Fig. 6l–p; n = 16 nerves). However, in contrast to wild type, growth cones in sox10−/− mutants continued to extend in all directions, without establishing a growth preference toward their original path (Fig. 6l–p). Furthermore, axons that initially grew in the appropriate ventral direction rarely extended ventrally beyond the level of the lateral line and instead grew along ectopic lateral trajectories (Fig. 6l–p). Importantly, average forward growth rates and maximum forward growth rates of axons in mutants and siblings were similar (wild type, 0.22 mm/d average with a maximum rate of 0.50 mm/d, n = 8 axons from 8 nerves in 6 animals; sox10−/− mutants, 0.23 mm/d average rate with a maximum rate of 0.44 mm/d, n = 10 axons from 9 nerves in 6 mutants). Thus, in the absence of Schwann cells, regenerating axons retain the capacity to sprout growth cones and extend, but they stray onto multiple, aberrant trajectories, failing to stabilize toward their original developmental path. Combined, these genetic analyses provide compelling evidence that, within a few hours after axons sprout from the nerve stump, Schwann cells provide regenerating growth cones with guidance critical for axons to navigate toward the original path.

Axonal scaffolds fail to compensate for Schwann cell-dependent guidance

Our data are consistent with the idea that, as growth cones sprout from the proximal nerve stump, Schwann cells direct regrowing axons onto their original path. Conceptually, this could be accomplished through several mechanisms. The prevailing view in the literature suggests that denervated Schwann cells and their surrounding basement membrane act as a substrate, sufficient to...
outline a path toward their original targets (Cajal, 1928; Ide et al., 1983; Hall, 1986a; Chen et al., 2005; McDonald et al., 2006; Par- rivello et al., 2010). Alternatively, denervated Schwann cells in the distal nerve stump might produce factors that direct regrowing axons onto the original path, but the identity of such factors has remained mostly elusive (Politis et al., 1982; Kuffler, 1986; Heumann et al., 1987). During development, when differentiated Schwann cells are absent, axons fasciculate with and grow along other axons (Tessier-Lavigne and Goodman, 1996; McDonald et al., 2006), suggesting that axons form a suitable substrate to support axonal growth. Furthermore, our live-cell imaging reveals that regenerating axons fasciculate with pioneering axons (Fig. 6D–H).

To determine whether a physical scaffold delineating the original path is sufficient to guide motor axons, we generated two experimental paradigms that provide a permissive scaffold devoid of Schwann cells. First, we took advantage of a transgenic line we had generated previously, in which axonal fragmentation is delayed for >1 week as a result of overexpression of the Wallerian degeneration slow (Wld<sup>s</sup>) protein in motor neurons (Rosenberg et al., 2012). Therefore, we transected nerves in larvae lacking Schwann cells and expressing Wld<sup>s</sup> selectively in motor axons (Tg(mnx1:GFP; Wld<sup>s</sup>–eGFP); sox10<sup–/–</sup>) to generate an axonal scaffold distal to the transection gap completely lacking Schwann cells (Fig. 7). Consistent with previously published results (Lunn et al., 1989; Rosenberg et al., 2012), we find that, in the presence of Schwann cells, regenerating axons extended alongside and intermingled with Wld<sup>s</sup>-positive axons, demonstrating that they are not inhibitory to axon regrowth (Fig. 7E–H). Despite the presence of an axonal scaffold positioned in close proximity to the proximal stump (Fig. 7I–L), in larvae lacking Schwann cells, regenerating axons still failed to navigate onto their original path and instead extended along ectopic trajectories identical to what we observed in sox10<sup–/–</sup> mutants (Fig. 7, compare A–D, I–L, quantified in U).

Crossing the transection gap is a well known challenge for regenerating peripheral axons (Zochodne, 2008), presenting one possible explanation why regenerating axons extended along ectopic trajectories despite the presence of an axonal scaffold along their original path. To test whether a permissive scaffold spanning the injury gap is sufficient for proper regenerative growth, we provided regenerating axons with a continuous axonal scaf-
fold that spanned the injury gap and extended along the entirety of the original path. We generated this uninterrupted axonal scaffold devoid of Schwann cells by transecting approximately half of the 60–80 motor axons in the motor nerve (Fig. 7). In wild-type siblings, the vast majority of regrowing axons in partially transected nerves regrew along their original trajectory, with few axons straying from appropriate trajectories at 48 hpt (Fig. 7M–P, quantified in V). In mutants lacking Schwann cells, we observed axons regenerating along ectopic trajectories in ~50% of partially transected nerves (n = 22 of 44), down from ~90% in fully transected nerves lacking Schwann cells (Fig. 7Q–T, quantified in U,V). Thus, the presence of a continuous axonal scaffold that bridges the transection gap can only partially compensate for the role of Schwann cells in directing regrowing axons onto their original path. Combined, our data provide compelling evidence that, in vivo, Schwann cells serve regrowing axons with more than a permissive substrate that simply connects regenerating growth cones with the denervated Schwann cells along their original path. Instead, we propose that Schwann cells actively guide axons across the injury gap and toward their original path.

The DCC guidance receptor is required for regenerative axon guidance

We next sought to define the role of canonical axon guidance systems in Schwann cell-dependent guidance during regeneration. Several canonical axon guidance systems, including Netrin...
and its receptor DCC have been implicated in promoting the extent of axon regeneration (Madison et al., 2000; Tanno et al., 2005; Gabel et al., 2008; Webber et al., 2011). However, an in vivo function for DCC in regenerative axon guidance has not been defined. In situ hybridizations at 5 dpf revealed that dcc mRNA is expressed throughout the ventral spinal cord (Fig. 8A) and that dcc mRNA colocalizes with GFP-positive motor neurons in Tg(mnx1:GFP) larvae, as well as with Schwann cells along the motor nerve in Tg(-7.2sox10:Gal4VP16;UAS:GFP) larvae, similar to what has been reported in mouse (Fig. 8B–D,G,H; Webber et al., 2011). Netrin1a mRNA is ubiquitous throughout the periphery, both before and after transection (data not shown; Fig. 8L), whereas netrin1b mRNA is concentrated in the ventral spinal cord but also colocalizes with Schwann cells along the motor nerve before and after transection (data not shown; Fig. 8I–K). Importantly, dcc mRNA in GFP-positive motor neurons was detectable during the period of initial axonal regrowth at 6 and 9 hpt (Fig. 8C–F; data not shown).

To test whether DCC is required for motor axon regeneration in vivo, we transected nerves in dcc zm130198 mutants. The dcc zm130198 allele carries a retroviral insertion 106 bp upstream of the start codon, resulting in a 90% reduction of dcc mRNA (Jain et al., 2014). Importantly, motor axons and Schwann cells develop normally in dcc zm130198 mutants, and at 5 dpf, they are morphologically indistinguishable from those in wild-type siblings (Fig. 8N,R; data not shown). By 48 hpt, we found that 78% of transected dcc zm130198 mutant nerves extended motor axons along their original path (n = 46 of 59 nerves), similar to what we...
observed in wild-type siblings (90%, n = 28 of 31 nerves). However, in ~40% of the transected nerves in dcczm130198, regenerating motor axons extended not only along their original path but also along ectopic lateral trajectories (Fig. 8T, U, quantified in M). Importantly, misdirected axons deviated from the original path at the level of the injury gap and extended into lateral territories, albeit less frequently, but similar to what we observed in mutants lacking Schwann cells (Fig. 3). Thus, dcc is required in vivo to guide regenerating motor axons across the injury gap toward their original trajectory. Combined, our results demonstrate that both Schwann cell- and dcc-dependent guidance is required during the early steps of axon regeneration to direct regenerating growth cones toward their original path.

### Discussion

Axons in the peripheral nervous system can reestablish functional connections with their original synaptic targets, primarily because the PNS environment promotes regrowth (Aguayo et al., 1981; David and Aguayo, 1981). Thus, to understand how peripheral axons regenerate, it is fundamentally important to visualize how injured axons interact with their cellular environment in vivo. We previously developed a system to record the process of motor axon degeneration in vivo and to document the intricate cellular interactions between injured motor axons and macrophages (Rosenberg et al., 2012). Here, we use this system to examine how Schwann cells respond to nerve injury. We find that...
Schwann cells react to injury in synchrony with dynamic changes in axonal morphology, demonstrate that Schwann cells provide more than a simple permissive substrate to direct regenerating axons, and illustrate a role for *dcc*-dependent signaling in guiding sprouting growth cones across the injury gap and toward their original path.

**Changes in Schwann cell morphology and axonal fragmentation are highly synchronized**

Schwann cells are the principle glia of the peripheral nervous system and are known to promote axonal regeneration. After injury, Schwann cells collaborate with macrophages to degrade axon and myelin debris, clearing the path for axon regeneration. They also undergo dramatic morphological changes, reflecting their dedifferentiation to a more immature state, critical for their own proliferation and production of diffusible factors that promote axon outgrowth (Holtzman and Novikoff, 1965; Scherer and Easter, 1984; Jessen and Mirsky, 1999, 2008). However, whether axonal degeneration is synchronized with the various changes in Schwann cell behavior has not been examined in live intact animals. We find that, as axons fragment, distal Schwann cells change their morphology and over the next 24 h retain this rounder appearance (Fig. 1C–H). During the same time period, Schwann cells also become motile, extending toward and sometimes across the lesion site (Fig. 2). The functional significance of this early migration is unclear, because ~80% of transected nerves regenerated, whereas Schwann cell migration was detectable in only 42% of these nerves.

Earlier work suggested that Schwann cell proliferation is important for nerve regeneration, but the time period during which proliferation is critical for regeneration has remained elusive (Hall, 1986a; Scaravilli et al., 1986). We find that, within the first 10–13 hpt, Schwann cell proliferation was only evident in 14% of transected nerves, consistent with the idea that Schwann cell proliferation is less important when axons cross the injury site and extend toward their original trajectory but might become important later to replace lost Schwann cells and remyelinate regrown axons. Finally, after axons crossed the injury gap and extended over the denervated Schwann cells along the original path, distal Schwann cell membranes began to revert to a smoother, pre-lesion appearance (Fig. 1I–L). Thus, after nerve injury, axon and Schwann cell behaviors change dramatically and with remarkable synchronicity.

**Schwann cells direct regenerating axons**

Peripheral axons often regenerate successfully after crush injuries, as the basal lamina remains essentially intact at the injury site and is continuous with the band of Bungner, which can serve as a substrate (Cajal, 1928; Scherer and Easter, 1984; Nguyen et al., 2002). In contrast, complete nerve transection disrupts Schwann cells and the basal lamina, resulting in a gap at the injury site. Although peripheral axons can regenerate across the transection gap, removal of the distal stump or increasing the size of the gap diminishes the ability of axons to regenerate, indicating that the distal nerve provides critical signals that support regeneration (Lundborg et al., 1981, 1982, 1986). One perspective that dominates the literature is that Schwann cells produce neurotrophic factors that promote axon regrowth (for review, see Vargas and Barres, 2007).

We assessed regeneration in four mutants that lack Schwann cells without affecting development or morphology of motor axons (Fig. 3) or Wallerian degeneration (Rosenberg et al., 2012). Our time-lapse movies demonstrate that, early in regeneration, growth cones sprout from *sox10*+/− nerve stumps with the same latency and extend at similar growth rates compared with wild type, but along ectopic routes ignoring their original trajectory (Fig. 6). Although we only analyzed axonal growth rates until 13 hpt, our data suggest that, early during the regeneration process, pioneering motor axons can rely on cell types other than Schwann cells as their source of growth-promoting factors. For example, neurotrophins are known to also be expressed by skeletal muscle (Griesbeck et al., 1995), and in denervated gastrocnemius muscle, BDNF mRNA levels increase (Fukakoshi et al., 1993). At the same time, our results also provide compelling *in vivo* evidence that Schwann cells provide directionality to axons as they cross the injury site and navigate onto their original trajectory.

The prevailing view in the literature is that denervated Schwann cells themselves, or in conjunction with fibroblasts, form a bridge across the lesion site that provides regenerating axons with a permissive substrate that leads to the denervated Schwann cells in the distal nerve stump along the original trajectory (Cajal, 1928; Ide et al., 1983; Hall, 1986a,b; Chen et al., 2005; McDonald et al., 2006; Parrinello et al., 2010). Although even basal lamina extracted from peripheral nerve segments and then implanted as a scaffold at the proximal nerve is able to provide an effective pathway for regrowing axons (Ide et al., 1983), if and to what extent purely axonal scaffolds can serve as a regenerative substrate *in vivo* is unclear. We find that, in the presence of Schwann cells, a continuous physical bridge consisting of non-transected axons was permissive to regrowth. In the absence of Schwann cells, such axonal scaffolds improved directional regrowth by 50% compared with when no bridge was provided (Fig. 7Q–T, and V compared with A–D, U) but failed to fully compensate for the absence of Schwann cells and resulted in twice the frequency of misguided axons (~50%) compared with partially transected wild-type nerves (~20%; Fig. 7M–P, U). Thus, *in vivo*, permissive Schwann cell free substrates support partial axonal regeneration yet fail to compensate for the robust guidance provided by Schwann cells. Importantly, our experiments do not distinguish between a contact-dependent role for Schwann cells in directing regenerating axons, e.g., by providing a permissive substrate unique to Schwann cells, and a contact independent role for Schwann cells, e.g., in providing directional guidance cues emanating from Schwann cells distal to the lesion site.

**DCC directs axons early during regeneration**

Complete nerve transection resulting in an injury gap resembles development, when pioneering axons navigate through a changing environment. Expression of the canonical guidance molecules Netrin1 and Slit2 are upregulated in the distal nerve stump after transection (Wang et al., 1999; Madison et al., 2000; Tanno et al., 2005; Park et al., 2007), as are NGF and GDNF, which can act as neurotropic cues (Cajal, 1928; Letourneau, 1978; Gundersen and Barrett, 1979; Schuster et al. 2010; Arthur-Farraj et al., 2012). During development, DCC is expressed on axonal growth cones and responds to Netrin secreted from midline glia cells (Kennedy et al., 1994; Chan et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996; Lauderdale et al., 1998). After sciatic nerve transection, *netrin-1* mRNA levels are upregulated in the band of Bungner Schwann cells (Madison et al., 2000). After peripheral nerve transection in mammals, regenerating axons and Schwann cells express DCC (Madison et al., 2000; Webber et al., 2011). Furthermore, siRNA knockdown of *dcc* reduces the number and extent of regenerating axon fibers (Webber et al., 2011), and in *netrin-1* heterozygous mice, the extent of peripheral nerve regeneration is significantly reduced (Jaminet et al., 2013). However, if and to what extent Netrin/DCC signaling guides regenerating axons has not been examined *in vivo*. 

### References

- Cajal, 1928
- Ide et al., 1983
- Hall, 1986a,b
- Chen et al., 2005
- McDonald et al., 2006
- Parrinello et al., 2010
- Arthur-Farraj et al., 2012
- Kennedy et al., 1994
- Chan et al., 1996
- Kolodziej et al., 1996
- Mitchell et al., 1996
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- Madison et al., 2000
- Tanno et al., 2005
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- Gundersen and Barrett, 1979
- Schuster et al. 2010
- Letourneau, 1978
- Maddison et al., 2000
- Ide et al., 1983
- Rosen et al., 1982
- Lauderdale et al., 1998
- Arthur-Farraj et al., 2012
- Jaminet et al., 2013
Although we were not able to resolve differences in mRNA expression before and in the hours after nerve transection, we find that the components of this signaling system are present in the cells important for axon regeneration. Consistent with a potential role in motor axon regeneration, we find that netrin1b mRNA colocalizes with Schwann cells, and that besides the widespread distribution of dcc mRNA in the spinal cord, dcc also localizes to motor neurons and Schwann cells (Fig. 8). We find that motor axons in dcc+/- mutants regenerate, yet a significant portion of motor axons extended along ectopic trajectories, somewhat similar to what we observed in animals lacking Schwann cells. Future experiments will determine whether in zebrafish dcc function is required in regenerating motor axons to respond to Netrin, possibly secreted from distal denervated Schwann cells, to attract axons across the injury site. Alternatively, dcc might function in proximal nerve stump Schwann cells, drawing these Schwann cells across the injury site, thereby forming a physical bridge that regenerating axons use to cross the injury gap. Our combined results reveal a novel and definitive role for Schwann cells and dcc-mediated guidance early in peripheral nerve regeneration to direct growth cones across the transection gap and onto their original axonal trajectory.

Notes

Supplemental material for this article is available at http://www.med.upenn.edu/granato/publications.shtml. Movie 1, Axonal and Schwann cell behaviors after nerve transection. Movie 2, Schwann cell migration after nerve transection. Movie 3, Axonal regeneration in wild type. Movie 4, Axonal regeneration in sox10+/− mutants lacking Schwann cells. This material has not been peer reviewed.

References


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