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Neuron

The *lh3* Glycosyltransferase Directs Target-Selective Peripheral Nerve Regeneration

Highlights

- Regenerating zebrafish motor axons exhibit target-selective innervation in vivo
- The glycosyltransferase *lh3* directs growth and guidance of regenerating axons
- Post-injury and Schwann cell *lh3* expression restore target-selective regeneration
- *lh3* acts through its substrate *collagen4a5* to destabilize mistargeted axons

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In Brief

Since Cajal's observations, there has been intense debate as to whether axons regenerate randomly or with preference for their original targets. Isaacman-Beck et al. identify a glycosyltransferase-dependent pathway acting in Schwann cells to convey target-selective regeneration by destabilization of mistargeted axons.

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The *lh3* Glycosyltransferase Directs Target-Selective Peripheral Nerve Regeneration

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SUMMARY

Functional PNS regeneration requires injured axons to return to their original synaptic targets, yet the mechanisms underlying target-selective regeneration have remained elusive. Using live-cell imaging in zebrafish we find that regenerating motor axons exhibit a strong preference for their original muscle territory and that axons probe both correct and incorrect trajectories extensively before selecting their original path. We show that this process requires the glycosyltransferase *lh3* and that post-injury expression of *lh3* in Schwann cells is sufficient to restore target-selective regeneration. Moreover, we demonstrate that Schwann cells neighboring the transection site express the *lh3* substrate *collagen4a5* and that during regeneration *collagen4a5* destabilizes axons probing inappropriate trajectories to ensure target-selective regeneration, possibly through the axonal repellant *slit1a*. Our results demonstrate that selective ECM components match subpopulations of regenerating axons with their original targets and reveal a previously unappreciated mechanism that conveys synaptic target selection to regenerating axons in vivo.

INTRODUCTION

Axons of the peripheral nervous system have the remarkable ability to regenerate following injury and to form functional connections with their original targets. Damage to peripheral nerves such as trauma, disease, or chemical insult triggers the well-characterized program of Wallerian degeneration that results in axonal fragmentation and debris clearance involving immune and Schwann cells (Vargas and Barres, 2007; Waller, 1850). Concomitantly, denervated Schwann cells de-differentiate to support axonal regrowth from the proximal nerve stump (Rosenberg et al., 2014; Zochodne, 2008). There, intrinsic and extrinsic factors promote sprouting of axonal growth cones, which then begin to re-establish functional connections with their original synaptic targets (reviewed in Brushart, 2011; Zochodne, 2008).

Not surprisingly, the degree of functional regeneration depends largely on the type of injury (Kruspe et al., 2014). For example, crush injuries leave the nerve-ensheathing basal lamina intact, providing an uninterrupted tube-like substrate leading regenerating axons back to their appropriate targets (Haftek and Thomas, 1968; Scherer and Easter, 1984; Sketelj et al., 1989; Westerfield and Powell, 1983). In contrast, nerve transections disrupt the continuity of the nerve and nerve basal lamina, forcing regenerating axons to navigate across the injury gap through an acellular environment (Forman and Berenberg, 1978; Forman et al., 1979). This challenge is even greater in cases when regenerating axons encounter a nerve branch choice point distal to the injury site. Axons that fail to select appropriate branch-specific trajectories frequently miss their original targets, thereby decreasing the degree of functional regeneration (reviewed in Brushart, 2011). Moreover, misguided axons can innervate inappropriate targets, leading to involuntary muscle contractions such as those observed in facial palsy (Kimura et al., 1975; Spector et al., 1991). Several studies argue that this sparse and/or ectopic axonal reinnervation is the result of regenerating axons selecting their path at branch points in a stochastic manner (English, 2005; Scherer, 1986; Westerfield, 1987; Westerfield and Powell, 1983), while others conclude that regenerating axons somehow "recognize" their original trajectory (Brushart, 1988; Grimm, 1971; Kuffler, 1986a; Lee and Farel, 1988; Mark, 1965; Sperry and Arora, 1965; Stephenson, 1979). However, the mechanisms and molecules that enable regenerating axons to select their original trajectory at branch choice points in vivo have remained elusive.

Extracellular matrix (ECM) components and their modifying enzymes are known to provide critical guidance to developing axons, and while several ECM components are transcriptionally upregulated following peripheral nerve injury, their roles in axonal regeneration have not been well defined in vivo (Chen et al., 2011; Kubo et al., 2002; Nix et al., 2014). In regenerating peripheral nerves, ECM components are the second most upregulated class of genes, and though regenerating axons associate with the ECM as they return to their targets (Chen et al., 2011; Chernousov and Carey, 2000; Kubo et al., 2002; Nix et al., 2014), the role of ECM components and their modifying enzymes has not been fully elucidated in genetic loss-of-function studies due to their frequent essential requirement during development (George et al., 1993; Guo et al., 1991; Löhler et al., 1984; Myllyharju and Kivirikko, 2004; Pöschl et al., 2004; Ruotsalainen et al., 2006; Smyth et al., 1999). Here, we take advantage of the optical transparency and stereotyped peripheral motor nerve architecture in larval zebrafish to determine the role of ECM components in target-specific regeneration of spinal motor axons. We find that the collagen-modifying glycosyltransferase *lysyl*

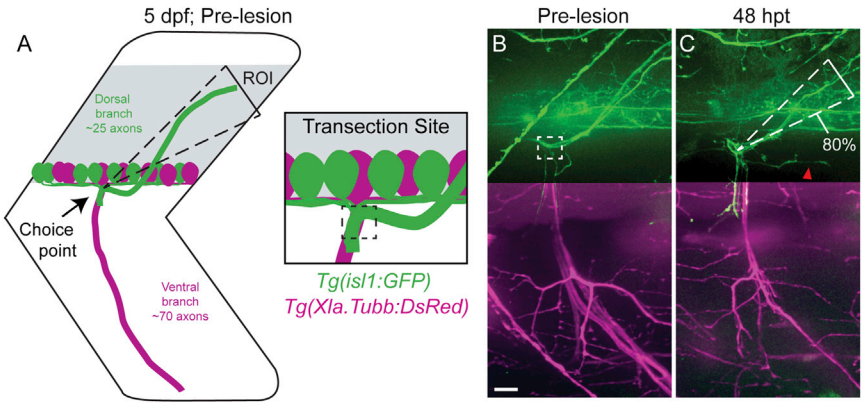

Figure 1. Regenerating Motor Axons Select Their Original Trajectory with High Fidelity

(A and B) A schematic (A) and in vivo imaging (B) demonstrate that zebrafish peripheral motor axons traverse a common path then diverge to innervate functionally distinct myotomal regions (dashed triangle, dorsal ROI; dashed boxes, nerve transection site; scale bar, 10 μm).

(C) By 48 hpt, dorsal axons regrow with great fidelity to the original trajectory (80% of fascicles that developed in the dorsal ROI regrew on the dorsal path; n = 14 larvae, 26 nerves; red arrowhead, misguided regrowth).

In (B) and (C), we omitted *Tg(Xla.Tubb:DsRed)* signal from the dorsal panel as expression in the spinal cord overwhelms the max projection image. The ventral panel shows expression from both transgenes.

hydroxylase 3 (*lh3*) is critical for regenerative growth and guidance of axons of the dorsal, but not ventral, nerve branch and that *lh3* expression during regeneration and in Schwann cells is sufficient to restore dorsal regeneration. Furthermore, we show that in vivo *lh3* exerts its function at least in part through its well-established substrate *collagen4a5* (Ruotsalainen et al., 2006; Wang et al., 2000) and that following nerve transection *collagen4a5* mRNA is selectively upregulated in Schwann cells at the lesion site. Combined, our results revise the widely held assumption that during regeneration ECM components serve primarily as permissive substrates and reveal an underappreciated, yet specific, role in directing regenerating axons toward their original targets.

RESULTS

Regenerating Motor Axons Select Their Original Trajectory with High Fidelity

Following complete nerve transection, peripheral axons can successfully traverse a short, acellular injury gap, yet whether axons randomly extend toward their original targets when confronted with a path choice or whether mechanisms for target-selective innervation exist has long been a point of contention (Brushart, 1993; English, 2005; Kuffler, 1986a; Scherer, 1986; Westerfield and Powell, 1983). As a first step to distinguish between these possibilities in a live vertebrate system, we took advantage of the simple architecture of larval zebrafish peripheral motor nerves. Each motor nerve consists of approximately 100 fasciculated axons, which separate into two main nerve branches shortly after exiting from the spinal cord—a ventral nerve branch consisting of 60–80 axons with synaptic targets in the ventral myotome, and a dorsal nerve branch consisting of 20–30 axons innervating the dorsal myotome (Myers et al., 1986; Westerfield, 1987; Westerfield et al., 1986 and Figure 1A).

To test whether regenerating motor axons preferentially select their original branch-specific nerve path, we laser transected the entire motor nerve proximal to where the trajectories of the dorsal and ventral branches diverge, creating an ~9 μm gap between the proximal and distal nerve stumps (Binari et al., 2013; Lewis and Kucenas, 2014; Rosenberg et al., 2014). We labeled both ventral and dorsal nerve axons using the *Tg(Xla.Tubb:DsRed)* transgene (Peri and Nüsslein-Volhard, 2008) and selectively labeled the dorsal branch with the *Tg(isl1:GFP)* transgene (Uemura et al., 2005), thereby enabling us to monitor target-selective regeneration in vivo (Figures 1A and 1B). Prior to nerve transection (pre-lesion), the majority of *Tg(isl1:GFP)*-labeled fascicles extend within a very narrow area that spans 20° of the dorsal myotome (Figures 1A and 1B). 48 hr post nerve transection, 80% of these fascicles regenerated to this original area (Figure 1C; see Experimental Procedures for more details on quantification). This is a significantly higher fraction than the 50% expected for a "random" mechanism given a binary choice between the 20° dorsal target area and regions outside, demonstrating that following complete nerve transection, regenerating axons of the dorsal nerve branch retain the ability to select their original branch-specific trajectory. Furthermore, transection of only the dorsal nerve branch resulted in the same degree of branch-specific regrowth of *Tg(isl1:GFP)*-positive fascicles (data not shown), indicating that target-selective regeneration of dorsal nerve axons occurs independently of injury to ventral nerve axons. Together these results reveal that when confronted with a choice point, regenerating zebrafish motor axons select their original path with high fidelity, consistent with the existence of non-random genetic mechanisms that promote target-selective regeneration.

Regenerating Axons Probe the Transection Gap Extensively before Selecting Their Original Path

We next used live-cell imaging to examine the behavior of regenerating axons as they encounter a branch choice point. One possibility is that regenerating axons exclusively rely on a predetermined intrinsic program that instructs growth cones to extend rapidly onto the appropriate path without probing the environment at choice points. Alternatively, regenerating growth cones might integrate extrinsic cues to select their appropriate path. A prediction for this latter scenario is that as regenerating growth cones cross the injury gap and encounter a choice point, they would extensively probe their environment for instructive cues. To determine the extent to which regenerating growth cones probe their environment, we transected the dorsal nerve branch and monitored in vivo growth cone dynamics of pioneering

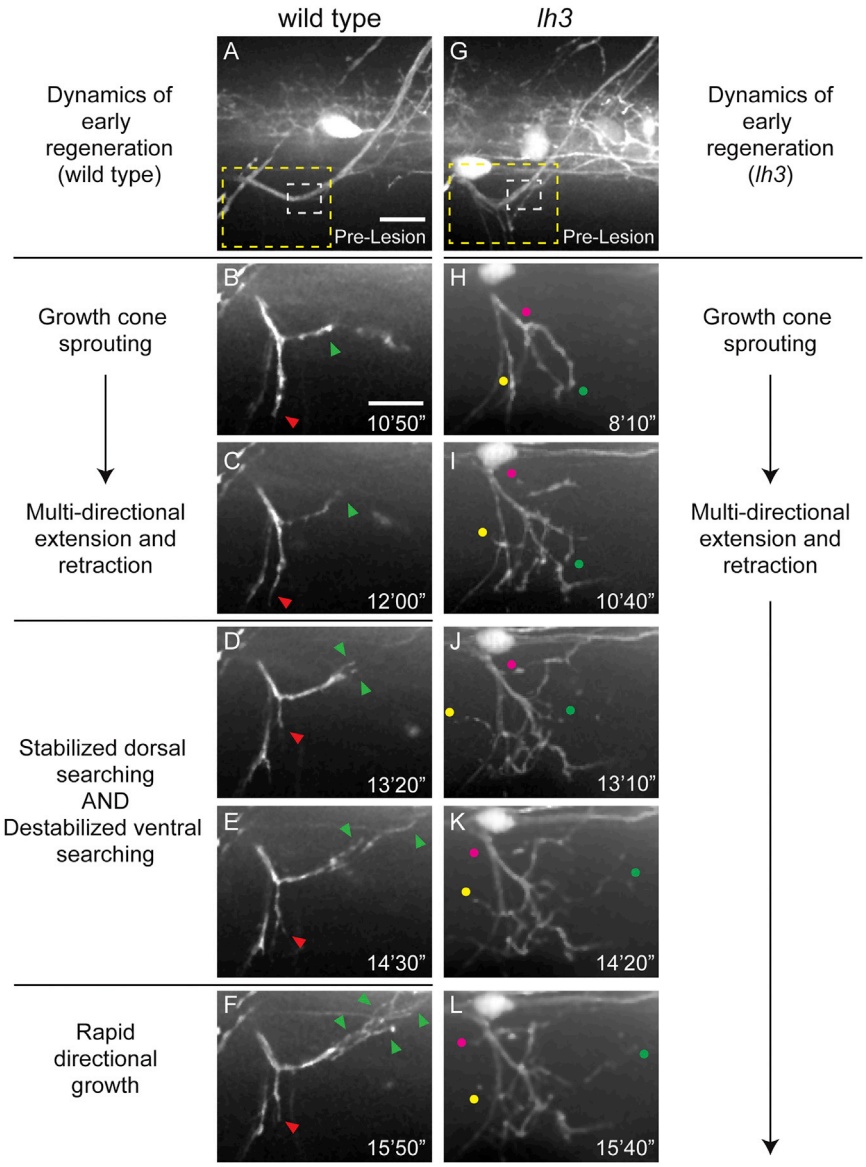

Figure 2. *Ih3* Is Required for Pathway Stabilization in Early Regeneration

(A) Wild-type dorsal nerve prior to nerve transection (white dashed box, transection site; yellow dashed box, region magnified in B–F; scale bar, 10 μm).

(B and C) Regenerating wild-type dorsal axons sprout growth cones (B) and probe the injury gap (C) through multi-directional extension and retraction (red arrowhead, ventral probing; green arrowhead, dorsal probing; scale bar, 10 μm).

(D–F) Axons then destabilize non-dorsal searching and stabilize dorsal searching at ~13 hpt (D) to ~14 hpt (E) leading to rapid directional growth (F; n = 8 larvae, 15/16 nerves).

(G) Conditional *Ih3* mutant dorsal nerves develop indistinguishably from wild-type (white dashed box, transection site; yellow dashed box, region magnified in H–L).

(H–L) In the absence of *Ih3*, axons sprout growth cones (H) after transection. Axons probe the myotome multi-directionally at 10 hpt (I), 13 hpt (J), 14 hpt (K), and 15 hpt (L; magenta, yellow and green dots track individual fascicles) but fail to stabilize dorsal searching and destabilize ventral searching (n = 11 larvae, 10/26 nerves). When *Ih3* mutant axons stabilized growth, these axons often grew on non-dorsal paths (n = 8/26 nerves, $p < 0.001$).

axons as they cross the injury gap and encounter the branch choice point (Figures 2A–2F; Movie S1). Following transection, axons proximal to the injury site retracted, while distal axons underwent Wallerian degeneration, which is conserved in zebrafish (Cajal, 1928; Gaudet et al., 2011; Lewis and Kucenas, 2014; Martin et al., 2010; Rosenberg et al., 2012, 2014; Vargas and Barres, 2007; Waller, 1850). Between 7 and 11 hr post-transection (hpt) we observed the first axons sprouting growth cones into the injury gap, where they probed the environment with short bursts of extension and retraction. These bursts of extension and retraction occurred at almost equal frequencies toward the correct dorsal path and toward the incorrect ventral path (Figures 2B and 2C; Movie S1; dorsal: 17.1 ± 1.46 bursts; ventral: 16.9 ± 1.68 bursts; n = 20 nerves; see Experimental Procedures for quantification). Over the next 2–4 hr, ventrally directed axons extended and collapsed frequently, while growth cones extending along the correct dorsal path stabilized (Figures 2D and 2E; n = 15/16 nerves; Movie S1). These stabilized, dorsally directed axons then extended rapidly, eventually reaching their original synaptic target regions in the dorsal myotome (Figure 2F; Movie S1). Thus, as regenerating axons of the dorsal nerve branch encounter the nerve branch point, they explore both the correct dorsal and incorrect ventral path before ultimately selecting the path to their original synaptic targets. This extensive probing behavior strongly supports the idea that regenerating growth cones rely on extrinsic cues to navigate their branch choice point.

Ih3 Is Required for Growth of Regenerating Axons and Target-Selective Regeneration

Regenerating axons exhibit highly dynamic behaviors as they probe the transection gap, suggesting that cues in the extracellular environment might lead them back to their original trajectory. Therefore, we chose to test components of the extracellular matrix (ECM) for specific roles in this process. Collagens are abundant in the ECM, and it has long been noted that axons regenerate along basal laminae rich in Collagens (Carey et al., 1983; Chernousov and Carey, 2000; Martin and Timpl, 1987). Given that vertebrate genomes express a large number of Collagen-encoding genes (28 in mammals, 42 in zebrafish), we decided to test the role of Collagens in nerve regeneration by

analyzing a single gene whose function is critical for post-translational Collagen modifications. Collagens are modified by ~20 isoenzymes, including glycosyltransferases whose functions are critical for collagen assembly, secretion, and function (Myllyharju and Kivirikko, 2004). Of these, *lysyl hydroxylase 3* (*lh3*) is a well-characterized glycosyltransferase that modifies a known set of Collagens for proper secretion and deposition in the ECM (Norman and Moerman, 2000 and Figure S3; Ruotsalainen et al., 2006; Sipilä et al., 2007).

To bypass the requirement of *lh3* during development (Schneider and Granato, 2006; Zeller and Granato, 1999), we generated a conditional, heat-inducible *Tg(hsp70l:lh3myc)* transgene to restore early motor nerve development in *lh3* mutants and then examined dorsal nerve regeneration in animals lacking *lh3* during regeneration (hereafter "conditional *lh3* mutants"; see Supplemental Experimental Procedures and Figure S1). At 5 days post fertilization (dpf), peripheral motor nerves in these conditional *lh3* mutants were indistinguishable from those of wild-type siblings, including the presence of closely associated Schwann cells (compare Figures 2A and 2G, and data not shown). Following dorsal nerve transection in conditional *lh3* mutants, we observed distal motor axon fragmentation and axonal debris removal, followed by proximal growth cones sprouting with kinetics comparable to those observed in wild-type siblings (Figures 2H and 2I; Movie S2 and data not shown). Like in wild-type, regenerating *lh3* mutant growth cones probed the transection gap through multi-directional extension and retraction (Figures 2I–2L). However, in contrast to wild-type growth cones, many *lh3* mutant axons failed to consolidate onto their original dorsally directed path and instead repeatedly exhibited short bursts of extension and retraction, which lasted for the duration of recording (10 hr; n = 10/26 nerves; Movie S2). In cases where *lh3* mutant axons stabilized growth, these axons often grew into aberrant regions of the myotome (n = 8/26 nerves; data not shown). To quantify the role of *lh3* beyond the early stages of axonal regrowth, we first analyzed dorsal axon regrowth extent at 48 hpt, when wild-type peripheral motor axons have regrown sufficiently to restore neuromuscular function (Rosenberg et al., 2012). In wild-type siblings, over 80% of dorsal nerves regrew axons into the dorsal myotome. In contrast, in *lh3* mutants only 60% of dorsal nerves regrew axons into the dorsal myotome, demonstrating that *lh3* is required to support growth of regenerating dorsal nerves in vivo (Figures 3A–3D, quantified in G "dorsal" using categories described in G inset and Supplemental Experimental Procedures; regrowth = extent categories 3–5; $p < 0.001$).

We noticed that instead of returning to their original dorsal muscle targets, regenerating dorsal nerve axons in *lh3* mutants frequently invaded lateral as well as ventral regions of the myotome (Figure 3D). To quantify the precision with which dorsal nerve axons regrew to their original dorsal targets, we applied a modified Sholl analysis (Li and Hoffman-Kim, 2008; Sholl, 1953). In wild-type siblings, 68% of regenerating fascicles from the dorsal nerve regrew and formed synapses on muscle fibers within a 20° region of their original synaptic target area, while in *lh3* mutants regrowth to this area was reduced to 26%, resulting in increased ectopic regrowth either adjacent to their original target area or into the ventral myotome (Figures 3H, 3I, and S2). To also take into account the number of fascicles present prior to nerve transection, we introduced a directionality ratio (percent of fascicles in target area pre-transection ÷ percent of fascicles in target area post-transection normalized to wild-type; Experimental Procedures). This confirmed that in *lh3* mutants the proportion of regenerating axons that extend along their original trajectory is significantly decreased (Figure 3K). We noted that in *lh3* mutants some fascicles regrew just outside the 20° region. Including these fascicles in our analysis did not change the statistical significance between mutants and wild-type ($p < 0.001$). Thus, *lh3* plays dual roles in regeneration by promoting the overall growth of regenerating dorsal nerve axons and by directing their growth to their original target area.

We next asked whether *lh3* is required for regeneration of all motor axons or if *lh3* function is selective for dorsal nerve regeneration. For this, we transected ventral nerves in conditional *lh3* mutant larvae. We detected no significant difference in the extent or fidelity of *lh3* ventral nerve regeneration when compared to wild-type siblings (Figure 3G "ventral" and data not shown). Combined, these data demonstrate that *lh3* selectively promotes regeneration and target selectivity of regenerating dorsal nerve axons.

Finally, we tested if *lh3* functions during the process of regeneration. To address this, we induced *lh3* expression from *Tg(hsp70l:lh3myc)* ~6 hr after dorsal nerve transection, just before the first regenerating growth cones emerge. This almost completely restored the extent of dorsal nerve outgrowth at 48 hr post-transection in *lh3* mutants (Figures 3E–3G) and significantly increased the ability of regenerating axons to return to their original target area (Figures 3J and 3K, $p < 0.001$). Combined, these data provide compelling evidence that *lh3* functions to promote regrowth and target-selective regeneration of dorsal nerve axons during regeneration.

The *lh3* Substrate *collagen4a5* Directs Regenerating Dorsal Nerve Axons

To identify the relevant in vivo *lh3* substrates for dorsal nerve regeneration, we took a candidate approach. *lh3* predominantly glycosylates fibrillar collagens and basal laminar collagens (Anttinen et al., 1978; Sipilä et al., 2007; Wang et al., 2000), and we therefore examined mutants for three *lh3* basal laminar collagen substrates. We focused on *collagen4a5* (*col4a5*) and *collagen18a1* (*col18a1*), because their expression is upregulated following peripheral nerve injury (Arthur-Farraj et al., 2012; Kubo et al., 2002; Siironen et al., 1992), and also included *collagen19a1* (*col19a1*) because of its role in motor axon guidance during zebrafish development (Beattie et al., 2000; Hilario et al., 2010). Each of these collagens contains several predicted *lh3* glycosylation sites required for proper secretion and deposition into the ECM (Figure S3 and Hautala et al., 1992; Ruotsalainen et al., 2006; Wang et al., 2000). Consistent with this, we found that transgenic Col4a5 expression in mutants lacking *lh3* activity, but not in wild-type embryos, caused aberrant Col4a5 protein localization, providing direct evidence that in zebrafish *lh3* is required for Col4a5 localization (Figure S3). To determine the in vivo roles of the three collagens in peripheral nerve regeneration, we obtained existing zebrafish *col4a5* and *col19a1* mutants (Hilario et al., 2010; Xiao and Baier, 2007) and generated

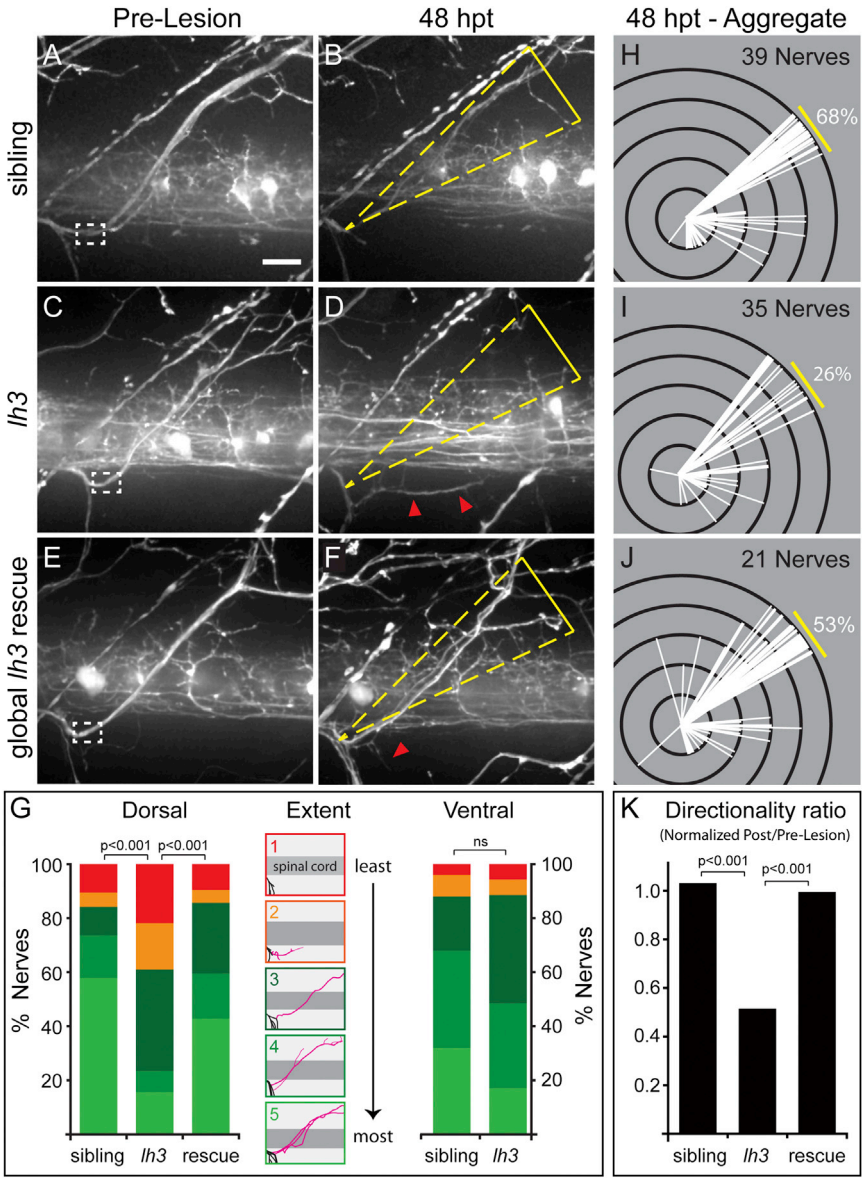

Figure 3. *lh3* Is Required for Regenerative Axonal Growth and Target-Selective Regeneration

(A–D) Examples of pre-lesion (A) and 48 hpt (B) wild-type motor nerve show robust and directional regrowth. In contrast, pre-lesion (C) and 48 hpt (D) conditional *lh3* mutant motor nerve examples show diminished regrowth that often targeted non-dorsal regions (white dashed box, transection site; yellow triangle, dorsal ROI; red arrowheads, misguided fascicles; scale bar, 10 μm).

(E and F) *lh3* expression after transection rescued this defect; compare pre-lesion (E) to 48 hpt (F) examples.

(G) *lh3* is required for regenerative growth across populations (wild-type sibling, n = 13 larvae, 39 nerves; *lh3*, n = 13 larvae, 35 nerves; global *lh3* rescue, n = 8 larvae, 21 nerves). Camera lucida tracings of regrowth "extent" categories described in Supplemental Experimental Procedures (black, uninjured axons; pink, regenerated axons). Regenerating ventral axons do not require *lh3* function (sibling, n = 9 larvae, 25 nerves; *lh3*, n = 16 larvae, 35 nerves).

(H–K) Modified Sholl analysis reveals that in comparison to siblings (H), fewer *lh3* fascicles (I) regrew to the dorsal myotome. This defect was partially rescued by ubiquitous *lh3* transgene expression during regeneration (J). (K) These differences were statistically significant after adjusting for developmental dorsal axon patterning in the directionality ratio.

several TALEN-induced *col18a1* mutations predicted to abolish *col18a1* function (see Supplemental Experimental Procedures).

Like *lh3* mutants, none of these mutants exhibited defects in ventral axon regeneration (data not shown). Importantly, dorsal axon regeneration was unaffected in *col18a1* and *col19a1* mutants, demonstrating that the mere removal of a basement membrane collagen is not sufficient to disrupt regeneration (Figure S3). Furthermore, dorsal nerve development in *col4a5* mutants was indistinguishable from that in wild-type siblings (compare Figures 4A and 4D). However, similar to *lh3* mutant fascicles, 57% of *col4a5* mutant fascicles regenerated into aberrant lateral and ventral regions of the myotome, demonstrating that *col4a5* is critical for dorsal axon regeneration in vivo and that *lh3* operates—at least partially—through *col4a5* to mediate peripheral nerve regeneration (Figures 4E–4G). Finally, we asked when and where *col4a5* is expressed during nerve regeneration (Figures 4H–4K). In the absence of nerve transection, *col4a5* mRNA was detectable in spinal cord cells adjacent to the central canal (data not shown) but was undetectable along the ventral or dorsal nerve path (Figure 4I, data not shown). In contrast, between 8 and 15 hpt, *col4a5* mRNA was upregulated in Schwann cells just ventral and ventrolateral to the transection site (Figures 4H, 4J, and 4K). Thus, *col4a5* mRNA is upregulated precisely when and where regenerating dorsal nerve axons select their original trajectory.

Col4a5 Promotes Target-Specific Regeneration by Destabilizing Misdirected Axons

We next wanted to understand how *col4a5* directs dorsal nerve regrowth. Given that *col4a5* is a constituent of the basement membrane, we first examined basement membrane integrity in *col4a5* mutants. Immunohistochemistry and electron microscopy revealed no difference between wild-type siblings and *col4a5* mutants in the basal lamina directly at the dorsal nerve choice point or at the neuromuscular junction, arguing against a significant defect in basal lamina integrity causing the observed regeneration phenotype (Figure S4). Regenerating axons extend in close association with the remaining basal lamina of the injured nerve, and we therefore asked if and to what

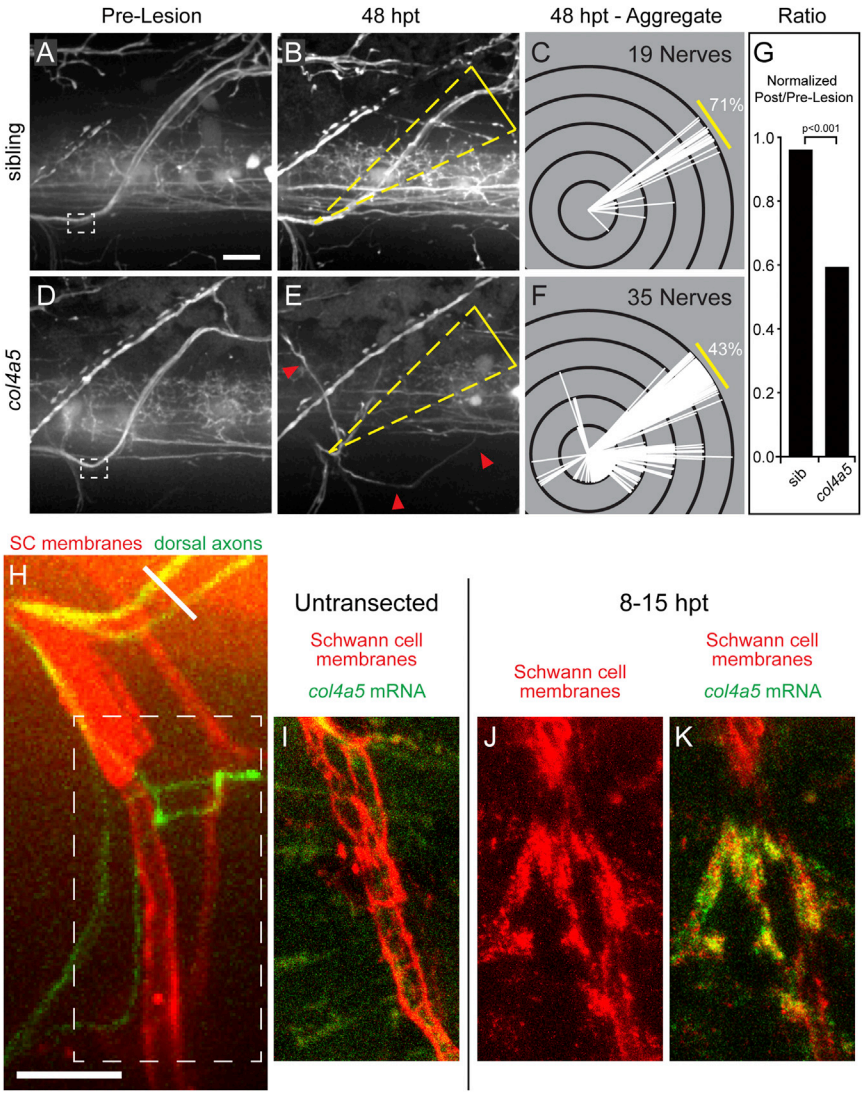

Figure 4. The *lh3* Substrate *collagen4a5* Is Upregulated after Nerve Transection and Directs Regenerating Dorsal Nerve Axons

(A–F) Compared to pre-lesion (A), 48 hpt sibling nerves (B) regenerate to the original outgrowth pathway. Modified Sholl analysis (C; n = 7 larvae, 19 nerves) reveals that the majority of sibling nerves regenerate on the correct path. In contrast, pre-lesion (D) and 48 hpt (E) *col4a5* nerve examples and Sholl analysis (F; n = 12 larvae, 35 nerves) show that mutant nerves frequently regrow into aberrant regions of the myotome (yellow triangles, dorsal ROI; red arrowheads, misguided fascicle).

(G) These differences were statistically significant after adjusting for developmental dorsal axon patterning in the directionality ratio.

(H) Region of transected nerves showing *col4a5* mRNA signal in (J) and (K) (oblique white line, transection site; white dashed box, region of nerve shown in I–K).

(I) *col4a5* in situ hybridization in untransected hemisegments revealed sparse signal (n = 12 larvae, 36/54 nerves).

(J and K) 8–15 hr post-transection, *col4a5* mRNA (J) was upregulated in Schwann cells (K) ventral and ventrolateral to the transection site (n = same 12 larvae, 52/60 nerves; $p < 0.001$). All scale bars, 10 μm.

extent regenerating axons in *col4a5* mutants retained their ability to grow. For this we quantified average and maximum forward axonal growth rates in *col4a5* mutants following nerve transection. In *col4a5* mutants, regenerative growth rates were indistinguishable from those in wild-type animals (wild-type: average = 0.15 mm/day; maximum = 0.51mm/day; n = 11 fascicles in 11 nerves; *col4a5*: average = 0.14 mm/day; maximum = 0.48 mm/day; n = 17 fascicles in 17 nerves; Experimental Procedures).

We therefore considered that rather than regulating growth rates, *col4a5* might promote target-specific regeneration by destabilizing axons probing incorrect trajectories. For this we analyzed growth cone behaviors of regenerating *col4a5* dorsal nerve axons in vivo. Similar to wild-type axons, between 7 and 11 hpt regenerating *col4a5* axons sprout growth cones into the injury gap, and like wild-type axons, they immediately and extensively probe their environment (compare Figures 5A–5C to Figures 5F–5H; Movie S3). As they explored their environment, *col4a5* mutant axons extended and retracted onto the dorsal path with the same frequency as when compared to wild-type axons (Figure 5K; see Experimental Procedures for growth index quantification). In contrast, unlike in wild-type animals, regenerating axons in *col4a5* mutants that probed ventral or ventrolateral territories frequently stabilized and grew (Figures 5D, 5E, 5I, and 5J; Movie S3; quantified in Figure 5K). Importantly, a significant fraction of these aberrantly projecting axons were the first to enter the transection gap (38%, 5/13 nerves). While we cannot formally exclude a potential role for *col4a5* in axonal fasciculation, our data are consistent with the idea that rather than defasciculating from axons directed toward the correct dorsal targets, these axons lacked proper guidance early in regeneration. Thus, during regeneration *col4a5* controls not axonal growth rates, but instead axonal directionality. These data provide compelling evidence that as dorsal axons navigate their branch choice point, *col4a5* destabilizes misdirected dorsal axons to promote regeneration toward the original trajectory.

Finally, we asked whether destabilization of misdirected axons correlates with the expression of repulsive guidance cues. Given their well-established roles in growth cone repulsion and their ability to bind to *col4a5*, we focused on Netrin and Slit (Xiao et al., 2011; Yebra et al., 2003). We have previously shown that both of the zebrafish *netrin* homologs are expressed in motor neurons and Schwann cells in 5 dpf larvae and that the *netrin* receptor *dcc* is required for ventral nerve regeneration (Rosenberg et al., 2014). To test whether Netrin-DCC signaling is required for dorsal nerve regeneration, we transected dorsal nerves in *dcc* mutant larvae. We did not observe any defects in *dcc* mutant

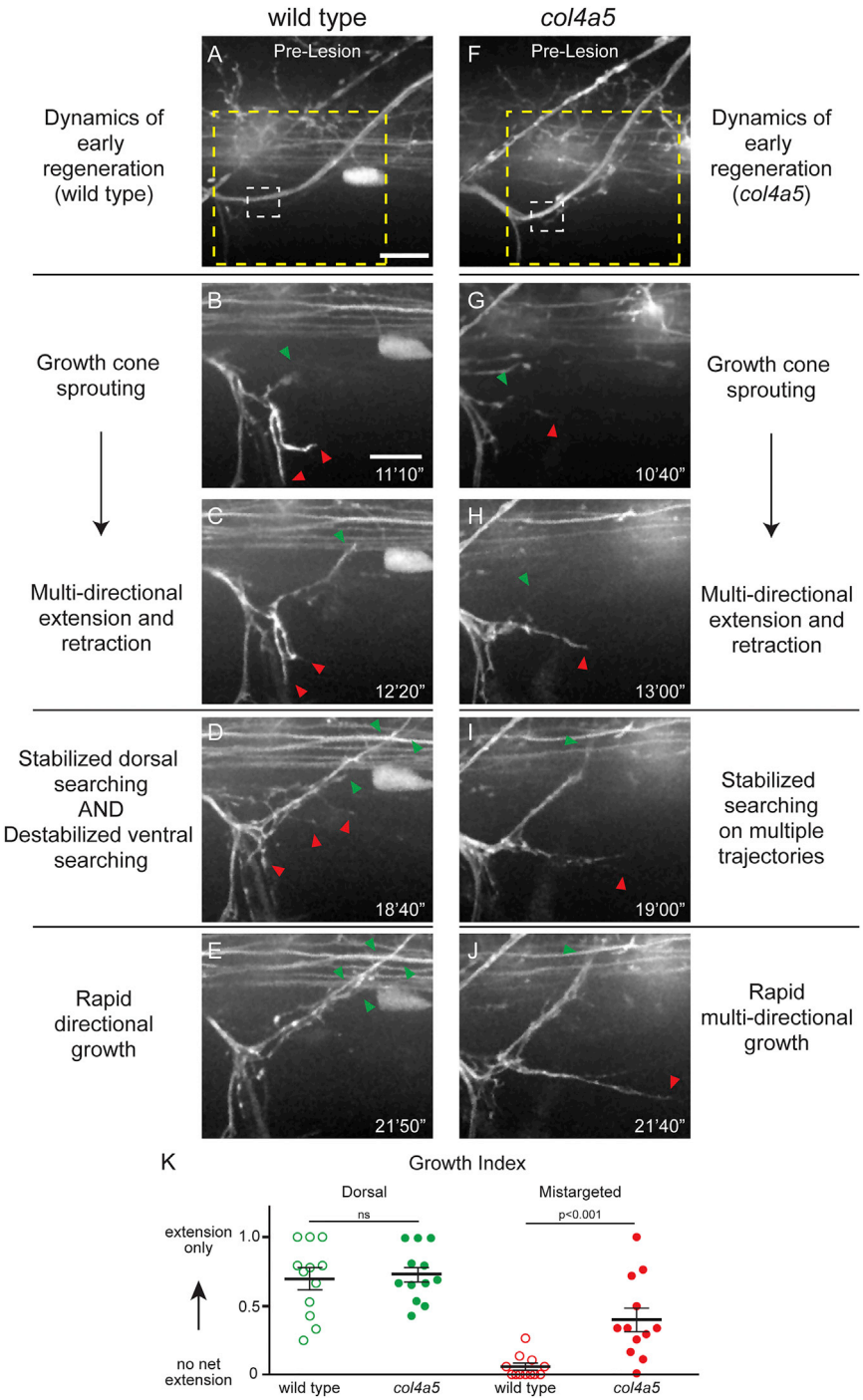

Figure 5. *col4a5* Destabilizes Aberrant Growth Early in Regeneration

(A) Wild-type dorsal nerve prior to nerve transection (white dashed box, transection site; yellow dashed box, region magnified in B–F; scale bar, 10 μm).

(B and C) Wild-type axons sprout growth cones (B) and probe all regions of the injury gap (C).

(D and E) Over time, axons destabilize searching on non-dorsal paths and stabilize searching on the dorsal path (D), leading to rapid directional growth (E; n = 8 larvae, 15/16 nerves).

(F) *col4a5* dorsal nerves develop indistinguishably from wild-type siblings (white dashed box, transection site; yellow dashed box, region magnified in H–L).

(G and H) Regenerating *col4a5* axons sprout growth cones (G) and search all regions of the injury gap (H).

(I) Over time, axons stabilize searching on the dorsal path but fail to destabilize searching on non-dorsal paths.

(J) This leads to invasion of non-dorsal regions of the myotome (n = 8 larvae, 17/25 nerves; $p < 0.0001$). Red arrowheads, ventral searching; green arrowhead, dorsal searching; scale bar, 10 μm.

(K) The net proportion of extension and retraction movements was similar between wild-type and *col4a5* fascicles on the dorsal path. In contrast, while wild-type fascicles extended and retracted with equal frequency, mistargeted *col4a5* fascicles extended more frequently and stabilized to grow on aberrant trajectories. All error bars indicate ±SEM.

nerve regeneration (data not shown). We therefore examined the expression of the four *slit* homologs in zebrafish: *slit1a*, *slit1b*, *slit2*, and *slit3*. Prior to and following dorsal nerve transection, we observed *slit1b*, *slit2*, and *slit3* mRNAs in the spinal cord but failed to detect expression in transected dorsal nerves (data not shown). In contrast, at 8–15 hpt we observed robust upregulation of *slit1a* mRNA expression ventral and ventrolateral to the lesion site, in the same regions we observed *col4a5* mRNA (Figures 6A–6C). Double in situ hybridization confirmed that *col4a5* and *slit1a* mRNA expression strongly co-localizes to a small group of Schwann cells (Figures 6D and 4H–4K). Thus, *col4a5* is required to direct dorsal nerve regeneration, and together with the guidance repellent *slit1a* is upregulated in a small group of Schwann cells located ventral and ventrolateral to the transection gap, suggesting a pivotal role for Schwann cells in *col4a5*-dependent regeneration.

lh3 Function in Peripheral Glia Directs Regenerating Axons

To identify the cell types relevant for *lh3* and *col4a5* in axonal regeneration, we employed a transgenic rescue strategy. Like in mammals, zebrafish peripheral motor nerves consist of several cell types, most prominently neurons, perineural glia, and Schwann cells (Brushart, 2011; Kucenas et al., 2008; Lyons and Talbot, 2015; Zochodne, 2008). In addition, peripheral nerves are in close contact with muscle fibers. Given that during regeneration *col4a5* mRNA expression localizes to Schwann cells (Figures 4J and 4K), we tested whether transgenic expression of the *col4a5*

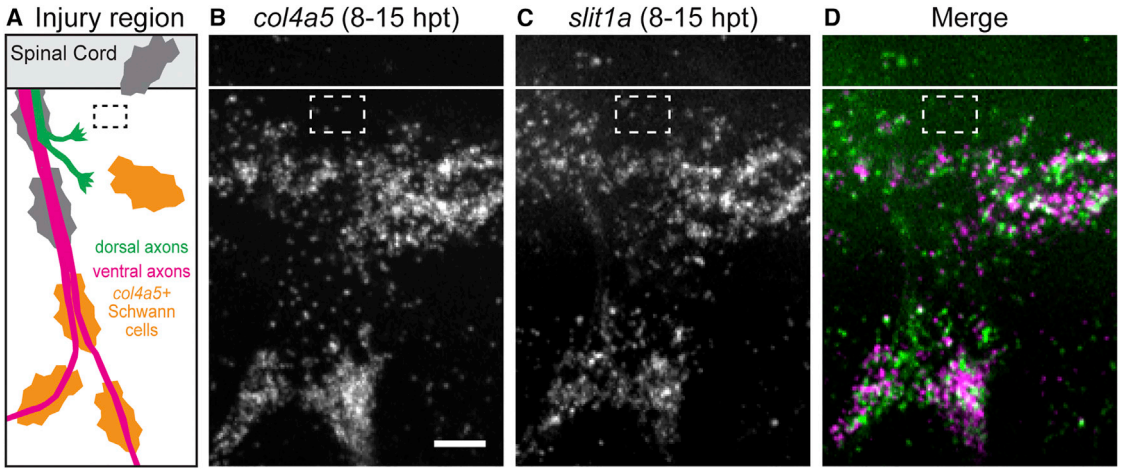

Figure 6. *Slit1a* Is Upregulated with *collagen4a5* after Nerve Transection

(A) Schematic showing approximate region imaged for in situ hybridization (black dashed box, transection site).

(B–D) *col4a5* mRNA (B) and *slit1a* mRNA (C) are co-expressed (D) ventral to the spinal after nerve transection (n = 6 larvae, 27/30 nerves). White line, dorsal aspect of spinal cord; white dashed box, approximate transection site; scale bar, 10 μm.

glycosyltransferase *lh3* in Schwann cells is sufficient to restore dorsal nerve regeneration in *lh3* mutants. As a control, we also generated transgenic lines expressing *lh3* in somitic muscle directly adjacent to the path of regenerating axons. Though *lh3* expression from the *Tg(aActin:lh3-mkate)* transgene was detectable in muscle cells adjacent to the nerve path, this was insufficient to restore *lh3* axon regeneration (Figures 7A, 7B, and 7E). In contrast, Schwann cell-specific expression of *Tg(sox10:lh3-mkate)* in *lh3* mutants restored axon regeneration (Figures 7C–7E). Importantly, in *lh3* mutants the number and position of $Sox10^{+}$ Schwann cells along the dorsal motor nerve were indistinguishable from those in wild-type siblings (*lh3* = 6.49 ± 0.16, n = 81 nerves; wild-type = 6.46 ± 0.14, n = 90 nerves). Thus, *lh3* function in Schwann cells—but not in muscle—is sufficient to direct dorsal nerve axons during regeneration. Combined, our data suggest a model in which *lh3* functions in a small group of Schwann cells to ensure proper secretion and/localization of Col4a5; *col4a5* de-stabilizes incorrectly projecting axons—possibly through *slit1a*—thereby promoting target-specific regeneration (Figure 7F).

DISCUSSION

Non-neuronal cells, including fibroblasts and Schwann cells, are known to generate an extrinsic milieu that promotes axon regeneration (Paíno et al., 1994; Parrinello et al., 2010; Richardson et al., 1980; Schröder et al., 1993; Xu et al., 1997), but whether this environment also provides regenerating axons with target specificity has been controversial. Here, using live imaging of regenerating vertebrate axons, we demonstrate that following nerve transection, axons confronted with a trajectory choice select the appropriate path back to their original targets, and this process depends on extrinsic cues. We identify a molecular pathway by which Schwann cell expression of the glycosyltransferase *lh3* and expression of *lh3* post-transection are required to convey target specificity. We show that one *lh3* substrate, *col4a5*, is upregulated in a defined subset of Schwann cells when regenerating axons select their original trajectory and that *col4a5* destabilizes mistargeted axons to provide target specificity to a subset of regenerating axons in vivo. Finally, we find that nerve transection induces upregulation of the canonical axon guidance repellent *slit1a* in cells expressing *col4a5*, providing a potential mechanism by which *col4a5* promotes target-selective regeneration (Figure 7F). Together, our results provide compelling evidence that regenerating axons targeted to different synaptic sites utilize specific ECM components that direct them back onto their original trajectories.

Zebrafish Spinal Motor Axons Regenerate to Their Original Developmental Targets

Since Ramon y Cajal's original experiments demonstrating axonal misdirection during PNS regeneration (Cajal, 1928), it has become clear that the degree of target-selective reinnervation varies. For example, fully transected sciatic nerve axons of the peroneal and tibial branches regenerating through a 5 mm Y-shaped tube displayed no preferential regeneration toward the appropriate distal nerve stump (Abernethy et al., 1992), while transection and surgical apposition of transected mouse sciatic nerves resulted in ~85% of the common fibular branch axons reinnervating their original muscle targets (English, 2005). In contrast, crushing the motor nerve such that the perineurium and the distal Schwann cell tubes remained intact resulted in over 90% of regenerating motor axons innervating their original muscle fibers (Nguyen et al., 2002). Thus, depending on the location and severity of the injury, regenerating axons display varying degrees of target-selective reinnervation. However, the in vivo behaviors of regenerating axons as they negotiate branch points have remained elusive.

In this study, we fully transected motor nerves and generated an ~9 μm injury gap, which destroys Schwann cells in the injury gap and induces characteristic regeneration-associated morphological changes in Schwann cells neighboring the lesion

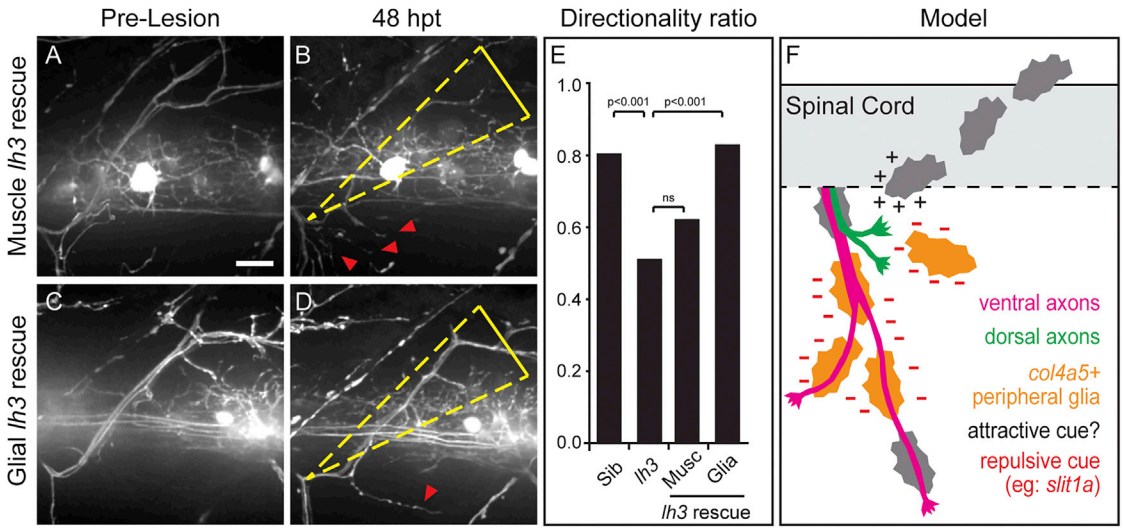

Figure 7. *lh3* in Peripheral Glia Rescues *lh3* Dorsal Axon Regeneration Defects

(A–E) Pre-lesion (A) and 48 hpt (B) examples reveal that expression of *lh3* in all muscles *Tg(aActin:lh3mkate)* fails to rescue *lh3* axon regeneration. In contrast, pre-lesion (C) and 48 hpt (D) examples demonstrate that transgenic expression of *lh3* in peripheral glia *Tg(sox10:lh3mkate)* significantly rescued these guidance defects (E).

(F) After nerve transection, regenerating motor axons cross the injury gap to return to distal Schwann cells and reinnervate dorsal muscle targets. *lh3* glycosylation of ECM components and the *lh3* substrate *col4a5* are required for targeting dorsal, but not ventral, motor axon regeneration. *Col4a5* is upregulated in ventral and ventrolateral Schwann cells where it may act to present canonical guidance cues, such as *slit1a*, to destabilize regenerating axons. Dashed white boxes outline the nerve transection site; dashed yellow triangle, dorsal ROI; red arrowheads, aberrant regrowth; scale bar, 10 μm.

site (Lewis and Kucenas, 2014; Rosenberg et al., 2012). We find that under these conditions axons retain a high degree of target specificity (80%), indicating a non-random mechanism of reinnervation, consistent with previous reports (Brushart, 1988; Grimm, 1971; Kuffler, 1986a; Lee and Farel, 1988; Mark, 1965; Sperry and Arora, 1965; Stephenson, 1979). We observed that regenerating axons initially extend highly dynamic growth cones randomly toward both correct and incorrect targets before selecting their appropriate path. Moreover, our in vivo studies revealed that misprojecting growth cones collapse in one of the first morphological steps toward target selectivity, consistent with repulsive forces playing a role in this process. Thus, live-cell imaging reveals that target-specific innervation is a multistep process that includes extensive interactions of regenerating axons with their environment. Indeed, endpoint analysis of transected mouse sciatic nerve axons (Witzel et al., 2005) revealed similar pathway sampling, suggesting that this is an evolutionarily conserved mechanism.

lh3 Reveals a Novel Role for Collagens in Target-Selective Peripheral Nerve Regeneration

Components of the extracellular matrix including heparan sulfate proteoglycans, collagens, and enzymes that modify them post-translationally have well-documented roles in developmental axon guidance (Ackley et al., 2001; Bülow and Hobert, 2006; Poulain and Chien, 2013; Xiao et al., 2011). With a few exceptions (Chen and Strickland, 2003; Edwards and Hammarlund, 2014), the in vivo roles of ECM components and their modifying enzymes in axonal regeneration are less established. This is in part because genetic knockouts of ECM components often have developmental phenotypes that preclude the analysis of nerve regeneration at later stages (George et al., 1993; Guo et al., 1991; Löhler et al., 1984; Myllyharju and Kivirikko, 2004; Pöschl et al., 2004; Ruotsalainen et al., 2006; Smyth et al., 1999). In vitro, there is compelling evidence that ECM molecules of the nerve basal lamina facilitate regrowth (Forman and Berenberg, 1978; Kuffler, 1986b; Martini, 1994; Nathaniel and Pease, 1963; Pollard and Fitzpatrick, 1973; Scherer and Easter, 1984). For example, axons from an excised mouse sciatic nerve grow on acellular Schwann cell basal lamina, suggesting that ECM components are sufficient to support axonal regrowth (Ide et al., 1983). These and other ex vivo experiments have contributed to the notion that during regeneration components of the ECM serve as permissive substrates (Uziyel et al., 2000; Wang et al., 1992a, 1992b; Werner et al., 2000). However, our genetic and live-cell imaging data indicate a much more directive role for the ECM during in vivo regeneration.

Using an inducible transgene, we demonstrate that *lh3* is required during nerve regeneration independent of its role during development. During development, *lh3* is required in a subset of muscle cells to guide motor axons from the spinal cord to their targets, independently of *col4a5* (Zeller and Granato, 1999). Following nerve transection, we find that *lh3* expression in Schwann cells is required for target selectivity of the dorsal, but not ventral, nerve axons and that this process also requires the *lh3* substrate *col4a5* (Figures 3, 4, 5, and 7). Importantly, *col4a5* does not regulate axonal growth rates but instead directs regenerating axons toward their original targets by destabilizing mistargeted axons (Figure 5, Movie S3). Thus, independent of their developmental roles, *lh3* and *col4a5* provide regenerating

axons with target specificity, demonstrating that in vivo ECM collagens provide more than a permissive substrate for axon regeneration.

lh3 and *col4a5* Reveal a Schwann Cell-Dependent Repair Mechanism that Ensures Target Selectivity

Our data provide compelling evidence that *lh3* and *col4a5* specifically direct regenerating axons of the dorsal nerve branch, matching these axons with their original targets and thereby achieving target-selective regeneration. While *lh3* promotes growth and directionality of dorsal nerve axons, *col4a5* appears critical only for axonal directionality, consistent with the idea that *lh3* exerts its various functions through different substrates, including *col4a5*. Given the large number of collagens in the vertebrate genome, it is unclear precisely which other collagen or group of collagens play critical roles in peripheral nerve regeneration. Furthermore, although the exact contribution of individual glycosylation sites on collagens are not well established, collagens are glycosylated by additional glycosyltransferases such as GLT25D1 and GLT25D2 (Schegg et al., 2009), increasing the complexity of this system.

How do *lh3* and *col4a5* selectively direct dorsal motor axons? While expression of *lh3* in all Schwann cells restores target selectivity, the relevant substrates, including *col4a5*, might be expressed only in a relevant subset of these cells. In fact, we find that *col4a5* is upregulated in Schwann cells ventral and ventrolateral to the injury gap (Figures 4 and 6). These data are consistent with rodent studies demonstrating that following peripheral nerve transection *collagen4* is upregulated in Schwann cells and that Schwann cells respond to injury with independent expression phenotypes depending on the nerve they associate with and their proximity to the injury site (Brushart et al., 2013; Höke et al., 2006; Siironen et al., 1992). The spatially restricted expression of *col4a5* also suggests a local mechanism by which *col4a5* might either directly or indirectly guide regenerating axons. For example, Collagen4 subunits can bind Integrin receptors and Discoidin Domain Receptors (Leitinger and Hohenester, 2007), and regenerating axons express Integrins (Lefcort et al., 1992; Vogelezang et al., 2001), providing a compelling scenario by which Schwann cells expressing Collagen4a5 might selectively guide dorsal nerve axons through Integrin receptors expressed on these, but not on ventral nerve axons. Alternatively, Collagen4a5 might bind and concentrate axonal guidance ligands to direct regenerating growth cones expressing the cognate guidance receptor. In fact, Col4a5 can bind Netrin and Slit, which are both upregulated after peripheral nerve transection in rodents (Xiao et al., 2011; Yebra et al., 2003). We find that Netrin-DCC signaling is dispensable for dorsal nerve regeneration but that *slit1a* is upregulated with *col4a5* in Schwann cells ventral and ventrolateral to the transection site. Thus, one possible scenario is that, in response to injury, Schwann cells ventral to the transection site secrete Collagen4a5, which binds and accumulates Slit, thereby forming a repulsive barrier to direct dorsal axons onto their original, dorsal path (Figure 7F). Although future studies are required to determine whether these mechanisms operate in isolation or in combination, our data reveal for the first time that in vivo, distinct ECM components serve to selectively direct a subpopulation of regenerating axons toward their original targets. Moreover, our results provide a compelling mechanistic framework underlying target-selective regeneration.

EXPERIMENTAL PROCEDURES

Zebrafish Genetics and Transgenes

Transgenic lines were generated in the Tübingen or Tupfel longfin (TLF) genetic background (see Supplemental Experimental Procedures) and maintained as previously described (Mullins et al., 1994). The following mutant strains were used: *lh3*^{TV2O5} (Schneider and Granato, 2006), *col4a5*^{s510} (Xiao and Baier, 2007), *col19a1*^{b393} (Hilario et al., 2010); *col18a1* mutants were generated via TALEN injection, and multiple alleles were identified as described (Dahlem et al., 2012). All zebrafish work was conducted in accordance with Institutional Animal Care and IACUC regulatory standards.

Whole-Mount Fluorescent In Situ Hybridization and Immunohistochemistry

Nerve transections were performed in 5 dpf *Tg(isl1:GFP)*; *Tg(nkx2.2a:GFP)* or *Tg(isl1:GFP); Tg(sox10:mRFP)* larvae to fluorescently label the dorsal nerve branch and surrounding peripheral glia, respectively (Kucenas et al., 2008; Uemura et al., 2005). Larvae were fixed between 8 and 15 hpt for 2 hr in 4% PFA in PBS, and in situ hybridization was performed using RNAscope (ACDbio; Gross-Thebing et al., 2014). Nerves were imaged in 1 μm sections on a 60× immersion lens on an Olympus Spinning disk confocal microscope using Slidebook Software and processed for analysis as described below. Anti-Sox10 (1:2,000, gift from S. Kucenas) and anti-Laminin (1:100, Sigma) were used to stain 5 dpf larvae as described (Rosenberg et al., 2014; Wolman et al., 2015). Nerves were imaged in 0.5–1 μm sections with a 40× water immersion lens on a Zeiss LSC 710 confocal scanning microscope.

Nerve Transection

Transection of both ventral and dorsal peripheral motor nerves was performed as previously described, resulting in an ~9 μm injury gap measured between proximal and distal nerve endings immediately following transection (Rosenberg et al., 2012). For Figure 1, dorsal and ventral nerves were transected on the common path, ~5 μm from the spinal cord exit point. For Figures 2, 3, 4, 5, 6, and 7, only the dorsal nerves were transected ~10 μm from the spinal cord exit point.

Live-Cell Imaging

Anesthetization, mounting, and imaging of embryos were carried out as previously described (Rosenberg et al., 2012).

Image Processing

For live imaging (Figures 1, 2, 3, 4, 5, and 7), image stacks were compressed into maximum intensity projections (MIPs) and processed using ImageJ and Adobe Photoshop to normalize brightness and contrast. For fixed imaging (Figures 4, 6, and S1–S4) MIPs were adjusted to equivalent brightness and contrast in ImageJ for comparison.

Axon Regeneration Quantification

Axon growth extent was quantified as described in the Supplemental Experimental Procedures. Axon growth directionality was quantified 48 hr post-transection using a modified Sholl analysis (Li and Hoffman-Kim, 2008; Sholl, 1953) as illustrated in Figure S2. Line thickness was selected proportional to the number of fascicles that crossed at a given intersection point. The proportion (p) of fascicles (F) within the ROI (25°–45° from the horizontal) at 48 hpt was divided by the proportion that developed in this ROI pre-lesion. In wild-type (WT) animals, ~70% of fascicles regrew into the dorsal ROI at 48 hpt, and the directionality ratio for a given genotype "X" was defined in relation to this as follows:

$$\frac{p(F,X)48\ hpt \div p(F,X)pre\text{-}lesion}{p(F,WT)48\ hpt \div p(F,WT)pre\text{-}lesion.}$$

Axon extension and retraction bursts were defined as growth or retraction of >1 μm between time-lapse frames (10 min), and the frequency was defined

as the cumulative number of these bursts counted until an axon remained on the same trajectory >1 hr. Axon growth rates were calculated as previously described (Rosenberg et al., 2014). To define a growth index, axons were monitored for direction and were labeled "dorsal" if they extended to dorsal regions or "mistargeted" if they extended to non-dorsal regions. Axons were scored "1" if they extended >1 μm or "−1" if they retracted >1 μm between frames (extension/retraction rarely exceeded 1.5–2 μm between frames) and were scored "0" if they moved <1 μm. We defined the growth index for a given fascicle as follows:

$$\sum(\text{extensions} + \text{retractions}) \div \sum(\text{observed movements}).$$

In this case, extension only has a growth index of 1, and no net growth has a growth index of 0.

Statistical Analysis

Fisher's exact and Student's t tests were performed on all applicable datasets.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2015.10.004.

AUTHOR CONTRIBUTIONS

J.I.-B. and M.G. designed research; J.I.-B., C.F.-A., and V.S. performed research; J.I.-B. and C.F.-A. analyzed data; J.I.-B. and M.G. wrote the paper.

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