Peripheral nerves are divided into multiple branches leading to divergent synaptic targets. This poses a remarkable challenge for regenerating axons as they select their original trajectory at nerve branch-points. Despite implications for functional regeneration, the molecular mechanisms underlying target selectivity are not well characterized. \textit{Danio Rerio} (zebrafish) motor nerves are composed of a ventral and a dorsal branch that diverge at a choice-point, and we have previously shown that regenerating axons faithfully select their original branch and targets. Here we identify \textit{robo2} as a key regulator of target-selective regeneration (sex of experimental subjects unknown). We demonstrate that \textit{robo2} function in regenerating axons is required and sufficient to drive target-selective regeneration, and that \textit{robo2} acts in response to glia located precisely where regenerating axons select the branch-specific trajectory to prevent and correct axonal errors. Combined, our results reveal a glia-derived mechanism that acts locally via axonal \textit{robo2} to promote target-selective regeneration.

**Key words:** collagen 4a5; peripheral nerve regeneration; \textit{robo2}; schwann cells; target selection; zebrafish

### Significance Statement

Despite its relevance for functional recovery, the molecular mechanisms that direct regenerating peripheral nerve axons toward their original targets are not well defined. Zebrafish spinal motor nerves are composed of a dorsal and a ventral branch that diverge at a stereotyped nerve branch-point, providing a unique opportunity to decipher the molecular mechanisms critical for target-selective regeneration. Using a combination of live cell imaging and molecular-genetic manipulations, we demonstrate that the \textit{robo2} guidance receptor is necessary and sufficient to promote target-selective regeneration. Moreover, we demonstrate that \textit{robo2} is part of a genetic pathway that generates transient, spatially restricted, and tightly coordinated signaling events that direct axons of the dorsal nerve branch toward their original, pre-injury targets.

### Introduction

The peripheral nervous system (PNS) has a remarkable capacity for axonal regeneration, and yet functional recovery from peripheral nerve injury is rare because axons regrow very slowly and often toward inappropriate targets. Supplementing pro-regenerative factors to regenerating PNS axons accelerates their growth rates yet falls short of providing spatial information to direct axons toward their original, pre-injury targets (Gordon and English, 2016). While components of many developmental axon guidance pathways are upregulated after nerve injury, their functional roles in directing regenerating axons are largely unknown (for review, see Giger et al., 2010). Thus, how regenerating axons navigate an environment that differs drastically from the embryo is not well understood.

In vertebrates, peripheral nerves exiting from the spinal cord divide repeatedly into progressively smaller branches, each carrying axons that innervate distinct synaptic targets (Lance-Jones and Landmesser, 1981). Depending on the type and location of a nerve injury, regenerating axons encounter a series of branch-points as they extend toward their original targets. For regenerating axons, repeatedly selecting the appropriate path at each branch-point, while critical to ensure functional regeneration, poses a formidable challenge. Although branch-specific regeneration of peripheral axons was first demonstrated $>50$ years ago (Mark, 1965; Politis, 1985), the molecular mechanisms that underlie branch-selective regeneration are largely unknown.

We previously established the optically transparent larval zebrafish as model to study the cellular and molecular mechanisms of target-selective peripheral nerve regeneration in \textit{vivo} (Rosenberg et al., 2012). Zebrafish spinal motor nerves are composed of functionally distinct axonal populations that share a
common path before diverging into two major branches: a ventral nerve branch (see Fig. 1A–E, magenta) consisting of ~50 motor axons that innervate ventral muscles, and a dorsal nerve branch (see Fig. 1A–E, green) consisting of ~20 motor axons that innervate dorsal muscles (van Raamsdonk et al., 1983; Myers et al., 1986; Westerfield et al., 1986; Svara et al., 2018). Following dorsal nerve transaction, the distal nerve undergoes Wallerian degeneration and is cleared away by macrophages, and the proximal nerve stump retracts into the spinal cord (see Fig. 1C). By 8 h post transaction (hpt), the axons of the proximal stump regrow toward the nerve branch-point where they select their regenerative path (see Fig. 1D).

We have previously shown that regenerating spinal motor axons reliably choose the nerve branch that leads to their original muscle targets (Rosenberg et al., 2012). Live cell imaging revealed that regenerating axons of the dorsal nerve branch pause at the nerve branch-point, exploring both the incorrect ventral path and their original dorsal path before selecting the correct dorsal path. During this exploratory period, a small group of Schwann cells at the nerve branch-point simultaneously upregulate the extracellular matrix (ECM) component col4a5 and the repulsive axon guidance cue slit1a (Isaacman-Beck et al., 2015), which bind each other with high affinity (Xiao et al., 2011). Moreover, we previously demonstrated that col4a5 is required to guide regenerating dorsal axons, and proposed a model by which Schwann cell-derived Col4a5 scaffolds Slit at the nerve branch-point to prevent regenerating dorsal nerve branch axons from inappropriately entering into and extending along inappropriate trajectories (see Fig. 1D) (Isaacman-Beck et al., 2015).

To test this model, we used genetic mutants of the Slit-Robo signaling pathway. We find that the Slit-receptor roundabout2 (robo2) and the Slit-Robo coreceptor heparan sulfate are dispensable for target-selective regeneration of ventral nerve axons, but required to direct regenerating dorsal nerve axons at the branch choice-point. Moreover, we find that robo2 is expressed in dorsal nerve neurons, that expressing robo2 in ventral nerve neurons is sufficient to redirect their regenerating axons into the dorsal nerve branch, and that this process requires col4a5. Finally, using live cell imaging, we demonstrate that during regeneration robo2 functions at the nerve branch-point, preventing aberrant extension of dorsal nerve axons, thereby promoting growth toward their original, dorsal targets. Combined, our results reveal a previously unappreciated role for Slit-Robo signaling critical to ensure branch-selective and hence target-selective regeneration.

Materials and Methods

Fish lines and maintenance. All fish lines were maintained in Tübingen or Tüpfel longfin backgrounds as previously described (Mullins et al., 1994). We used the following mutant alleles, which were genotyped as previously described: robo2-ti272ze (Fricke et al., 2001), extl3tm70g (Lee et al., 2004), and col4a5s510 (Xiao and Baier, 2007). The Tg(sox10:col4a5-myc) line was generated as previously described (Isaacman-Beck et al., 2015) and genotyped by amplifying the Myc transgene using the following primers: 5′-GACTACAAGGATGACG-3′ (forward) and 5′-TTCCTCCATAGTCCGGTAC-3′ (reverse). For in vivo imaging, the following transgenic lines were used: Tg(mnx1:GFP)tm351 (Flanagan-Steed et al., 2005) to visualize motor axons in both dorsal and ventral nerve branches, Tg(is1l1:GFP)tm25 (Uemura et al., 2005) to visualize dorsal nerve branch axons alone. Zebrafish veterinary care was performed under the supervision of the University Laboratory Animal Resources at the University of Pennsylvania. All zebrafish work was performed in accordance with protocols approved by the University of Pennsylvania Institutional Animal Care and Use.

Nerve transaction. Dorsal and ventral nerves were transected using a nitrogen-pulsed dye (440 nm) laser as previously described (Rosenberg et al., 2012). Briefly, one of the two nerve branches (dorsal for Figs. 1–3, and 5 or ventral for Figs. 4, 6, and 7) was transected ~5 μm from the nerve branch-point (10-15 μm from the motor exit point [MEP]), leaving the other nerve branch intact and a ~5 μm gap between proximal and distal stumps of the transected nerve branch.

Before transection, larvae were genotyped by fin clipping at 3 dpf post fertilization (dpf) to ensure a mix of genotypes (~50% siblings and ~50% mutants) in each experiment. For this, caudal fins were resected using a microscalpel distal to the notochord and without breaching the caudal artery/vein loop. This method removes less tissue than published methods commonly performed before rearing larvae and is widely accepted not to affect gross development (Kosuta et al., 2018). After larvae were selected based on their genotypes, larvae were pooled and then randomly selected to blind the researcher to genotype during nerve transaction. In extl3−/− larvae, dorsal nerves reach dorsal muscle targets, but a small subset grow along an aberrantly lateral trajectory. This phenotype is variably penetrant, affecting 0%-50% of nerves per larva and <20% of all nerves across the mutant population (n > 50 larvae; P.L.M., unpublished observations). Thus, for dorsal nerve transactions in progeny from extl3−/− parents, we carefully selected phenotypically normal nerves for transaction.

Quantification of axon regeneration. Dorsal axon guidance before and after transection was quantified using modified Sholl analysis, as previously described (Isaacman-Beck et al., 2015), with the exception that line thickness in Sholl diagrams here does not correlate to fascicle thickness. When performing these analyses, nerves were deidentified and gene expression IDs that could later be reassociated with their meta-data, so that researchers were blinded to genotype while scoring images. To calculate the regeneration error rate for each nerve, we divided the number of fascicles that regenerated outside the dorsal ROI ("errors") by the total number of fascicles that regenerated by 48 hpt. To determine the angle of ventral nerve extension, we measured the angle between two consistent points along the trunk of the nerve (50 and 100 μm from the MEP). The extent of ventral nerve regeneration was scored by counting the number of fascicles which regenerated at least 50 μm from the MEP by 48 hpt.

Immunohistochemistry and whole-mount FISH. To visualize robo expression after nerve transection, dorsal nerves were transected in 5 dpf Tg(is1l1:GFP) larvae. At 0-10 hpt, larvae were fixed in 4% PFA in PBS with 0.1% Tween-20 overnight at 4°C. Antisense robo2 probe was synthesized from pBluescript-robo2 linearized with EcoRI using T3 RNA polymerase (Promega); sense robo2 probe was synthesized from pBluescript-robo2 linearized with Xhol using T7 RNA polymerase (Promega). Probes were hydrolyzed in 0.6 x sodium carbonate and 0.4 x sodium bicarbonate at 60°C for 11 min to yield 300-500 bp fragments. Whole-mount ISH was performed as previously described (Thiese and Thiese, 2006) with the following modifications: 5 dpf larvae were permeabilized using 1X saline with 0.1% Triton X-100; 1% streptavidin-Biotin (Roche) was applied to the sections; and endogenous peroxidases were quenched by incubating in 0.3% H2O2 for 30 min before adding anti-digoxigenin antibody; for blocking and antibody incubation, we used 2% Blocking Reagent (Roche) in PBS with 0.1% Tween-20; probes were detected using sheep anti-digoxigenin POD Fab fragments (Roche) and developed for 2 min using Tyramide Signal Amplification (TSA Plus kit, PerkinElmer). We stained for Tg(is1l1:GFP) using chicken anti-GFP (1:500, Aves Labs) detected by donkey anti-chick AlexaFluo-488 (1:500, Jackson Immunol) and secondary antibodies (anti-Tg, Cy3; anti-myc, Alexafluor594). Signals were imaged using the Zeiss LSM 880 laser scanning confocal microscope and Zeiss Zen software or using a 40x water immersion lens and 1 μm optical sections on a Carl Zeiss LSM 880 laser scanning confocal microscope and Zeiss Zen software or using a 40x water immersion lens and 1 μm optical sections on an Olympus spine confocal microscope and 3i Slidebook Software. Overlap between GFP antibody and AlexaFluo488 images in Fiji in the following way: motor pools of transected nerves were isolated by cropping optical sections in 3D; motor pools were then compressed into maximum intensity projections (MIPs); GFP and robo2 probe signals were separated and converted into binary masks using the K (entropy) and moments for 2-D methods; percent particle overlap between the two masks was calculated using the GDSC colocalization plugin; percent particle overlap was...
Figure 1. Spatiotemporal restriction of *col4a5* to the nerve branch-point is required for target-selective axon regeneration. Top, In 5 dpf larval zebrafish (schematized in A), dorsal (green) and ventral (magenta) motor nerves exit the spinal cord in each body hemisegment via the ventral MEP and diverge at a stereotyped branch-point to innervate the dorsal and ventral muscles, respectively. B, Magnified schematic of single motor nerve in dashed box in (A) pre-transection, showing dorsal (green) and ventral (magenta) branches of the spinal motor nerve and associated Schwann cells (orange). Purple box represents transection site. C, By 4-6 hpt, the nerve distal to the transection site degenerates and is cleared away, and the proximal nerve retracts into the spinal cord. D, At 8-15 hpt, growth cones enter the transection gap, and a small subset of Schwann cells at the nerve branch-point upregulate *col4a5* and *slit1a* (blue with red stripes). E, By 48 hpt, the majority of dorsal axons regenerate into the dorsal ROI, defined as 20°-50° with respect to spinal cord. Bottom, Representative images of *Tg(isl1:GFP)*+ dorsal nerves at 5 dpf and 48 hpt, respectively, in wild-type (WT) siblings (F,G) and *Tg(sox10:col4a5-myc)* larvae (H). Yellow boxes represent transection site. Green arrowheads indicate dorsal regrowth. Magenta arrowheads indicate "errors" that regrow along nondorsal paths. Scale bars, 10 μm. Sholl diagrams showing overlay of all *Tg(isl1:GFP)* fascicle trajectories at 48 hpt in WT siblings (G, n = 96 fascicles) and *Tg(sox10:col4a5-myc)* larvae (J, n = 133 fascicles). For WT siblings, fascicles were counted for *n* = 37 nerves in 11 larvae; for *Tg(sox10:col4a5-myc)* larvae, fascicles were counted for *n* = 40 nerves in 11 larvae. Green lines indicate fascicles inside dorsal ROI. Magenta lines indicate fascicles outside of the dorsal ROI. Proportion of fascicles inside the dorsal ROI at 48 hpt was compared between *Tg(isl1:GFP)* and WT siblings using one-tailed Fisher’s exact test (*p* = 0.0002). K, Graph of regeneration error rate for nerves in WT siblings (No Tg) and *Tg(sox10:col4a5-myc)* larvae (*Tg*”) compared using one-tailed t test (*t*<sub>130</sub> = 3.807, *p* = 0.0001). Each dot represents error rate for one nerve. +, Mean. ***p < 0.001.
then normalized across samples using the number of cell bodies manually counted in each motor pool.

**Plasmid construction.** The mnx1:mKate, mnx1:robo2 plasmid was constructed using Gateway cloning (Kwan et al., 2007) using pME:robo2 (Campbell et al., 2007) and pl-Scel mnx1:mKate, mnx1:DEST, which encodes two mnx1 promoters in tandem (Bremer and Granato, 2016), to insert the robo2 coding sequence behind the second mnx1 promoter. To construct plasmid for synthesis of robo2 in situ probe (pBluescript-robot2), the full-length robo2 coding sequence was amplified from the mnx1:mKate, mnx1:robo2 plasmid using the following primers 5'--AGTCAGCTC GAGAAACGTGTTCTGGGGTTGAGA-3'.

**Sparse axonal labeling.** We used mnx1:mKate, mnx1:robo2 and mnx1:mKate, mnx1:DEST to label small numbers of ventral motor neurons by injecting 50-100 pg of plasmid DNA into one-cell stage embryos with Isce-I, as previously described (Downes et al., 2002). We have previously validated that both mnx1 promoters in this construct are active and drive comparable levels of expression (Bremer and Granato, 2016).

At 3 dpf, injected larvae with mKate expression were screened for ventral nerves with very few labeled axons using a 40<sup>x</sup> water immersion lens on Olympus spinning disk confocal microscope using 3i Slidebook software. When examining branch-specific labeling in development and regeneration, larvae and nerves were deidentified and given unique IDs that could later be reassociated with their meta-data, so that researchers were blinded to the injection condition and genotype while scoring.

Nerves were scored as "dorsal" if any mKate<sup>+</sup> fascicles were present along the dorsal branch, regardless of whether mKate<sup>+</sup> fascicles were also present along the ventral branch.

It was technically challenging to sparsely label ventral axons in sufficient numbers for transection experiments without labeling multiple neurons per motor pool. We could almost always count multiple (2-6) dorsal axons, we believe it is very likely that this reflects the regeneration of multiple labeled axons.

**Live imaging.** Larvae were anesthetized, mounted in agarose, and imaged on a spinning disk confocal microscope as previously described (Rosenberg et al., 2012). We began our time-lapse experiments at 7-9 hpt and filmed regeneration for 12-15 h. Because of variability in time when axons started regrowing (8-14 hpt), we quantified axon dynamics starting when the first regenerating fascicle reached the nerve branch-point, ending up to 10 h later. We analyzed regenerating fascicles for a total of 5940 min in siblings (n = 9 nerves) and 5030 min in mutants (n = 10 nerves). When analyzing regeneration dynamics, nerves were deidentified and given unique IDs that could later be reassociated with their meta-data, so that researchers were blinded to genotype while scoring.

**Image processing.** For ventral nerves (see Fig. 4) and fixed samples (see Fig. 2), Z stacks were compressed into MIPs. Brightness and contrast were automatically optimized based on the image histogram in Fiji ImageJ (National Institutes of Health). The dorsal nerve branch wraps around the spinal cord, closely apposed to motor neuron cell bodies, which are labeled brightly by our transgenic lines. To visualize the dorsal nerves independently of neuron cell bodies (see Figs. 1, 3, 5-7), we used Fiji to create multiple MIPs from the same Z stack, including only optical sections that contained the dorsal nerve without neuron cell bodies in each XY position. These MIPs were adjusted to equivalent brightness and contrast and then stitched together using the Pairwise Stitching plug-in (Preibisch et al., 2009).

**Experimental design and statistical analysis.** For all experiments, desired sample sizes were defined before beginning data collection. For dorsal nerve transection and live imaging experiments, we determined appropriate sample sizes based on those in previously published experiments that were able to detect effects of the sizes that we expected. For stochastic labeling experiments, sample sizes were determined via power analyses using G*Power (Faul et al., 2007). Zebrafish sex determination is polygenic and at the larval stage (28 dpf or earlier) requires multiple qPCRs that have only recently been identified (King et al., 2020). Therefore, we were unable to control for the sex of our experimental subjects.

Continuous data (see Fig. 5) were analyzed using one- or two-tailed t<sup>t</sup> tests, as dictated by experimental design and indicated in the figure legends. Categorical data (see Figs. 1, 3, 6, 7) were analyzed in contingency tables using one- or two-tailed Fisher’s exact tests for proportionality, as indicated in the figure legends. Count data (see Fig. 5) were analyzed using two-tailed Mann–Whitney tests.

**Results**

**Col4a5 upregulation at the nerve branch-point is critical to guide regenerating dorsal axons.** We previously demonstrated that the glycosyltransferase lysylhydroxylase-3 (Lh3) and its substrate collagen-4-α-5 (Col4a5) are required to direct regenerating dorsal nerve axons toward their
original targets (Isaacman-Beck et al., 2015). Lh3 is constitutively expressed at low levels and acts in Schwann cells to promote target-selective regeneration, while col4a5 expression is transiently upregulated 8-15 hpt in a small subset of Schwann cells near the nerve branch-point (Fig. 1D) (Isaacman-Beck et al., 2015), suggesting that col4a5 expression restricted to the nerve branch-point might be instructive in directing regenerating axons. To test this idea, we generated a transgenic line, Tg(sox10:col4a5-Myc), in which col4a5 is now expressed in all Schwann cells, before and following peripheral nerve transection (Isaacman-Beck et al., 2015).
Beck et al., 2015). Before nerve transection, dorsal nerves in wild-type (WT) siblings appear indistinguishable from those in transgenic animals expressing col4a5 in all Schwann cells (compare Fig. 1F with Fig. 1I). Specifically, we quantified targeting of Tg(isl1:GFP)+ dorsal nerve axons before nerve transection in 5-d-old animals. Dorsal nerve axons tightly fasciculate with one another, precluding us from quantifying individual axons contained in Tg(isl1:GFP)+ dorsal nerves. We therefore quantified the number of discernable Tg(isl1:GFP)+ fascicles and determined the fraction of fascicles within the dorsal muscle target area, which we previously defined as spanning 30 deg before transection (dorsal ROI in Fig. 1E) (Isaacman-Beck et al., 2015). When we compared these fractions across genotypes, we found no significant difference in dorsal fascicles between Tg(sox10:col4a5-Myc) animals and WT siblings (n = 103 of 105 fascicles in dorsal ROI WT siblings, n = 99 of 101 in Tg(sox10:col4a5-Myc) larvae; p = 0.6746, one-tailed Fisher’s exact test), suggesting that, in Tg(sox10:col4a5-Myc) animals, dorsal nerve targeting during development is unaffected.

To determine whether spatially restricted expression of col4a5 is critical for target-selective regeneration, we laser-transected dorsal nerves in WT and Tg(sox10:col4a5-Myc) larvae and compared target-selective regeneration at 48 hpt, when WT motor axons have reestablished functional connections with their muscle targets (Rosenberg et al., 2012). In WT animals, 70% of fascicles containing dorsal nerve axons regenerated to their original dorsal target area (Fig. 1G,H; 96 fascicles, 37 nerves, 11 larvae), consistent with previous findings that regenerating axons readily select their original branch and targets (Isaacman-Beck et al., 2015). In contrast, transgenic expression of col4a5 in all Schwann cells reduced target-selective regeneration significantly. In Tg(sox10:col4a5-myC)-expressing larvae, only 46% of the fascicles containing dorsal nerve axons selected their original dorsal trajectory (133 fascicles, 40 nerves, 11 larvae), concomitant with an 1.8-fold increase from 30% to 54% of fascicles selecting incorrect ventral and lateral trajectories (Fig. 1J,K; p = 0.0002, one-tailed Fisher’s exact test). We next examined the regeneration error rate for individual nerves at 48 hpt and found that, compared with WT siblings, nerves in Tg(sox10:col4a5-myC)-expressing larvae displayed errors at a significantly higher rate (Fig. 1L; \( t(75) = 3.807, p = 0.0001 \), one-tailed t test). Thus, expressing col4a5 from a small subset of Schwann cells strategically positioned at the nerve branch region to all Schwann cells impairs target-selective regeneration. Moreover, the resulting phenotype closely mirrors the phenotype in mutants lacking col4a5 (Isaacman-Beck et al., 2015). These results support the idea that col4a5’s transient expression in a subset of Schwann cells at the nerve branch-point where regenerating axons of the dorsal branch select their branch specific trajectory is of functional importance.

Slit-Robo signaling is required for target-selective regeneration

The same small group of Schwann cells that upregulates col4a5 after injury concurrently upregulates slitta (Isaacman-Beck et al., 2015), suggesting that, similar to col4a5, slitta might play a functional role in target-selective regeneration. Slitta encodes a canonical ligand for the Roundabout (Robo) family of repulsive axon guidance receptors (Blockus and Chédotal, 2016) and is the only one of the four Slit ligands whose injury-induced expression mirrors that of col4a5 (Isaacman-Beck et al., 2015). Moreover, in the developing zebrafish visual system, Col4a5 directly binds to, and is required for, basement membrane anchoring of Slit, which guides laminar targeting of retinal ganglion cell axons through robo2 (Xiao et al., 2011). We therefore wondered whether robo2 is involved in guiding regenerating dorsal axons. Using whole-mount FISH, we detected robo2 mRNA expression in Tg(isl1:GFP)+ motor neurons of the dorsal nerve before transection and also during regeneration (Fig. 2A–C, unct: n = 65 nerves, 1220 motor neurons unct; 0–4 hpt: n = 28 nerves, 499 motor neurons; 4–6 hpt: n = 16 nerves, 365 motor neurons; 6–10 hpt: n = 22 nerves, 469 motor neurons).

We next asked whether Slit-Robo signaling plays a functional role in target-selective regeneration. For this, we examined dorsal nerve regeneration in genetic mutants for two Slit-Robo signaling components: exostosin-like-3 (ext3), a glycosyltransferase required for the biosynthesis of heparan sulfate proteoglycans, which are critical to stabilize Slit-Robo binding (Hussain et al., 2006); and the Robo-receptor robo2. We first examined dorsal nerve regeneration in ext3 mutants, which at 5 dpf lack detectable levels of heparan sulfate (Lee et al., 2004). Before nerve transection, targeting of dorsal nerve axons in 5 dpf ext3 mutants was indistinguishable from their siblings (compare Fig. 3A–D, n = 103 of 108 fascicles in dorsal ROI in ext3+/−/+;+, n = 43 of 44 in ext3++, p = 0.4399, one-tailed Fisher’s exact test). In WT siblings, 68% of regenerating dorsal nerve fascicles returned to their original, dorsal targets (Fig. 3B,C), while 32% selected ventral and ventrolateral trajectories (38 nerves from 19 larvae). In contrast, in ext3 mutants, we observed a 1.6-fold increase (from 32% to 52%) of dorsal nerve fascicles failing to select their dorsal trajectory, instead extending along erroneous ventral or ventrolateral trajectories (23 nerves from 7 larvae) (Fig. 3E,F; p = 0.0105, one-tailed Fisher’s exact test). Similarly, compared with WT siblings, individual nerves in ext3 mutant formed errors at a significantly higher rate at 48 hpt (Fig. 3G; \( t_{(58)} = 2.862, p = 0.0031 \), one-tailed t test).

We next examined the role of Robo2 in dorsal nerve regeneration. Before transection, dorsal nerve targeting in robo2 mutant animals was slightly lower than what we observed in robo2 WT siblings (compare Fig. 3H–K; n = 103 of 108 fascicles in dorsal ROI in robo2+/−/+;+, n = 67 (79 in robo2+/−−;+, p = 0.0548, one-tailed Fisher’s exact test), yet still within the range we have previously observed in WT animals (Isaacman-Beck et al., 2015). Following nerve transection in WT siblings, 34% of dorsal nerve fascicles failed to select their original trajectory, while the vast majority returned to their original target area (66%; Fig. 3L,J). In contrast, in robo2 mutants only 50% of regenerating dorsal nerve fascicles returned to their original target area, an almost 1.5-fold increase of Tg(isl1:GFP)+ fascicles now extending along aberrant ventral or ventrolateral trajectories (Fig. 3LM; \( p = 0.0134 \), Fisher’s exact test). Similarly, compared with nerves in WT siblings at 48 hpt, individual nerves in robo2 mutants exhibited significantly higher error rates (Fig. 3N; \( t_{(57)} = 2.333, p = 0.0116 \), one-tailed t test). Together, these results demonstrate that Slit-Robo signaling plays a functional role in directing regenerating dorsal nerve axons along their original, pre-injury trajectories. Finally, to determine whether ext3 and robo2 play a selective role in promoting target selection of dorsal, rather than ventral, nerve axons, we transected ventral nerves in ext3 and robo2 mutants. At 5 dpf and 48 hpt, ventral nerves in ext3 and robo2 mutants are indistinguishable from their siblings (Fig. 4A–J). Similarly, compared with their siblings, we found no significant difference in the number (Fig. 4E,K) or angle of extension (Fig. 4FL) of ventral nerve fascicles in robo2 or ext3 mutants before or after injury. Together, these results demonstrate that Slit-Robo signaling is selectively required for dorsal nerve target-selective regeneration.
Robo2 promotes target-selective regeneration at the nerve branch-point by preventing and correcting errors

To further understand the cellular mechanisms by which robos2 promotes target-selective regeneration, we examined the dynamics of regenerating axons navigating the nerve branch-point in robos2 mutants. We have previously shown that, after dorsal nerve transection in WT larvae, regenerating axons pause at the nerve branch-point and extend growth cones toward their original dorsal targets.
as well as along erroneous ventral and lateral trajectories. Over the next few hours, erroneous projections are destabilized, while growth cones along the dorsal path stabilize and continue to extend toward their original targets (Isaacman-Beck et al., 2015). To determine whether robo2 directs regenerating dorsal nerve axons early in the process by minimizing the formation of erroneous projections, or subsequently by destabilizing already extending erroneous projections, we performed time-lapse imaging between 8 and 20 hpt as regenerating robo2 mutant dorsal nerve axons navigate the branch choice-point (Fig. 5A–H; Movies 1 and 2). From these movies, we quantified the number of erroneous projections (errors), defined as Tg(isl1:GFP)\(^+\) growth ≥1 μm that extended from the nerve branch-point along erroneous ventral or lateral trajectories (for more details, see Materials and Methods). Compared with WT siblings, robo2 mutants exhibit no significant difference in the number of errors that form at the branch-point (Fig. 5I), suggesting that robo2 does not play a role in error formation, whether it occurs by collateral sprouting or other mechanisms. To determine whether there was a deficit in error correction at the branch-point in robo2 mutants, we counted the number of errors (Fig. 5A–H; magenta arrowheads; Movies 1 and 2) that were corrected. Errors were counted as “corrected” if they retracted within <1 μm away from the nerve branch-point. Compared with siblings, robo2 mutants displayed a significant decrease in the percent of errors that were corrected during early regeneration (Fig. 5J).

Based on the well-characterized role of Slit-Robo in axon repulsion (Blockus and Chemodat, 2016), we considered whether in robo2 mutants the deficit in error correction at the nerve branch-point might be because of reduced error retraction. To test this, we measured in WT siblings and robo2 mutants the percent of time that errors spent retracting, extending, or being stable (no movement). We failed to detect any significant difference in relative time that errors spent retracting in robo2 mutants compared with WT siblings (Fig. 5K). Similarly, we did not observe any differences in the average speed of extension or retraction in robo2 mutants compared with siblings (Fig. 5L). Instead, compared with WT siblings, erroneous projections (errors) in robo2 mutants spent significantly more time extending (Fig. 5K). This is the result of a combined reduction in the time that errors spent retracting and stable in robo2 mutants, neither of which is statistically significant on its own (Fig. 5K). These results suggest that, rather than promoting axonal retraction along incorrect trajectories, robo2 promotes dorsal nerve target selection by preventing axon extension along erroneous ventral and lateral trajectories. Consistent with this idea, compared with WT siblings, erroneous projections in robo2 mutants grew longer distances (Fig. 5M). Combined with previous results this provides strong support for a model in which robo2 expression on regenerating dorsal nerve axons prevents and corrects errors at the nerve branch-point in response to Slit1a transiently produced by a small subset of adjacent Schwann cells and spatially scaffolded by Col4a5. By preventing error extensions, robo2 tilts the balance between extension and retraction such that errors retract more often than they extend. This results in shorter errors that are readily corrected through robo2-independent mechanisms of retraction, ultimately biasing regenerating dorsal nerve axons toward their original dorsal trajectory.

Robo2 expression drives target-selective regeneration

We next asked how Slit-Robo signaling selectively influences regeneration of dorsal, but not ventral, nerve axons. One possibility is that robo2 functions selectively in dorsal nerve axons, enabling regenerating axons of only the dorsal, but not the ventral, branch to mount a slit1a-dependent error response at the nerve branch-point. We hypothesized that, if this were the case, forcing robo2 expression in regenerating ventral nerve axons would redirect them onto a dorsal trajectory. To test whether robo2 expression is indeed sufficient to drive target-selective regeneration, we used the motor neuron-specific mnx1 promoter (Flanagan-Steeet et al., 2005) to transiently express mKate alone or mKate with robo2 in small subsets of motor neurons (for more details, see Materials and Methods). Importantly, compared with mKate expression, robo2-mKate expression in motor neurons did not affect their developmental bias in selecting a ventral or dorsal trajectory (for Tg(mnx1:mKate; mnx1:mKate), n = 23 of 41 nerves contained at least one visible mKate\(^+\) fascicle within the dorsal ROI at 5 dfp; for Tg(mnx1:mKate; mnx1:robo2), n = 22 of 41 nerves; p = 0.9999, Fisher’s exact test) nor did it impair their ability to regrow an axon (for Tg(mnx1:mKate; mnx1:mKate), n = 13 of 26 nerves regenerated by 48 hpt; for Tg(mnx1:mKate; mnx1:robo2), n = 15 of 32 nerves; p = 0.999, Fisher’s exact test). This is consistent with the absence of a developmental motor axon phenotype in robo2 mutants, further confirming that robo2 acts selectively during the regeneration process. To determine whether robo2 is sufficient to promote dorsal branch selection in regenerating axons, we screened for spinal motor nerves with small subsets of mKate\(^+\) axons along the ventral, but not the dorsal, branch (Fig. 6A,C). We laser-transected these ventral nerves and assessed the regeneration of mKate\(^+\) fascicles at 48 hpt. We found that regenerating mKate\(^+\) ventral nerve axons always selected a ventral path toward their original targets (Fig. 6B), consistent with previous results (Rosenberg et al., 2012). In contrast, forcing robo2-mKate expression in regenerating ventral nerve axons was sufficient to redirect them onto a dorsal trajectory (Fig. 6C,D; Fisher’s exact test \(p = 0.0069\)). Importantly, the trajectories taken by these axons were indistinguishable from those taken endogenously by dorsal nerve axons (compare with Fig. 3B,I). Thus, robo2 is both required and sufficient to drive target-selective regeneration.

Robo2 requires col4a5 function for target-selective regeneration

In robo2 and col4a5 mutants, ventral branch axons reliably regenerate along their appropriate ventral path (Fig. 4) (Isaacman-Beck et al., 2015), while dorsal branch axons frequently fail to select their original dorsal trajectory and instead extend along erroneous, ventral and lateral trajectories. Because of the similarities of their mutant phenotypes, we next asked whether robo2 and col4a5 act through two distinct pathways or whether they are both part of one common pathway. We reasoned that, if the latter was the case, then redirecting ventral nerve axons toward dorsal trajectories via forced robo2 expression should depend on col4a5 function. To test this hypothesis, we repeated the robo2 mis-expression experiment driving sparse expression of either mKate or robo2-mKate in small subsets of regenerating ventral nerve axons, but now in a col4a5\(^+\) mutant background. Before nerve transaction at 5 dfp, there was no difference between the branch-selection of sparsely labeled WT and robo2-expressing axons in col4a5\(^+\) siblings or mutants (for col4a5\(^+/+\); Tg(mnx1:GFP; mnx1:mKate), n = 39 of 89 nerves contained at least one visible mKate\(^+\) fascicle within the dorsal ROI at 5 dfp; Tg(mnx1:mKate; mnx1:robo2), n = 42 of 80 nerves, p = 0.2831, Fisher’s exact test; for col4a5\(^{-/-}\) larvae: Tg(mnx1:mKate; mnx1:mKate), n = 33 of 48 nerves, Tg(mnx1: mKate; mnx1:robo2), n = 13 of 26 nerves, p = 0.1364, Fisher’s
Figure 5. robo2 prevents error extension at the nerve branch-point during regeneration. Representative images of Tg(isl1:GFP) nerves in robo2^{+/+} (WT sibling) (A) and robo2^{−/−} (E) at 5 dpf and during regeneration (B–D, F–H). Dashed yellow box represents transection site. Dashed white box represents area enlarged 2× in B–D and F–H. Green arrowheads indicate dorsal regrowth. Magenta arrowheads indicate errors. B–D, F–H, Bottom right, White text indicates time after transection. Magenta text counts errors as a fraction of [errors formed/errors corrected]. Scale bars, 10 μm. I, Number of errors formed 8-20 hpt in WT siblings (robo2^{+/+}, n = 9 nerves) and robo2^{−/−} larvae (n = 10 nerves). Each dot represents one nerve. Ranks between genotypes were compared using two-tailed Mann–Whitney test (p = 0.6238). J, Percent of errors corrected 8-20 hpt in WT siblings (robo2^{+/+}, n = 9 nerves) and robo2^{−/−} larvae (n = 10 nerves). Errors were counted as “corrected” when their length measured from the nerve branch-point was < 1 μm. Each dot represents one nerve. Ranks were compared between genotypes.
using two-tailed Mann–Whitney test ($p = 0.0208$). K. Quantification of regenerating axon dynamics in WT siblings and rob2+/− larvae 8–20 hpt plotted by total time spent extending, retracting, and stable (no movement). Each dot represents one error (for siblings, $n = 23$ errors; for rob2+/−, $n = 34$ errors). Error movements were examined in 10 min intervals and classified as extensions when there was a $>1$ μm increase in error length measured from the MEP; movements were classified as retractions when $>1$ μm decrease in error length measured from the MEP; errors were classified as stable when no movement $\geq 1$ μm occurred. Line indicates mean. Error bars indicate 95% CI. M. Maximum length of errors in WT siblings and rob2+/− larvae in micrometers measured from the MEP. Each dot represents one error. +, Mean. Means were compared between genotypes using two-tailed $t$ test ($p = 0.0012$). **$p < 0.01$. *$p < 0.05$. [View online]

**Discussion**

Following peripheral nerve injury, regenerating axons face the challenge of navigating toward and reconnecting with their original synaptic targets. The difficulty of this task increases with the complexity of the injured nerve. After exiting the spinal cord, peripheral nerves repeatedly divide into progressively smaller branches leading to different targets (Lance-Jones and Landmesser, 1981). Regenerating axons may therefore encounter multiple nerve branch-points where they confront the choice to select their appropriate, pre-injury trajectory. Despite this
enormous navigational challenge, regenerating axons are able to preferentially select their original nerve branch (Politis, 1985; Brushart, 1993; Isaacman-Beck et al., 2015) and regrow toward appropriate targets (Sperry and Arora, 1965; Lundborg et al., 1986; Krarup et al., 2002; Nguyen et al., 2002). Previous studies strongly support the notion that regenerating axons are guided at nerve branch-points by dedicated molecular mechanisms, yet few such mechanisms have been described. Here we identify a molecular pathway critical for communication between glia located at a nerve branch-point and regenerating axons to direct axons of one nerve branch onto their pre-injury trajectory.

Specifically, our results provide compelling evidence for transient, spatially restricted, and tightly coordinated signaling events between col4a5/slit1a-expressing Schwann cells and robo2-expressing regenerating axons at a nerve branch-point critical to promote target-selective regeneration.

**Robo2 selectively destabilizes erroneous axonal extension at the nerve branch-point**

Live cell imaging experiments provide compelling evidence for a Robo2-dependent mechanism that directs regenerating axons into the appropriate nerve branch. As they encounter the nerve branch-point, regenerating dorsal nerve axons in both WT siblings and robo2 mutants initiate growth (~1 μm) along erroneous ventral and ventrolateral trajectories with similar frequencies (Fig. 5f). In robo2 mutants, axons are more likely to extend along these erroneous trajectories (Fig. 5K), ultimately forming errors 1.5-3 times longer than errors observed in WT larvae (Fig. 5M). This suggests that (1) robo2-independent mechanisms mediate error formation at the nerve branch-point, although it remains to be determined whether this occurs via collateral sprouting, misrouting of entire axons or both; and (2) rather than preventing their initial outgrowth, robo2 destabilizes axons on erroneous trajectories (>~1 μm), preventing their further growth. This is markedly different from the role that canonically repulsive axon guidance systems, including Slit-Robo, often play after injury (Hagino et al., 2003; Giger et al., 2010). For example, in Caenorhabditis elegans, Slit (slt-1) and Robo (sax-3) inhibit the extension of the mechanosensory PLM axon after transection, ultimately leading to reduced regeneration (Chen et al., 2011). In contrast, we find identical rates of regenerating axon outgrowth in robo2 mutants and WT animals (Fig. 5L). This provides strong evidence that, during zebrafish peripheral nerve regeneration, robo2-independent mechanisms promote axon outgrowth, while robo2’s role is to selectively bias regenerative growth of dorsal axons toward their original trajectory.

How similar is this robo2-dependent mechanism to other, well-documented mechanisms known to promote target-selective regeneration? In mammals, motor axons preferentially regenerate into their original nerve branches (Mark, 1965; Politis, 1985; Redett et al., 2005). This process is regulated by

![Figure 6](image-url)

**Figure 6.** robo2 is sufficient to promote dorsal branch-selection by regenerating axons. Representative images of Tg(hb9:GFP) (green) nerves with small numbers of fascicles expressing transient Tg(mnx1:mKate, mnx1:mKate) (A,B) or Tg(mnx1:mKate, mnx1:robo2) (C,D) (magenta) in small subsets of ventrally projecting motor axons. Merged GFP and mKate images shown at 5 dpf (A,C) and 48 hpt (B,D). mKate channel is shown alone at 5 dpf (A’,C’) and 48 hpt (B’,D’). In larvae expressing Tg(mnx1:mKate, mnx1:mKate), n = 13 of 13 nerves had only ventral regrowth of mKate+ fascicles, as in the example shown. In larvae expressing Tg(mnx1:mKate, mnx1:robo2), n = 7 of 15 nerves had dorsal regrowth of mKate+ fascicles, as in the example shown. Proportions of nerves with dorsal regrowth were compared between conditions using one-tailed Fisher’s exact test (p = 0.0054). Images were processed as described in Materials and Methods.

Dashed yellow boxes represent transection site. Scale bars, 10 μm.
\textbf{Figure 7.} \textit{co4a5} is required for the role of robo2 in branch-selective axon regeneration. Top, Representative images of \textit{Tg(hb9:GFP)} (green) nerves in \textit{co4a5}^{+/−} (WT sibling) larvae with fascicles expressing transient \textit{Tg(mnx1:mKate, mnx1:mKate)} (A,B) or \textit{Tg(mnx1:mKate, mnx1:robo2)} (C,D) (magenta) in small subsets of ventrally projecting motor axons. Merged GFP and mKate images shown at 5 dpf (A,C) and 48 hpt (B,D). mKate channel is shown alone at 5 dpf (A',C') and 48 hpt (B',D'). In larvae expressing \textit{Tg(mnx1:mKate, mnx1:mKate)}, \textit{n} = 12 of 12 nerves had only ventral regrowth of mKate′′ fascicles, as in the example shown. In larvae expressing \textit{Tg(mnx1:mKate, mnx1:robo2)}, \textit{n} = 5 of 14 nerves had dorsal regrowth of mKate′′ fascicles, as in the example shown. Proportions of nerves with ventral regrowth were compared between conditions using one-tailed Fisher’s exact test (\textit{p} = 0.0304). Bottom, Representative images of \textit{Tg(hb9:GFP)} (green) nerves in \textit{co4a5}^{−/−} larvae with fascicles expressing transient \textit{Tg(mnx1:mKate, mnx1:mKate)} (E,F) or \textit{Tg(mnx1:mKate, mnx1:robo2)} (G,H) (magenta) in small subsets of ventrally projecting motor axons.
Schwann cells and by nerve end-organs, such as muscle and skin (Madison et al., 2009; Abdullah et al., 2013). For example, after injury, Schwann cells in the distal nerve stump upregulate branch-specific neurotrophic factors and cell adhesion molecules (Höke et al., 2006; Jesuraj et al., 2012; Brushart et al., 2013; Wood and Mackinnon, 2015). These molecules support the outgrowth and maintenance of appropriate axonal populations (Martini et al., 1994; Franz et al., 2005) such that, when axons regenerate into inappropriate nerve branches, the resulting errors are pruned away over weeks or months (Brushart, 1993; Ghalib et al., 2001). Thus, in contrast to well-documented pruning mechanisms that occur long after axons have regenerated toward incorrect targets, the robo2-dependent mechanism we describe here is engaged during the time period when regenerating axons confront a choice-point, thereby promoting target-selective regeneration locally and on a much shorter timescale.

A co4a5/glia robo2/axon-dependent mechanism provides local guidance to promote target-selective regeneration

In response to peripheral nerve injury, Schwann cells distal to the injury site change their differentiation state to that of a repair Schwann cell through a well-characterized molecular pathway (Jessen and Mirsky, 2016, 2019). Repair Schwann cells exhibit location- and modality-specific differences in gene expression (Höke et al., 2006; Jesuraj et al., 2012; Brushart et al., 2013), yet the functional significance of these differences, including their roles in target-selective regeneration, have remained largely unknown. We previously reported that in response to peripheral nerve injury in zebrafish, a small group of Schwann cells (~1-3) located where the dorsal nerve branch deviates from the ventral nerve branch, upregulate col4a5 and its binding partner, the repulsive guidance cue slit1a (Xiao et al., 2011; Isaacman-Beck et al., 2015). While we had shown that col4a5 is critical for target-selective regeneration, whether the spatial restriction of col4a5 to just a few Schwann cells was important for target selectivity was unclear. Similarly, whether Slit1a played a functional role in this process and whether Slit1a and col4a5 expression were functionally related had not been defined.

Our results demonstrate that expanding the expression of col4a5 to all Schwann cells severely impacts target-selective regeneration (Fig. 1). Although we cannot exclude the possibility that overexpression of col4a5 affects Schwann cell development, the regeneration phenotype we observe in Tg(sox10:col4a5-myc) larvae is distinct from what we have previously reported in larvae with absent or immature Schwann cells (i.e., mutants for sox10 or erbB3) (see Rosenberg et al., 2014). Rather than extending along random trajectories, regenerating dorsal nerve axons in Tg(sox10:col4a5-myc) larvae extend along erroneous ventral and ventrolateral trajectories, similar to what we observe in mutants lacking col4a5, robo2, and extl3, respectively (Fig. 3) (Isaacman-Beck et al., 2015). Thus, in animals expressing col4a5 in all Schwann cells, regenerating dorsal nerve axons make errors precisely where they pause and explore the nerve branch-point before turning dorsally during regeneration (Fig. 5). Combined,
this provides compelling evidence that, rather than providing a permissive substrate and environment, spatially restricted col4a5 expression is critical to instruct regenerating dorsal nerve axons at the nerve branch-point.

Previous work in rodents and zebrafish has demonstrated that various collagens, including Collagen XV (Guillon et al., 2016), Collagen XIX (Hilario et al., 2016; Wakabayashi, 2021), as well as other ECM components and modifications, play attractive and repulsive roles in axonal development and regeneration (for review, see Chelyshev et al., 2020). Our results highlight a pivotal yet less appreciate role for collagens, not only as an ECM component critical for axonal regeneration, but also as part of an instructive signaling pathway to direct regenerating axons in vivo. Indeed, our data provide compelling evidence that precise spatial localization of col4a5 is critical for its role in peripheral nerve regeneration and suggest a potentially more specialized role for Collagen conduits in clinical applications to direct regenerating peripheral nerve axons toward their original synaptic targets.

Our results also reveal a previously uncharacterized role for Slit-Robo signaling in target-selective regeneration. Loss-of-function mutations in two Slit-Robo signaling components, exil3 and robo2, result in the same target-selective regeneration defects (Fig. 3). Conversely, we find that transgenic expression of robo2 in ventral nerve axons, which are unaffected by the loss of Slit-Robo signaling, is sufficient to redirect these axons onto a dorsal nerve branch specific route (Fig. 6), and that this process requires col4a5 (Fig. 7). Given the incomplete penetrance of the robo2 mutant phenotype in target-selective regeneration, we cannot exclude the possibility that one or multiple of the additional three zebrafish Robo receptors (Lee et al., 2001; Bedell et al., 2005) play a role in this process and hence partially compensate for the loss of robo2. Future experiments, including generating single- and double-mutant combinations for the other three Robo receptors as well for each of the four known Slit ligands (Hutson et al., 2003), will provide a comprehensive view on the role of Slit-Robo signaling during target-selective regeneration and inform the contribution of individual Robo receptors in this process.

Nonetheless, our results provide strong support for a mechanism in which glial-derived col4a5 expression restricted to the branch-point promotes dorsal turning of regenerating axons possibly via Col4a5-bound Slit1a. In the future, it will be important to further characterize the molecular identity of the col4a5/slit1a-expressing branch-point Schwann cells, particularly how they sense and respond to spinal motor nerve injury. Candidate regulators of this process include c-Jun and STAT3 (Gordon et al., 2016), which transiently express restricted to the nerve branch-point. These factors have been shown to regulate axon regeneration. J Neurosci 26:9646–9655.

References