Supporting Information

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SI Methods

Cloning and Virus Construction. All KLF7 mutants were created using PCR-based amplification, restriction digestion, and ligation into recipient vectors using restriction enzymes, 2× Phusion Master Mix, and T4 Ligase from New England Biologicals. Primer sequences are listed in Table S1.

EGFP-tagged KLF7 mutants. EGFP was cloned into a pAAV-MCS (Stratagene) using PCR primers that removed the stop codon and inserted in-frame Kpn1 and Xho1 sites at the 3′ end. Full-length KLF7 and truncation mutants of KLF7 were PCR amplified using primers that incorporated Kpn1 (5′) and Xho1 (3′) recognition sites, digested, and inserted 3′ to EGFP.

EGFP-tagged VP16-KLF7 and Engrailed-KLF7. The KLF7 zinc finger and adjacent nuclear localization signal was PCR amplified using primers that incorporated 3′ Kpn1 and BamH1, then ligated into AAV-EGFP. The VP16 activation domain was PCR amplified from the pYSTrp3 vector (Invitrogen), and the Engrailed repression domain was PCR amplified from Addgene plasmid 19715 (1) and ligated immediately upstream of the KLF7 ZF domain.

2A-mCherry constructs for in vivo experiments. A 2A plasmid was first constructed in an AAV backbone (Stratagene) by inserting a 2A peptide sequence upstream of mCherry. The 2A bridge was modified from the original sequence found in the Thosea asigna virus (2, 3). The amino acids comprising the 2A bridge facilitate a ribosomal pause and as such produce two individual proteins from a polycistronic mRNA. EBFP (Addgene plasmid 14891) (4), KLF7, VP16-KLF7, and Eng-KLF7 were then PCR amplified using primers that incorporated a 3× Flag signal at the 5′ end, digested, and ligated upstream of the 2A-mCherry sequence.

Cloning primers.

KLF7 (forward) GGTACCATGGACGTGTTGGCTAGTTATA
Δ7 KLF7 (forward) GGTACCATGAGTATATTCCAGGAGCTACAAC
Δ15 KLF7 (forward) GGTACCATGGTCCACGACACCGGCTAC
Δ23 KLF7 (forward) GGTACCATGGCTTTGCCATCCCTGGAG
Δ32 KLF7 (forward) GGTACCATGCAGCAGACATGCCTTGAGTT
Δ40 KLF7 (forward) GGTACCATGCGCTACCTCCAGACAGAAC
Δ76 KLF7 (forward) GGTACCATGGGATCCCTAGACCCCTTGCTGCTCC
KLF7 (reverse) CTCGAGTTAGATATGTCTCTTCATATGGA
KLF7 nls/zinc (forward) GGTACCATGGGATCCCCTGAAAACAAGAAGAGGGTC
KLF7 (reverse) CTCGAGTTAGATATGTCTCTTCATATGGA
VP16 (forward) GGTACCATGAATTGCATCGATACGGCCCC
VP16 (reverse) GGATCCCCCACCGTACTCGTCAATTC
Engrailed (forward) GGTACCATGGCCCTGGAGGATCGCTGC
Engrailed (reverse) GGATCCGATCCCAGAGCAGATTTCTCT

AAV8 virus production. Viral particles were produced using FPLC-based purification at the Miami Project Viral Vector Core, using AAV8 capsid plasmids obtained from the University of Florida. Using a qRT-PCR method, titers were measured in the range of 2.5–3 × 10^{14} viral particles per milliliter.

Luciferase Assays. 293T cells were cultured in DMEM containing 10% FBS, glutamine, sodium pyruvate (all from Invitrogen). Using Lipofectamine 2000 (Invitrogen), 293T cells were cotransfected at a 3:1 ratio with EGFP-tagged KLF constructs and a reporter plasmid with 970 bp of the P21 promoter, or scrambled control sequence, upstream of *Renilla* luciferase (SwitchGear Genomics). At 24 h after transfection, cells were lysed and supplied with luciferase substrate (SwitchGear Genomics) reagents, and luminescence was quantified using a Fluostar Omega Plate Reader (BMG Labtech).

Neuronal Cell Culture. Neurons were cultured, and neurite outgrowth was quantified as described previously (5). Briefly, rat cortical neurons from postnatal day 3 pups (Sprague–Dawley; Charles River) were dissociated by sequential incubation with 20 U/mL papain (Worthington) and 1 mL 2.5% trypsin (Gibco) and plated in 24-well plates (Falcon) coated with 100 μg/mL PDL (Sigma) and 10 μg/mL mouse laminin I (Cultrex). Cell density was 10,600 cells/well, and growth media were supplemented, glial-enriched neurobasal media (Invitrogen) (5). After 3 d, neurons were fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich)/4% sucrose (Sigma-Aldrich), and immunohistochemistry for neuron-specific βIII-tubulin was performed as previously described. Neurons were then imaged using an ArrayScan VTI HCS Reader automated microscope (Cellomics). Thresholds for EGFP detection were set such that <1% of nontransfected neurons were defined as expressing EGFP. Using the Neuronal Profiling Algorithm, neurite lengths were quantified for all EGFP+ neurons. Data were then exported to Spotfire software for analysis.

Cortical Slice Culture. P5 Sprague–Dawley rats were anesthetized on ice and killed in accordance with guidelines set by the University of Miami Institutional Animal Care and Use Committee. Brains were placed in dissection media [37.7 mL HBSS (GIBCO), 0.8 mL of 1 M Hepes (GIBCO), 1.1 mL of 1.2 M D-glucose (Sigma), and 0.4 mL of 1 M magnesium sulfate (Sigma)]. Meninges were removed and 350-μm coronal sections of cortex were prepared using a McIlwain tissue chopper. Sections were trimmed to prepare ∼2 × 2-mm sections of right and left cortex linked by an intact corpus callosum. Slices were cultured on 30 mm Organotypic PTFE 0.4-μm culture inserts (Millipore; PICMORG50) on 35 × 10-mm culture dishes (Falcon) with a culture media consisting of 50% Basal Medium Eagle (GIBCO), 18% HBSS (GIBCO), 2% SM1 50× supplement (StemCell), 4 mM L-glutamine (Invitrogen), 6 mg/mL 1.2 M D-glucose (Sigma), and antibiotic/antimycotic (Invitrogen). Two hours later, a 0.5-μL drop of viral particles was applied to each hemisphere. The virus mixture consisted of control treatment: AAV8-fEBFP-2A-mCh (control virus) + AAV8-EGFP (reporter virus) 1:1 ratio; fVP16-KLF7-2A-mCh treatment: AAV8-anti luciferase (reporter virus) + AAV8-fVP16-KLF7-2A-mCh (treatment) 1:1 ratio. The sections were rinsed the next day with culture media to remove the virus. Either immediately after rinsing, or 7 d later, virus-treated slices were transected along the midline, and then paired with slices that were prepared as described but not treated with viral particles. In the pairings, the right hemisphere of viral-treated cortex was paired with the left hemisphere of nontreated cortex such that the two sides of the severed corpus callosum were opposed,

and vice versa. Regardless of how long the virus-treated slices were maintained in culture before transection, the nontreated recipient slice was also prepared from a P5 animal and aged 1 d in culture. After pairing, cocultured slices were maintained an additional 7 d, with media refreshed every other day, and then fixed with 4% paraformaldehyde, 4% sucrose (Sigma) and mounted on Micro Slides (VWR) with Fluoro-Gel (Electron Microscopy Services). To quantify the growth of EGFP-labeled axons, 60× confocal pictures were taken through all Z-planes of the slice at a distance of 1 mm away from the tissue boundary, and the total number of axons was extrapolated by measuring the length of the boundary. Axons crossing a virtual line 1 mm from the boundary were manually counted using Olympus Fluoview FV1000 software.

Laser Capture Microdissection and qRT-PCR Analysis of KLF7 Expression.

Retrograde labeling of corticospinal tract neurons and laser capture microdissection. Eight-wk-old female C57/BI6 mice were anesthetized with ketamine and xylazine. The neck and back of mice were shaved and an incision made from approximately the occipital notch to the T2 dorsal vertebral. The vertebral column was exposed from T2 to C2 and clamped into a custom-built stabilizer (Ping Zheng, Norton Neuroscience Institute). Connective tissue and dura were removed to expose the dorsal surface of the spinal cord between C3 and C4, and 0.8 μL of 1% CTB-Alexa Fluor 555 (Invitrogen) was injected over 4 min at a depth of 0.8 mm, just left of the dorsal medial vein, through a Hamilton syringe with a pulled glass pipette driven by a Stoelting QSI Infusion Pump. One week later, mice were killed by C02 inhalation and brains were immersion fixed in zinc fixative (BD Biosciences) with 30% sucrose at 4 °C overnight. Brain were embedded in tissue-freezing medium (Triangle Biomedical Sciences) and frozen in liquid nitrogen. The 12-μm cryostat sections of frontal cortex were mounted on PEN-Membrane 2.0-μm slides (Leica), dehydrated for 30 min in a vacuum chamber with desiccant-anhydrous indicating drierite (W.A. Hammond Drierite Company), and CTB-Alexa Fluor 555-labeled cells were outlined and collected into extraction buffer (Arcturus PicoPure RNA Isolation Kit; Applied Biosystems) using a laser capture microscope (Leica LMD6000). RNA was then extracted following manufacturer instructions and eluted in a final volume of 15 μL elution buffer.

qRT-PCR. RNA was subjected to reverse transcription (iScript; Bio-Rad), and quantitative PCR reactions (SYBR Green) were performed using a Bio-Rad thermocycler with KLF7 and 18S primers. Three-repeat wells (technical replicates) were used for each condition in addition to no-enzyme control wells. Fold change was calculated according to primer efficiency as described previously (6). Each experiment was performed 3× with different pools of RNA (biological replicates). Primers were KLF7 forward: ttgctctctcgggacaagtt, reverse: gagctgagggaagccttctt. This primer set recognizes all known isoforms of KLF7 (University of California San Diego Genome Browser).

Spinal Cord Injury Experiments. All animal experiments were performed in accordance with guidelines set by the University of Miami Institutional Animal Care and Use Committee.

Viral injections. Female C57/Bl6 mice were anesthetized, a craniotomy was performed, and mice were placed in a stereotaxic frame (Stoelting Mouse Adaptor). Using a Hamilton syringe attached to pulled glass needle and a Stoelting QSI infusion pump, 0.5 μL of viral particles were injected over the course of 10 min into a single site located 1.5 mm lateral from Bregma (left), 0.5 mm anterior, and 0.55 mm below the surface of the brain. The needle was then withdrawn and the skin closed with staples.

Pyramidotomies. One week after virus injection, mice were reanesthetized and pyramidotomy was performed as previously described (5). Following craniotomy of the occipital bone using laminectomy forceps to expose the underlying pyramidal tract, a microfeather scalpel was used to puncture the dura and lesion the entire right pyramidal tract. Four weeks after pyramidotomy (5 wk after virus injection), mice were euthanized by transcardial perfusion.

Spinal cord injuries. Using the procedures described above for CTB-555 injection, 8-wk female C57/Bl6 mice were placed in a custom spinal stabilization device and the spinal cord between C4 and C5 was exposed. The right dorsal quadrant of the spinal cord was then transected using a custom-built Vibraknife (Ping Zheng and Christopher Shields, Norton Neurologicals), set to a depth of 0.9 mm below the dorsal surface of the spinal cord and 1 mm to the left of the midline and extending beyond the right edge of the spinal cord. After transection, while the blade was still in the spinal cord, the cord was carefully inspected for depression of the surface, which is indicative of distorted tissue and potentially incomplete transection. The blade was then removed, the muscle sutured, and the skin closed with staples.

Tissue processing and analysis. Mice were perfused with 250 mL PBS followed by 250 mL 4% PFA, and brains and spinal cord were postfixed in 4% PFA overnight at 4 °C. Cortex, medullas, and spinal cords from 2 mm rostral to 4 mm caudal from the injury site were embedded in 12% gelatin (Sigma; G2500), and 50 μm (brains and medulla) or 100 μm (spinal cord) free-floating sections were cut on a Leica VT100S Vibratome. Immunohistochemistry for GFAP was performed on free-floating sections using 20% normal goat serum/PBS block, DAKO rabbit anti-GFAP primary antibody (1:500), and goat anti-rabbit Alexa Fluor 647 (Invitrogen; 1:500). Sections were mounted on glass slides and viewed under fluorescence using an Olympus IX81 microscope. In the pyramidotomy experiment, transverse cryosections of the cervical enlargement (C4–C5) were quantified by measuring the axon density in the denervated gray matter (averaged over four sections) and normalized against total axon count in medulla to obtain the axon index, which is then plotted as a function of the distance from the midline. In the spinal cord injury experiments, axon growth was quantified using 100-μm sagittal sections of spinal cord, in which a virtual line was drawn at the site of injury, and the number of EGFP+ profiles that intersected that line were counted, as well as lines 250, 500, 1,000, or 1,500 μm caudal. All sections from the right (injured) side of the animal were analyzed, as well as the first section to the left of the left of the midline. The total number of labeled corticospinal tract fibers was quantified using transverse sections of medulla by sampling five regions of the pyramid at 60× magnification and then extrapolating based on total cross-sectional area. Fiber index was calculated as axon counts in the spinal cord divided by the total number in the pyramids.

1. Kim DS, Matsuda T, Cepko CL (2008) A core paired-type and POU homeodomain-containing transcription factor program drives retinal bipolar cell gene expression. *J Neurosci* 28:7748–7764.
2. Tang W, et al. (2009) Faithful expression of multiple proteins via 2A-peptide self-processing: A versatile and reliable method for manipulating brain circuits. *J Neurosci* 29:8621–8629.
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5. Blackmore MG, et al. (2010) High content screening of cortical neurons identifies novel regulators of axon growth. *Mol Cell Neurosci* 44:43–54.
6. Moore DL, et al. (2009) KLF family members regulate intrinsic axon regeneration ability. *Science* 326:298–301.

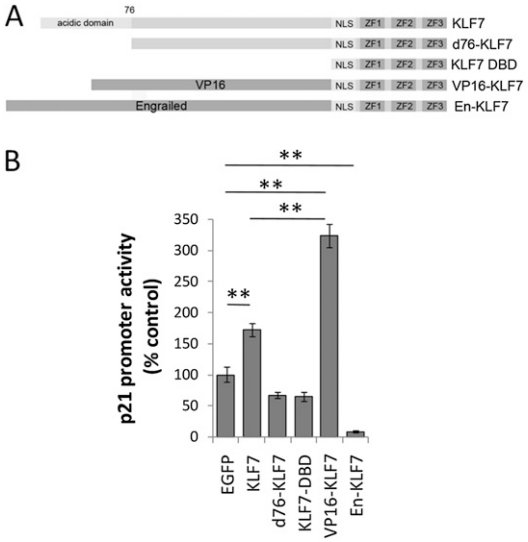

Fig. S1. Luciferase assays with the p21 promoter confirm transcriptional activity of KLF7 mutants. (*A*) Mutant forms of KLF7 were generated by domain deletion or by fusing VP16 activation or engrailed (En) repression domains to the KLF7 DNA-binding domain (DBD). (*B*) Luciferase assays show that KLF7 and VP16-KLF7 significantly induce, and En-KLF7 significantly represses, the p21 promoter. p21 promoter induction does not occur with KLF7 mutants that lack an acidic activation domain, or consisting only of the KLF7 DBD and adjacent nuclear localization signal. Luciferase signal in three replicate wells from three independent experiments and normalized to EGFP. $^{**}P < 0.01$, ANOVA with Tukey's post hoc comparison.

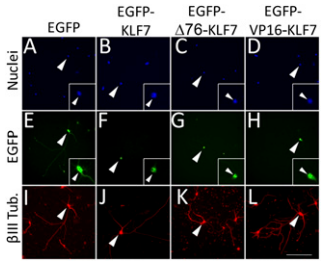

Fig. S2. Wild-type and mutant KLF7 localize to the nucleus. P3 cortical neurons were transfected with EGFP control or EGFP-tagged KLF7 mutants, cultured for 3 d on laminin substrates, then fixed and stained for Hoechst (nuclei; *A*–*D*) and neuronal-specific βIII tubulin (*I*–*L*). As expected, in EGFP-transfected neurons (*A*, *E*, and *I*) the EGFP is distributed throughout the cell body and processes. Wild-type EGFP-KLF7 (*B*, *F*, and *J*) appears concentrated in the nucleus (arrows, *Insets*), as does the d76 truncation mutant (*C*, *G*, and *K*) and VP16-KLF7 (*D*, *H*, and *L*). (Scale bar: 100 μm.)

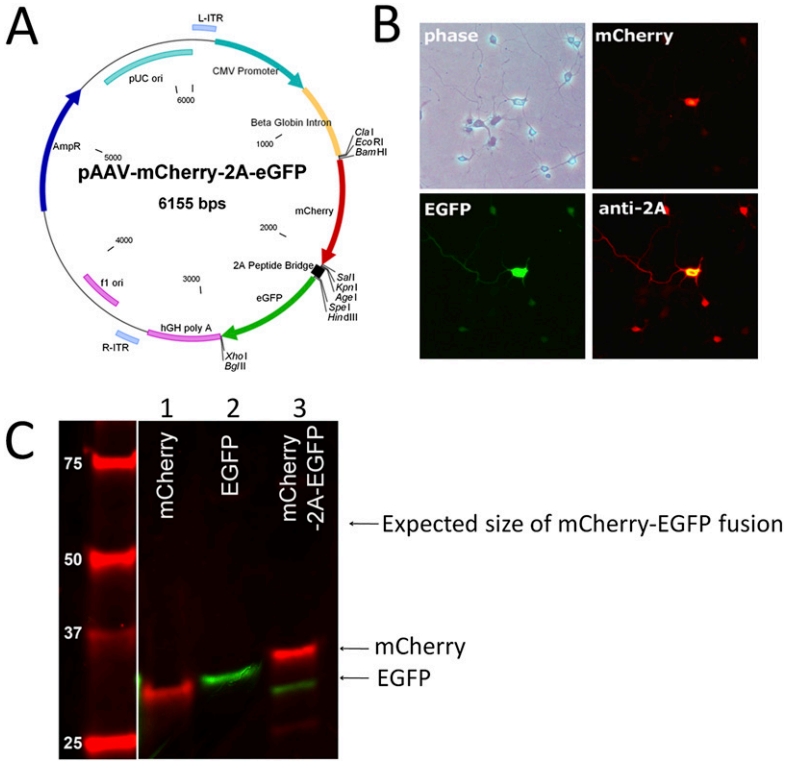

Fig. S3. A 2A peptide bridge plasmid drives expression of two separate proteins in neurons. (*A*) Schematic representation of a 2A bridge plasmid expressing mCherry and EGFP with an intervening 2A peptide sequence. (*B*) E18 hippocampal neurons were transfected with mCherry-2A-EGFP plasmid and cultured for 2 d. Fluorescent images demonstrate expression of both fluorescent proteins, as well as detection of a 2A peptide epitope tag on the first protein, which is readily detected with an antibody to the tag. (*C*) Western blotting confirms that E18 hippocampal neurons transfected with mCherry-2A-EGFP plasmid (lane 3) express two separate proteins. Blotting with antibodies to EGFP or to mCherry detects proteins of the expected sizes for mCherry (lane 1) and EGFP (lane 2), but does not detect an mCherry-EGFP fusion protein after transfection with mCherry-2A-EGFP (lane 3; mCherry = 30 kDa, EGFP = 27 kDa).

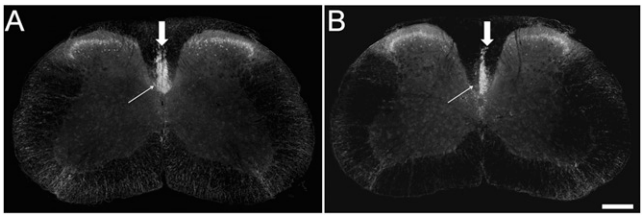

Fig. S4. PKCγ immunohistochemistry confirms complete pyramidotomy lesions. In uninjured animals (*A*), PKCγ expression can be detected bilaterally in the main CST (arrows) in the spinal cord. After a unilateral pyramidotomy (*B*), the injured side loses PKCγ expression, whereas the intact CST is not affected. Any animals without the loss of PKCγ expression in the injured CST were excluded from the study. (Scale bar: 200 μm.)

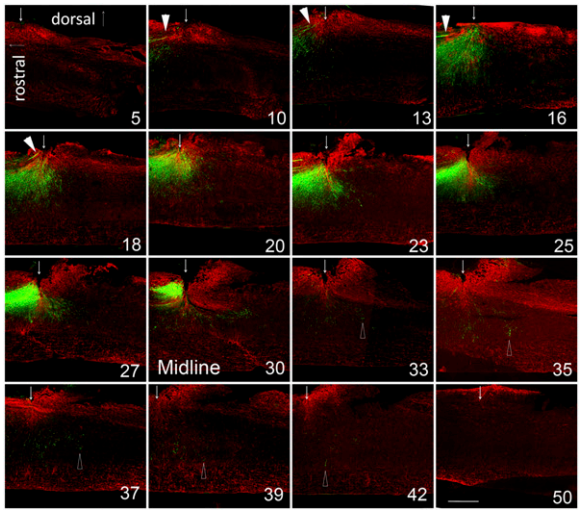

Fig. S5. Sagittal sections of injured spinal cord. Left motor cortices of adult mice were coinjected with AAV8-EGFP and AAV8-VP16-KLF7 (day 0), cervical spinal cords were injured by unilateral dorsal hemisection (day 7), and complete sagittal series of 30-μm sections of cervical spinal cords were analyzed (sacrifice at day 63). Numbers refer to lateral position (right edge = 1, left edge = 55), and arrows indicate the position of spinal injury. EGFP-labeled axons are green, GFAP is red. The main ventral CST tract is visible in sections 25–30. Dorsolateral CST axons are visible in sections 10–18 (arrowheads) and are confirmed to be severed by the injury. Labeled axons are not detected in ventral spinal cord. Collateral sprouts are present in the left side of the spinal cord (open arrowheads), but spared axons are not visible. Images are composites of multiple fields of view. (Scale bar: 500 μm.)

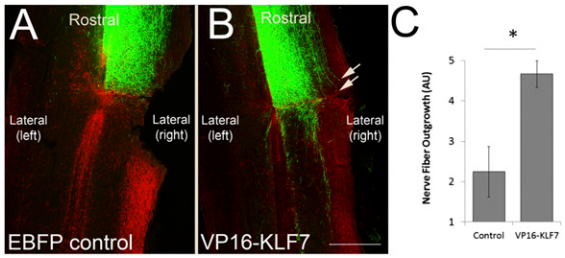

Fig. S6. Horizontal sections of injured spinal cord confirm promotion of CST regeneration by VP16-KLF7 and severing of dorsolateral CST axons. Left motor cortices of adult mice were coinjected with AAV8-EGFP and AAV8-VP16-KLF7-2A-mCherry or AAV8-EGFP and AAV8-EBFP-2A-mCherry (day 0), cervical spinal cords were injured by unilateral dorsal hemisection (day 7), and horizontal sections prepared (sacrifice at day 63). EGFP-labeled axons are green, GFAP is pseudocolored red. (*A*) In control animals, EGFP+ axons are mostly confined proximal to the lesion. (*B*) In animals treated with VP16-KLF7, EGFP+ axons extend caudal to the lesion. Dorsolateral axons are visible and clearly interrupted by the injury (arrows). (*C*) Sections were scored by a blinded observer for the degree of regeneration on a 1–5 scale. Sections from animals treated with VP16-KLF7 were scored as having higher regeneration (*$P < 0.02$, paired two-tailed *t* test). $n = 4$ EBFP, 3 VP16-KLF7. (Scale bar: 500 μm.)