Cell

EXTENDED EXPERIMENTAL PROCEDURES

Antibodies

Primary antibodies for Western blotting and immunofluorescence analysis were: anti-RT97 (kind gift of J. Woods), anti-neurofilament (Abcam), anti-S100β (DAKO), anti-$p75^{NGFR}$ (Chemicon), anti-fibronectin (Sigma), anti-prolyl-4-hydroxylase β (Acris), N-cadherin (BD transduction labs), anti-p-Eph (kind gift of K. Nobes), anti-EphB2 (R&D systems), anti-Sox2 (kind gift of M. Wegner).

Co-culture Modifications

Effects of fibroblast soluble, matrix and matrix+membrane fractions on Schwann cell clustering were assessed as follows. Schwann cells were seeded on the bottom chamber of a culture dish containing a transwell insert (Falcon) onto which confluent fibroblasts were seeded, or onto matrices prepared by culturing fibroblasts to confluency and then removing them from the dish with non-enzymatic cell dissociation buffers (Invitrogen) or lysing them with H2O for 1h at 37°C. For membrane fractionation experiments, purified membrane fractions were prepared as reported (Kapfhammer et al., 2007) and were added to Schwann cell cultures for 24-48h.

Quantification of Axonal Outgrowth In Vivo

To quantify axonal outgrowth after nerve cut in the absence or presence of recombinant EphB2, the whole sciatic nerve was sectioned, stained and examined. Representative sections, which were parallel to the length of the nerve as judged by the positioning of the uncut region, were further quantified for axonal length using the ImageJ macro NeuronJ (Meijering et al., 2004). Additionally, the number of axons growing at an angle smaller or greater than 45° to the nerve axis were counted for each sample. A total of 9 control-treated and 10-EphB2-treated animals were analyzed. Of the EphB2 group, 7 showed a clear phenotype, one could not be analyzed because of incorrect sectioning angle and 2 had no phenotype, most likely due to malfunctioning of the pump and/or delivery system. Of the control group, one nerve could not be scored for the same reasons as above. The remaining 8 animals showed no disruption in axonal outgrowth and had a phenotype indistinguishable from untreated nerves. For the experiments in mouse, 5 animals of each group were analyzed as above. All knock-out animals showed a strong phenotype.

Quantification of N-Cadherin Immunofluorescence

Junctional N-cadherin levels were quantified using Metamorph 6.0 software. Pixel intensity was measured at cell-cell contacts and normalized to contact area. A minimum of 80 junctions across multiple randomly selected images was quantified. The average of the total pixel intensity of 4-6 images was calculated per condition.

siRNA and qPCR

RNA silencing and quantitative RT-PCR were performed as reported previously (Parrinello et al., 2008). See also Table S2 and Table S3.

siRNA Rescue Experiments

All siRNA oligos used for rescue experiments were specific for rat sequences (see table, oligo 1). For N-cadherin and Sox2 rescue experiments, human and mouse genes respectively were delivered using adenoviral vectors 36 hr after siRNA transfection. 18 hr post-infection Schwann cells were co-cultured with fibroblasts for 6-8 hr. For EphB2 rescue experiments RNA oligos and plasmids were delivered simultaneously using attractene reagent (QIAGEN) according to the manufacturers instructions. A GFP-encoding vector was also co-transfected to label transfected cells. 36 hr later cells were co-cultured with fibroblasts for 18 hr and only GFP positive cells were scored. Efficacy of knockdown and overexpression of relevant genes was confirmed by Western blotting and qPCR (not shown).

SUPPLEMENTAL REFERENCES

Kapfhammer, J.P., Xu, H., and Raper, J.A. (2007). The detection and quantification of growth cone collapsing activities. Nat. Protoc. *2*, 2005–2011.

Meijering, E., Jacob, M., Sarria, J.C., Steiner, P., Hirling, H., and Unser, M. (2004). Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. Cytometry A *58*, 167–176.

Parrinello, S., Noon, L.A., Harrisingh, M.C., Digby, P.W., Rosenberg, L.H., Cremona, C.A., Echave, P., Flanagan, A.M., Parada, L.F., and Lloyd, A.C. (2008). NF1 loss disrupts Schwann cell-axonal interactions: a novel role for semaphorin 4F. Genes Dev. *22*, 3335–3348.

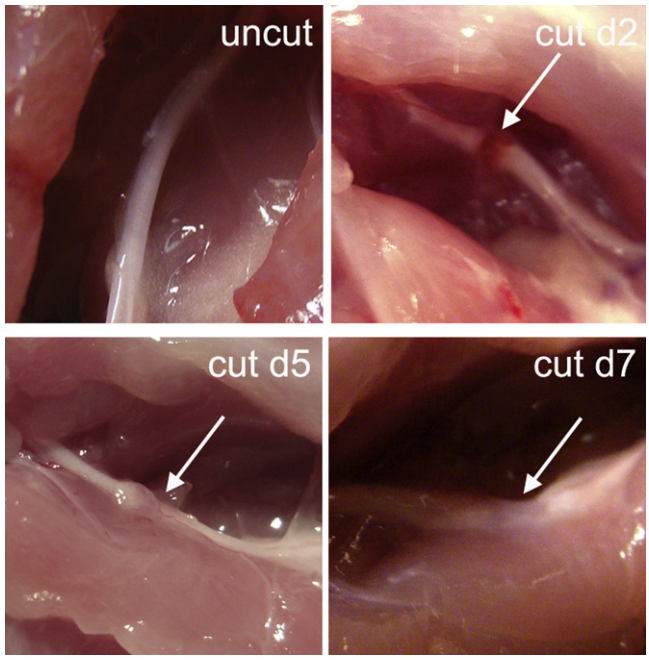

Figure S1. Rat Sciatic Nerves Reconnect Spontaneously after Full Transection, Related to Figure 1
Photographs of rat sciatic nerves prior (uncut) or following transection at the indicated days post-surgery.

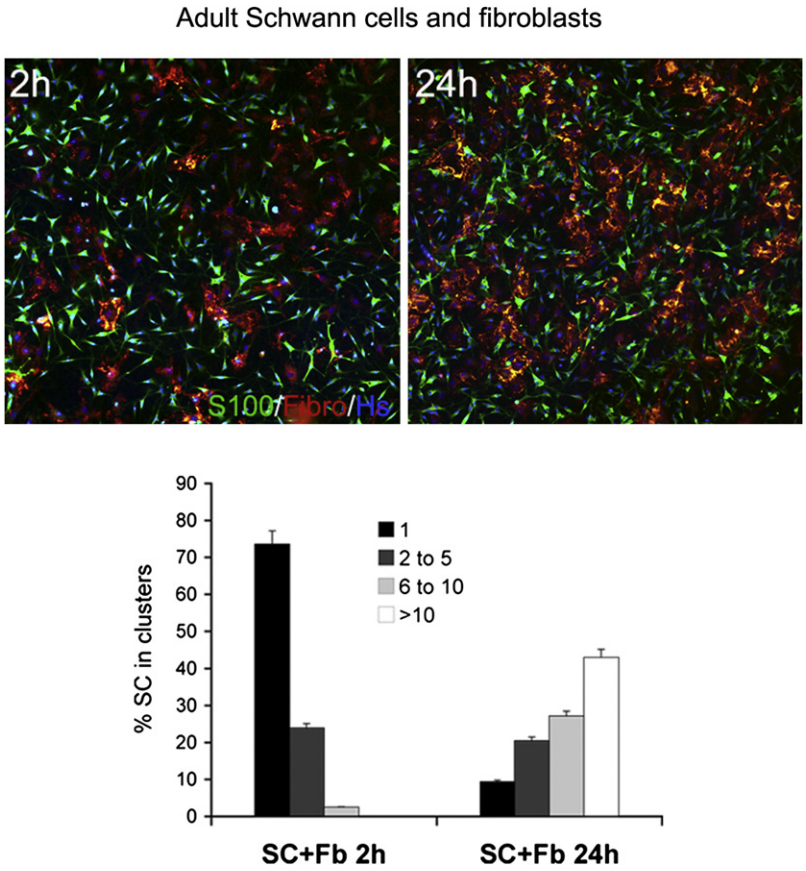

Figure S2. Adult Schwann Cell and Nerve Fibroblasts Undergo Cell Sorting in Culture, Related to Figure 2

Representative images and quantifications of primary Schwann cell/fibroblast co-cultures at 2 and 24 hr after seeding. Cells were isolated from 6 week old rat sciatic nerves and stained for S100β and fibronectin. Data are represented as mean ± SD.

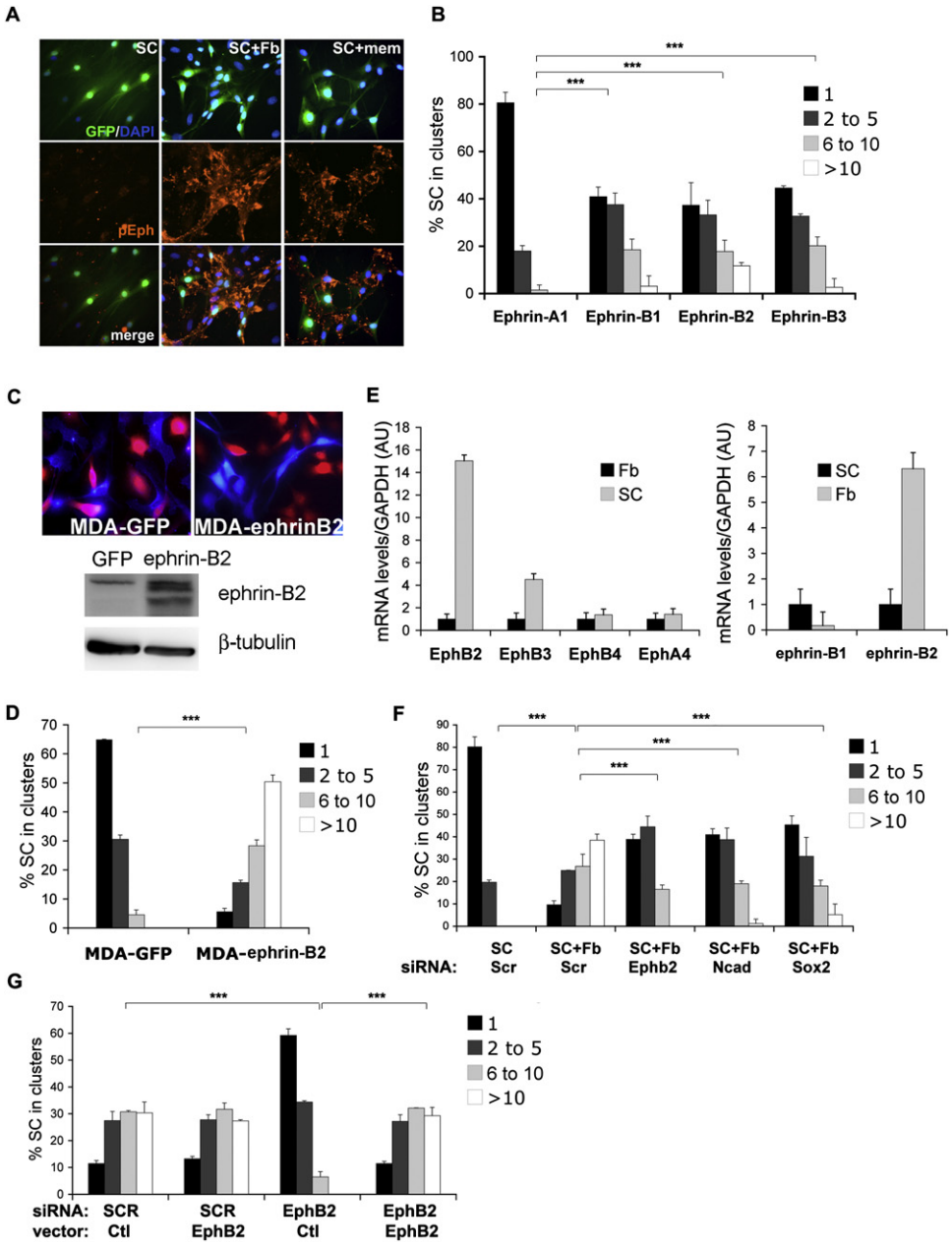

Figure S3. Ephrin-B/EphB Signaling Directs Schwann Cell Sorting in the Presence of Fibroblasts, Related to Figure 3

(A) Immunofluorescence analysis of phosphorylated Eph receptor (red) of GFP-labeled Schwann cells cultured on their own (SC), with fibroblasts (SC+Fb) or on fibroblast membranes (SC+mem) for 24 hr. Nuclei are counterstained with Hoechst.

(B) Quantification of clustering in Schwann cell monocultures treated with the indicated recombinant preclustered ephrin ligands for 24h. Shown is one experiment of three that gave similar results. Data for ephrin A1 stimulation are representative of all A ligands tested which had no clustering effect.

(C) Representative images of Schwann cells (blue) co-cultured with MDA-MB-435 breast cancer cells (red) engineered to express GFP (MDA- GFP) or ephrin-B2 (MDA- ephrinB2) and Western blots showing ectopic ephrin-B2 expression and β-tubulin as loading control.

(D) Quantification of Schwann cell clustering for experiment described in C.

(E) Quantitative RT-PCR analysis of relative expression levels of EphB receptors (left panel) and ephrin-B ligands (right panel) in Schwann cells and fibroblast monocultures as indicated. Bars represent average expression levels of three independent experiments. Error bars represent SEM.

(F) Quantification of clustering of scr siRNA-treated Schwann cells in the absence (SC Scr) or presence of fibroblasts (SC+Fb Scr) and EphB2, N-cadherin and Sox2 knock-down Schwann cells cultured in the presence of fibroblasts (SC+Fb N-cad) using a second independent siRNA oligo.

(G) Quantification of clustering of scr- or EphB2-siRNA treated rat Schwann cells co-cultured with fibroblasts and transfected with a control plasmid or a plasmid encoding mouse EphB2.

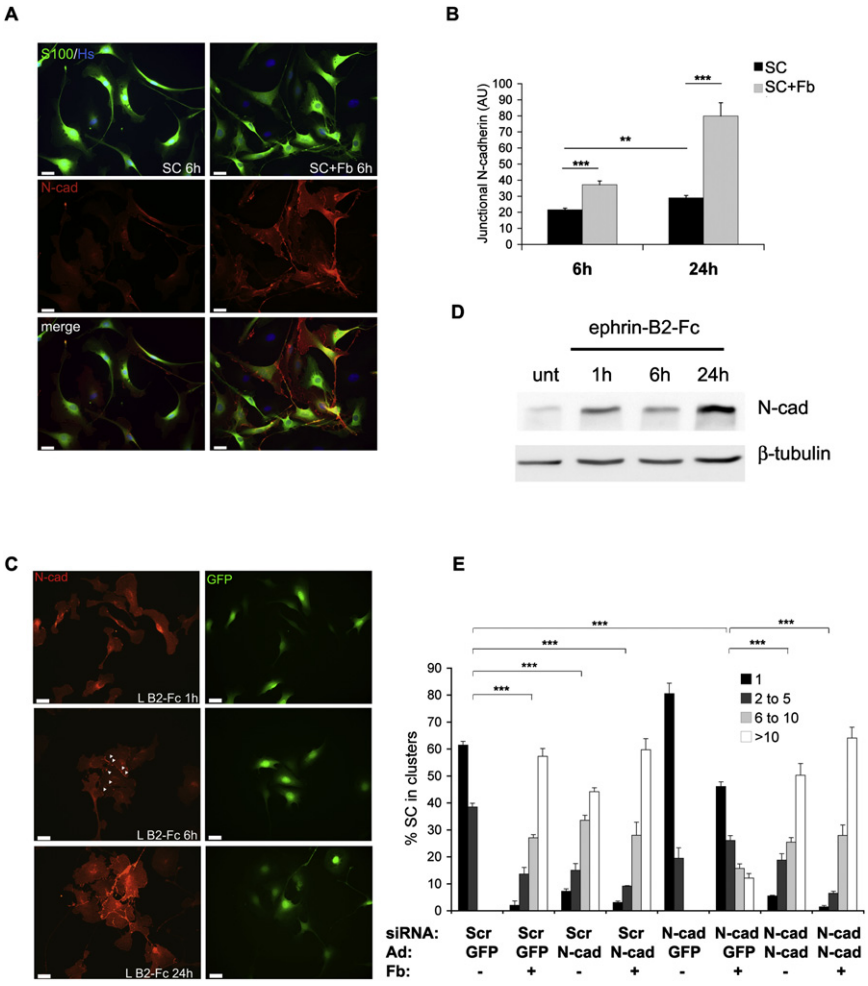

Figure S4. Sorting Results from the Rapid Relocalization of N-Cadherin to Cell Junctions upon Ephrin-B Stimulation, Related to Figure 5

(A) Immunofluorescence analysis for N-cadherin and S100β of Schwann cells cultured on their own or with fibroblasts for 6h. Nuclei are counterstained with Hoechst. Scale bar = 25 μm.

(B) Quantification of N-cadherin fluorescence at cell-cell contacts in Schwann cell cultured alone (SC) or in the presence of fibroblasts (SC+Fb) for 6 or 24h. Bars represent average pixel intensity normalized to junction area. Error bars depict SEM.

(C) Immunofluorescence analysis for N-cadherin of GFP-labeled Schwann cells treated with pre-clustered ephrin-B2-Fc for the indicated time intervals. Scale bar = 25 μm.

(D) Western blot analysis of N-cadherin expression in total protein lysates from Schwann cells treated as in C. β-tubulin was used as loading control.

(E) Quantification of clustering of scr- or N-cad-siRNA treated Schwann cells infected with GFP or siRNA-insensitive N-cadherin adenoviral vectors and cultured in the absence (-) or presence (+) of fibroblasts.

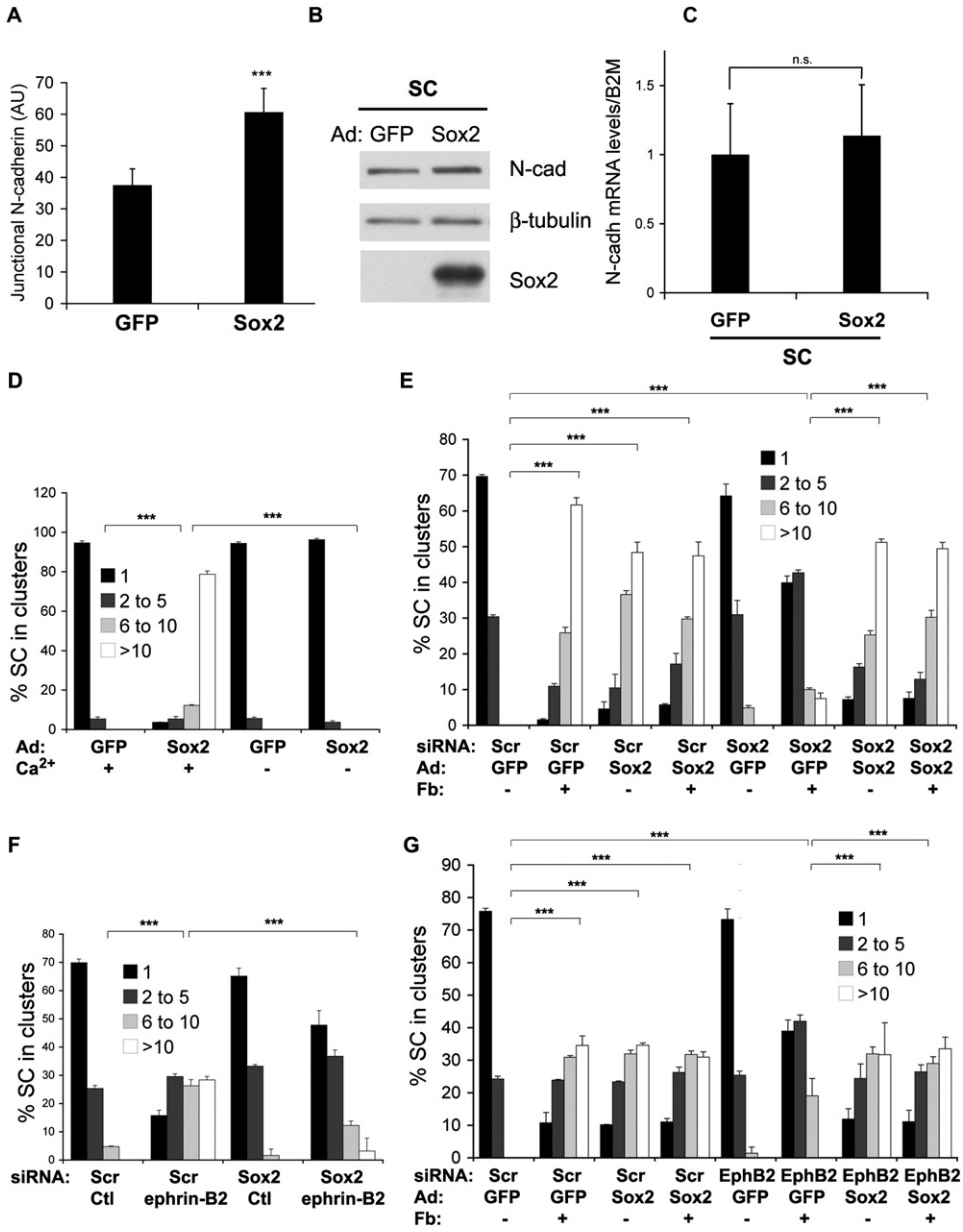

Figure S5. Sox2 Does Not Induce the Transcription of N-Cadherin, Related to Figure 6

(A) Quantification of N-cadherin staining at junctions in GFP and Sox2 overexpressing cells. Average pixel intensity at cell-cell contacts is shown. Error bars represent SEM.

(B) Western analysis of N-cadherin, β-tubulin and Sox2 levels in lysates of Schwann cell cultures overexpressing GFP or Sox2.

(C) Quantitative RT-PCR analysis of N-cadherin mRNA levels in Schwann cells engineered to overexpress GFP or Sox2. Shown is the average expression of three independent experiments normalized to GAPDH. Error bars depict SEM across experiments.

(D) Quantification of clustering of Schwann cell cultures infected with GFP or Sox2 adenoviral vectors and cultured in normal (+) or low Ca^{2+} (-) media.

(E) Quantification of clustering of scr- or Sox2-siRNA treated Schwann cells infected with adenoviral vectors encoding GFP or siRNA-insensitive mouse Sox2 and cultured in the absence (-) or presence (+) of fibroblasts.

(F) Quantification of clustering of Schwann cells transfected with scr- or Sox2-siRNA and treated with control proteins or ephrin-B2-Fc.

(G) Quantification of clustering of scr- or EphB2-siRNA treated Schwann cells infected with GFP or Sox2 adenoviral vectors and cultured in the absence (-) or presence (+) of fibroblasts.

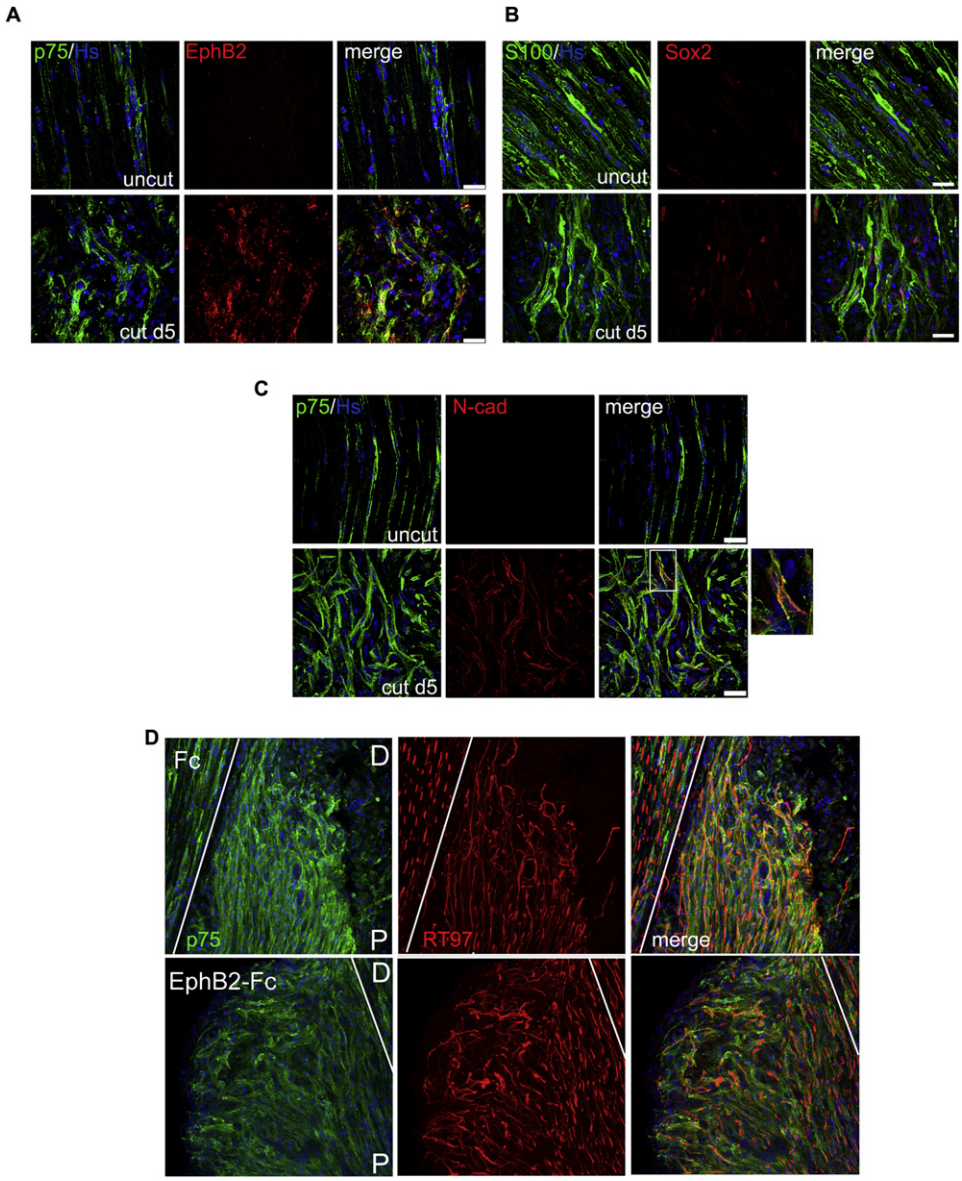

Figure S6. Schwann Cell Cords in the Nerve Bridge Express EphB2, N-Cadherin and Sox2, Related to Figure 7

(A–C) Immunofluorescence staining of sections of contralateral (uncut) and nerve bridges 5 days post transection (cut d5) for the indicated proteins. S100β and p75NGFR were used to co-stain Schwann cells. Nuclei were counterstained with Hoechst. Scale bar = 25 μm.

(D) Immunofluorescence analysis for Schwann cell and axonal markers p75NGFR and RT97. Shown are representative sections of proximal stumps of half-cut nerves exposed to Fc controls or EphB2-Fc inhibitor proteins for 4 days. Note the almost complete co-localization of regrowing axons and migrating Schwann cells (merge).