Supplemental Information

The *lh3* glycosyltransferase directs target-selective peripheral nerve regeneration

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SUPPLEMENTAL DATA INVENTORY

Figure S1, related to Figures 2 and 3, shows rescue of developmental axon guidance defects in *lh3* mutants by conditional global expression of *Tg(hsp70:lh3myc)* and shows the method by which loss of function of *lh3* was tested in peripheral nerve regeneration.

Figure S2, related to Figures 3, 4, and 7, shows methodology for quantification of guidance defects in peripheral nerve regeneration and shows that regenerating axons form new synapses in the target region.

Figure S3, related to Figures 4 and 5, shows that *lh3* modification is required for the *in vivo* localization of its substrate, Collagen4a5.

Figure S4, related to Figures 4 and 5, shows that neuromuscular basement membranes are indistinguishable between *col4a5* mutants and wild type siblings.

Movie S1, related to Figure 2, contains the movie which corresponds to the wild type dorsal nerve regeneration time series shown in Figure 2A-F.

Movie S2, related to Figure 2, contains the movie which corresponds to the *lh3* dorsal nerve regeneration time series shown in Figure 2G-L.

Movie S3, related to Figure 5, contains the movie which corresponds to the *col4a5* dorsal nerve regeneration time series shown in Figure 5G-L.

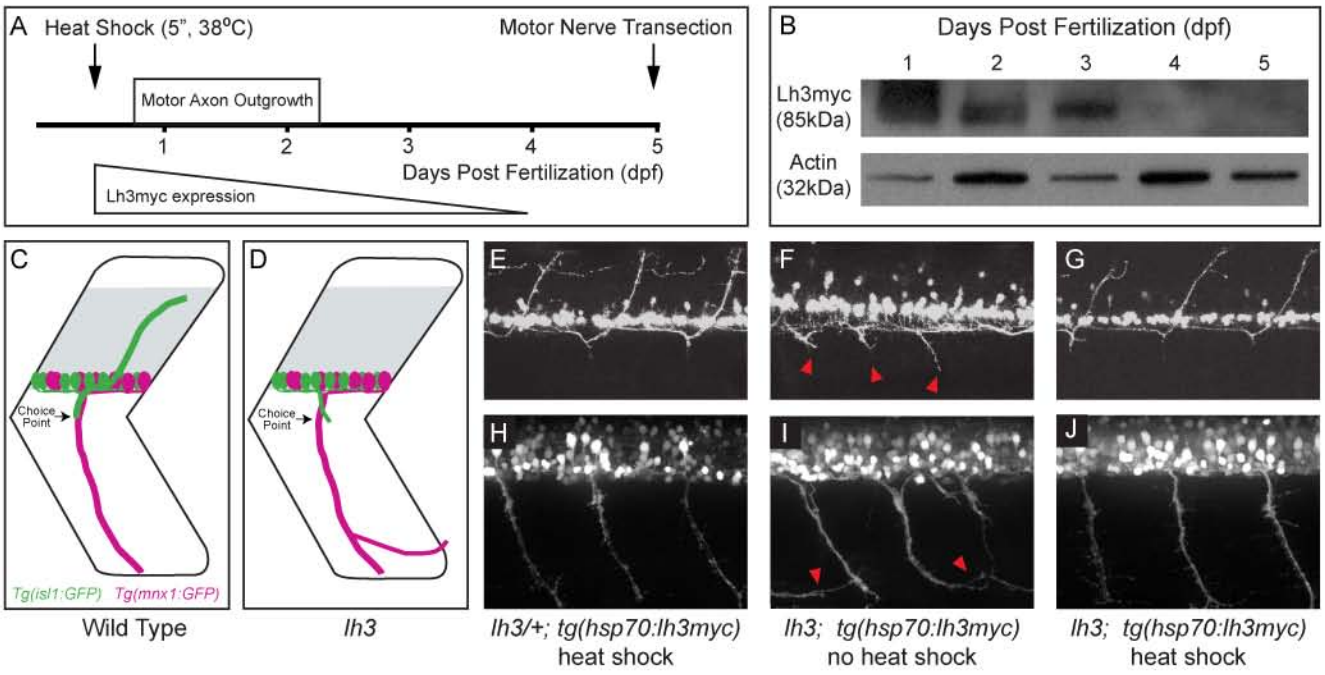

Figure S1. Conditional expression of *Tg(hsp70:lh3myc)* rescues *lh3* motor axon guidance defects, related to Figures 2 and 3. (A) Schematic showing conditional expression paradigm. **(B)** *Tg(hsp70:lh3myc)* is no longer detectable by western blot at 4 dpf. **(C,D)** Schematics showing patterns of dorsal and ventral motor axon outgrowth detected in **(E-G)** and **(H-J)** with *Tg(isl1:gfp)* and *Tg(mnx1:gfp)* respectively. **(E-G)** Dorsal (72hpf) and **(H-J)** ventral (52hpf) motor axon development was rescued by expression of *Tg(hsp70:lh3myc)* (n > 20 larvae, 200 nerves per condition). Red arrowheads show misguided mutant axons.

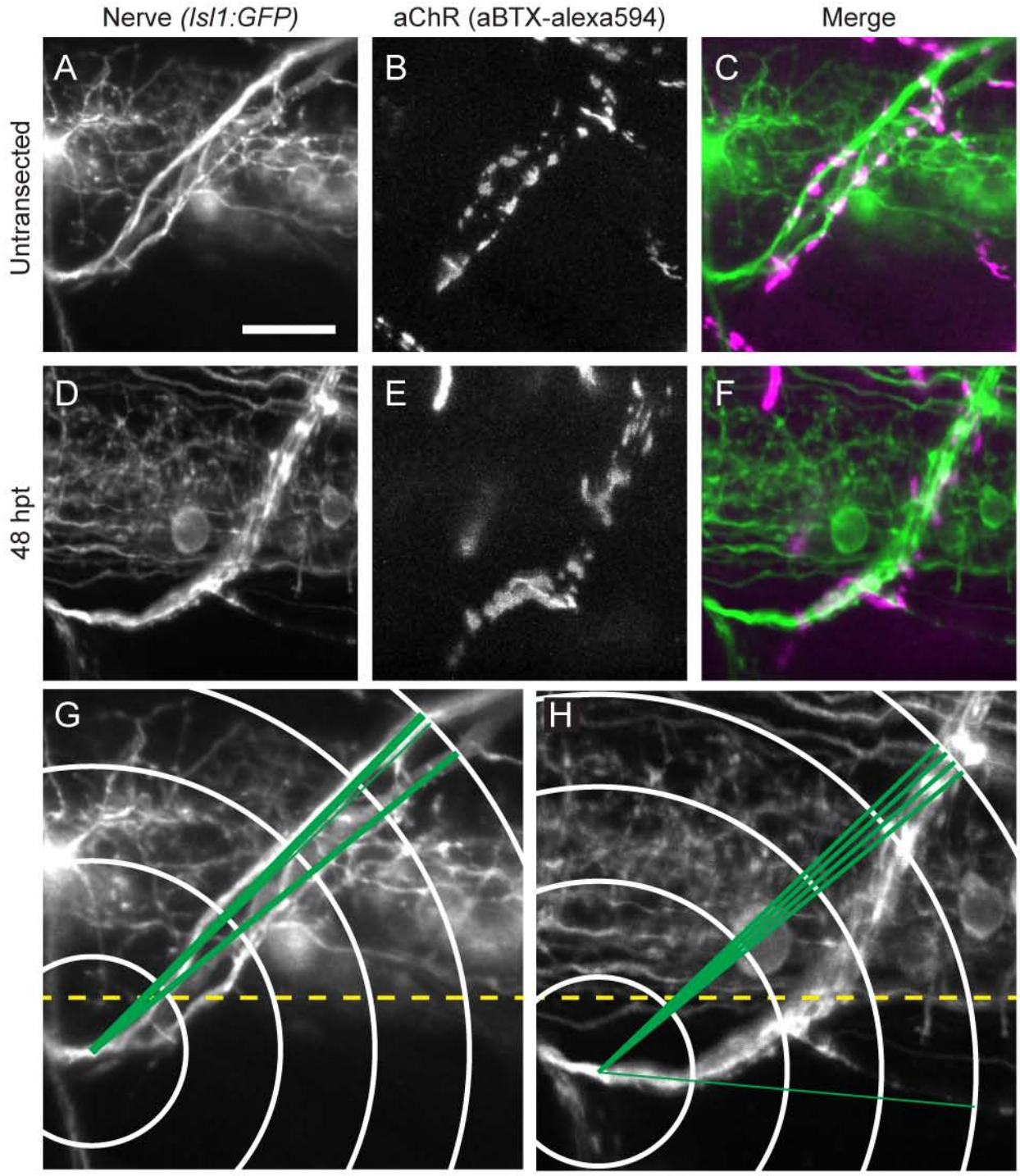

Figure S2. Modified sholl analysis of target selection before and after nerve transection, related to Figures 3, 4, and 7. (A-F) a-Bungarotoxin detection reveals that dorsal nerves form neuromuscular junctions along the main nerve trunk at 5DPF (A-C) and after nerve transection (D-F). **(G,H)** Sholl analysis on 5DPF (G) and 48 hpt (H) nerves. Images are rotated so the spinal cord is aligned on the horizontal axis. Concentric circles (white arcs) are drawn emanating from the dorsal turn and lines are drawn to the furthest point of intersection. Line thickness is determined by number of visible fascicles crossing at a given point. Scale bar = 10μm.

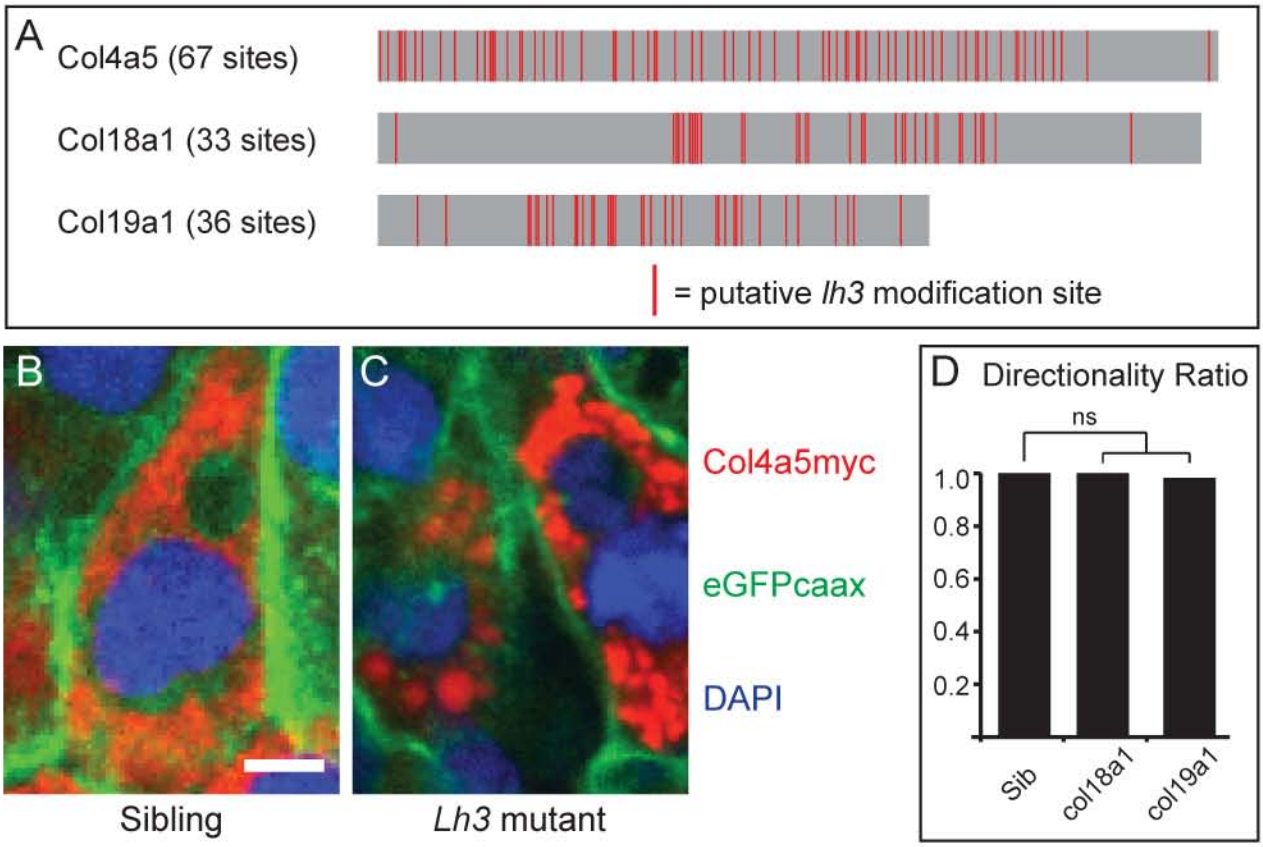

Figure S3. Lh3 modifies Collagens and is required for Col4a5 localization, related to figures 4 and 5 (A) Lh3 glycosylation sites are present in the three collagens that we tested for roles in motor axon regeneration (grey bars – schematic of protein; red stripes – putative *lh3* modification sites). **(B)** *Tg(sox10:col4a5myc)* is expressed diffusely throughout the cells in wild type siblings and associates with membranes, but in *lh3* mutants **(C)** Col4a5myc aggregates in large puncta in the cytoplasm (n > 10 embryos, 50 cells per condition; Scale bar = 5μm). **(D)** *col18a1* and *col19a1* are not required to guide regenerating dorsal fascicles (n > 20 larvae, 200 nerves per condition).

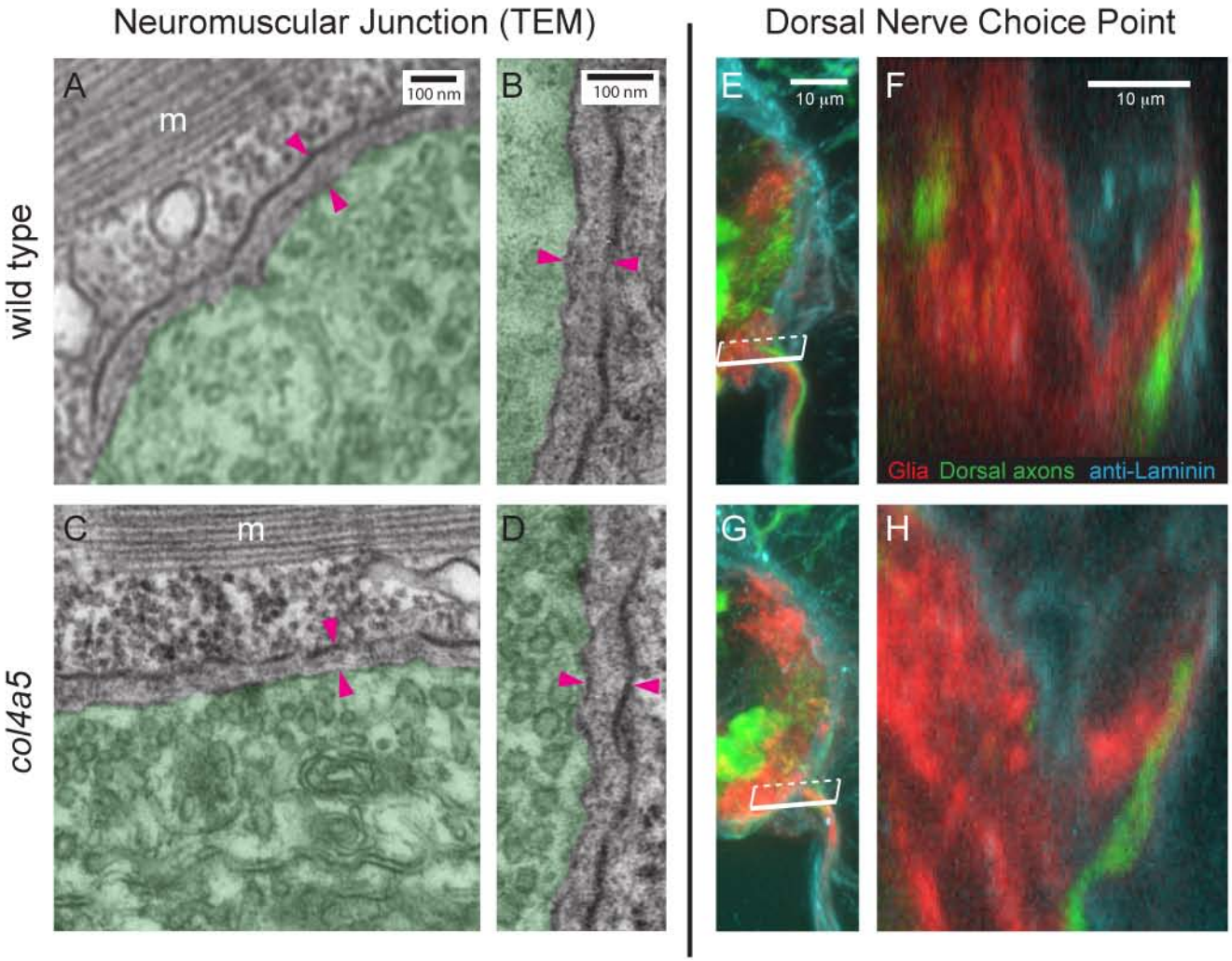

Figure S4. Basal laminae are intact in the *col4a5* mutant, related to Figures 4 and 5. (A) representative ultrastructure of wild type neuromuscular junction (n = 3 larvae, 23 NMJs). **(B)** higher magnification showing details of wild type junctional gap. **(C)** *col4a5* neuromuscular junction (n = 3 larvae, 17 NMJs). **(D)** higher magnification showing details of *col4a5* junctional gap. Green shading, axon; magenta arrowheads, plasma membranes enclosing basement membrane; m, muscle. **(E)** Representative cross-section of wild type dorsal nerve axons (green) exiting the spinal cord surrounded by glia (red) and basal lamina (cyan) (n = 10 larvae, 30 nerves). **(F)** Oblique optical cross section from (E) showing detail of dorsal axon exit and choice point. **(G)** Cross-section of *col4a5* dorsal nerve axons exiting with glia and basal lamina (n = 10 larvae, 30 nerves). **(H)** Oblique optical cross section from (G).

SUPPLEMENTAL MOVIE LEGENDS

Movie S1. The dynamics of wild type dorsal nerve regeneration, related to Figure 2. Regenerating axons of the dorsal nerve were imaged *in vivo* using *Tg(isl1:GFP)*. After growth cone sprouting, axons initially probe the transection gap multi-directionally (arrow, dorsal searching; arrowhead, ventral searching). Thereafter, axons stabilize searching on the dorsal path (bracket), and destabilize searching on the ventral path. Finally axons on the dorsal path rapidly extend into the dorsal myotome (arrow in final frames). Movie starts at ~11 hpt, images were taken every 10 minutes for ~7.5hrs as indicated by the time stamp. Images were processed as described in Experimental Procedures; scale bar = 10 μm; n = 8 larvae, 15/16 nerves.

Movie S2. The dynamics of *lh3* dorsal nerve regeneration, related to Figure 2. Regenerating axons of the *lh3* dorsal nerve were imaged *in vivo* using *Tg(isl1:GFP)*. After growth cone sprouting, axons probe the transection gap multi-directionally as in wild type siblings (arrow, dorsal searching; arrowhead, ventral searching). However, axons fail to stabilize on the dorsal path and fail to destabilize searching on the ventral path. Instead axons continue to probe aberrant regions of the myotome (red circles). Movie starts at ~8 hpt, images were taken every 10 minutes for ~10hrs as indicated by the time stamp. Images were processed as described in Experimental Procedures; scale bar = 10 μm; n = 7 larvae, 5/9 nerves.

Movie S3. The dynamics of *col4a5* dorsal nerve regeneration, related to Figure 5. Regenerating axons of the *col4a5* dorsal nerve were imaged *in vivo* using *Tg(isl1:GFP)*. After growth cone sprouting, axons initially probe the transection gap multi-directionally (arrow, dorsal searching; arrowhead, ventral searching). Thereafter, axons stabilize searching on the dorsal path (angled bracket), but fail to destabilize ventral searching (horizontal bracket). Finally, axons rapidly extend into both the dorsal myotome and ventral myotome (arrow and arrowhead in final frames). Movie starts at ~11 hpt, images were taken every 10 minutes for 11hrs as indicated by the time stamp. Images were processed as described in Experimental Procedures; scale bar = 10 μm; n = 8 larvae, 17/25 nerves.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Zebrafish genetics and transgenes:

Several transgenes were utilized for live visualization of different cell types *in vivo*: *Tg(mnx1:GFP)*^{ml2} (Flanagan-Steet et al., 2005), *Tg(Xla.Tubb:DsRed)*^{zf148} (Peri and Nüsslein-Volhard, 2008), and *Tg(CM-isl1:GFP)* (Uemura et al., 2005) were used to label spinal motor nerves, and the *Tg(sox10(7.2):mRFP* (Kucenas et al., 2008) was used to label peripheral glia. *lh3*^{tv2o5} mutants (Schneider and Granato, 2006) were used in Figures 1-4 and *col4a5*^{s510} (Xiao and Baier, 2007) and *col19a1*^{b393} (Hilario et al., 2010) mutants were used in Figure 5. The *Tg(hsp70:lh3-myc)* was generated by microinjection of hsp70:lh3-myc as previously described (Thermes et al., 2002). *Tg(sox10: lh3-mkate)*, *Tg(aActin:lh3-mkate)* and *Tg(sox10:col4a5myc)* were generated by microinjection of sox10:lh3-mkate, aActin:lh3-mkate, or sox10:col4a5myc plasmid DNA with *tol2* mRNA as previously described (Suster et al., 2009). *col18a1*$^{-/-}$ mutants were generated through TALEN injection and identified using high resolution melt analysis (HRMA) as described (Dahlem et al., 2012).

Genotyping

Genotyping protocols for the following mutants were performed as previously described: *lh3*^{TV2O5} (Schneider and Granato, 2006) and *col4a5*^{s510} (Xiao and Baier, 2007). *col19a1*^{b393} mutants were phenotyped for delay in ventral motor nerve outgrowth using the *Tg(Xla.Tubb:DsRed)*^{zf148} transgene (Beattie et al., 2000; Peri and Nüsslein-Volhard, 2008). Fluorescent transgenic expression was defined using an upright Olympus fluorescence dissection microscope. *col18a1* mutant alleles were amplified from exon4 using the following primers: 5' ACTACACCGAGCCTGATTCGCA 3' (forward) and 5' CCTCACTGCCATTTAACCCG 3' (reverse). Amplicons were digested using Afe-I which cuts the 308bp wild type band to 159bp and 149bp, but does not digest any of the wild type alleles. *Tg(hsp70:lh3-myc)* was genotyped by amplifying the myc transgene using the following primers: 5' CCGATTAACACACTACCACGAG 3' (forward) and 5' ATTAAGCTAGCGGTGAGGTCGCCCTAGCTCTCCAT 3' (reverse).

Conditional *lh3* expression

Embryos from matings between *lh3*^{TV205}/+; *Tg(hsp70l:lh3myc)* and *lh3*^{TV205}/+ adults were kept at 28ºC to the desired stage. For developmental heat shock (HS) treatments, 5 12 hpf embryos were placed in 150µL E3 medium in a single well of 96-well PCR plate, heat shocked for 5 min at 38ºC, and returned to 28ºC until 5 dpf. This treatment rescued developmental motor axon outgrowth in transgenic *lh3* embryos, failed to rescue non-transgenic embryos and had no effect on transgenic siblings (Figure S1). For post-transection HS, individual 6 hpt larvae were placed in 150µL E3 medium in a single well of a 96-well PCR plate, heat shocked for 30min at 38ºC and returned to 28ºC until observation at 48 hpt. This treatment rescued regenerative motor axon guidance in transgenic *lh3*

larvae, failed to rescue non-transgenic embryos and had no effect on transgenic siblings (Figure 3 and data not shown).

Western Blot

Embryos from the cross of *lh3*^{TV205}*/+; Tg(hsp70l:lh3myc)* to *lh3*^{TV205}*/+* were kept at 28ºC and were heat shocked for 5 min at 12hpf at 38ºC. Embryos were raised as before and were lysed in pools of 20 in lysis buffer (10mM Tris pH 7.5, 150mM NaCl, 5mM EDTA, 1% NP40, 10% glycerol) at 24hpf, 48hpf, 72hpf, 96hpf, and 120hpf and then were quickly frozen in liquid nitrogen. Western Blots were performed as previously described using the mouse monoclonal α-myc antibody 9E10 (Jing et al., 2010).

a-Bungarotoxin labeling

Laser-transected 5DPF larvae were fixed and peeled as previously described (Wolman et al., 2015) and were incubated with Alexa-594 conjugated a-Bungarotoxin (1:500, Molecular Probes, Eugene, Oregon) for 3hrs at 4ºC. Larvae were mounted and imaged as described in Experimental Procedures.

Plasmid Construction

Lh3-myc was cloned into pCS2+ as previously described (Schneider and Granato, 2006) and was subcloned into pzHSP70 (Halloran et al., 2000) via Cla-I and Apa-I sites. Infusion cloning (Clontech – Kit #638909) was used to make a c-terminally *mKate*-tagged *lh3* construct. pCS2+-Lh3-eGFP (Schneider and Granato, 2006) was used as a template for lh3 without a stop codon and with an upstream Cla-I site was amplified using the following primers: 5' TGCAGGATCCCATCGATGCCACCATGACACCGGTGCC 3' (forward) and 5' CAGCTCGCTCACCATGGTTGTGGCCATATTATCAT 3' (reverse). The c-terminal mKate fusion protein with downstream Xho-I site was amplified from pCS2+-V2AmKate using the following primers: 5' ATGGTGAGCGAGCTGATTA 3' (forward) and 5' GTTCTAGAGGCTCGAGTCATCTGTGCCCCAGTTTG 3' (reverse). The two amplicons were then directionally ligated into Cla-I and Xho-I sites in the pCS2+ MCS according to kit protocol. The Lh3-mkate fusion construct was then shuttled into the pENTR-d/topo plasmid (Lifetechnologies – K2400-20) according to kit protocol and then into pDestTol2pA2 behind either the sox10 promoter (Kucenas et al., 2008) orthe aActin promotor (Higashijima et al., 1997) using the Tol2kit for Multisite Gateway cloning (Kwan et al., 2007). Col4a5myc was constructed by amplifying mouse Col4a5 cDNA (NM_007736) from pCMV6-Col4a5-Kan/Neo (purchased from Origene, Rockville, MD) in 2 fragments. The 5' fragment of Col4a5 (nucleotides 1-1047) was amplified using the following primers: 5' AAAAATCGATGCCACCATGCAAGTGCGTGGAGTGT 3' (forward) and 5' GCTAGCCTTGTCATCGTCATCCTTGTAGTCAGGTGCAGGAATTACAAGTCCG 3' (reverse) – this includes a 1X Flag tag and a 3' Nhe-I restriction site. The 3' fragment of Col4a5 (nucleotides 1057 – 5077) was amplified using the following primers: 5' GCTAGCGTGACTATGGGAGAAAAAGGAAATATCGG 3' (forward) and 5' AAAACTCGAGTTATGTCCTCTTCATGCATACTTGACATCG 3' (reverse)

which incorporates a 5' Nhe-I site and a 3' Xho-I site. Both Col4a5 fragments were TOPO cloned into pCR2.1 (Lifetechnologies – K4510-20) to make pCR2.1-Col4a5-5'-Flag and pCR2.1-Col4a5-3'. Col4a5-3' was digested out of pCR2.1-Col4a5-3' using Nhe-I and Xho-I restriction sites and ligated into pCR2.1-Col4a5-5'-Flag to make pCR2.1- Col4a5-Flag. Col4a5-Flag was digested out of pCR2.1- Col4a5-Flag with Cla-I and Xho-I and ligated into pCS2+ to generate pCS2+-Col4a5-Flag. To insert a Myc tag into pCS2+-Col4a5-Flag we amplified 5x-Myc (with no stop codon) from pCS2+-Lh3-myc (Schneider and Granato, 2006) using the following primers: 5' AAAAAGCTAGCGGTGAGGTCGCCCTTGCT 3' (forward) and 5' AAAACCTAGCCGTAAGGTAAATCGATCG 3' (reverse) which adds both a 3' and 5' Nhe-I sites. We digested the 5x-Myc amplicon with Nhe-I and ligated 3' to the Flag tag in pCS2+-Col4a5-Flag to make pCS2+-Col4a5-Flag-5xMyc (now called pCS2+-Col4a5myc). Col4a5myc was then shuttled into the pENTR-d/topo plasmid (Lifetechnologies – K2400-20) according to kit protocol and then into pDestTol2pA2 behind the sox10 promoter (Kucenas et al., 2008).

Axon Growth Extent Quantification

The extent of nerve regrowth was defined by 5 categories: 1 - axons failed to regrow or did not extend dorsal to the spinal cord; 2 - one fascicle grew dorsal to the spinal cord but did not grow the entire length of the dorsal myotome; 3 - multiple fascicles grew partially through the dorsal myotome or a single fascicle grew through the entire myotome; 4 - multiple fascicles grew past the spinal cord and one grew through the entire length of the dorsal myotome; 5 - Two or more fascicles grew through the entire length of the dorsal myotome.

Electron Microscopy

Embryos were fixed at 5dpf in 9% Gluteraldehyde in 0.1M cocodylate for 1hr at RT and used immediately or stored for up to several days at 4ºC in the fixative. Larvae were pierced anterior to the yolk sac and the last 5 somites of tail tissue were removed within the first minutes of fixation to allow for better penetration of the fixative. Tails were post fixed, sectioned, observed and imaged as previously described (Rosenberg et al., 2012).

SUPPLEMENTAL REFERENCES

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