Supplementary information; Roberts et al.

Sox2 expression in Schwann cells inhibits myelination in vivo and induces influx of macrophages to the nerve.

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Supplementary Figures

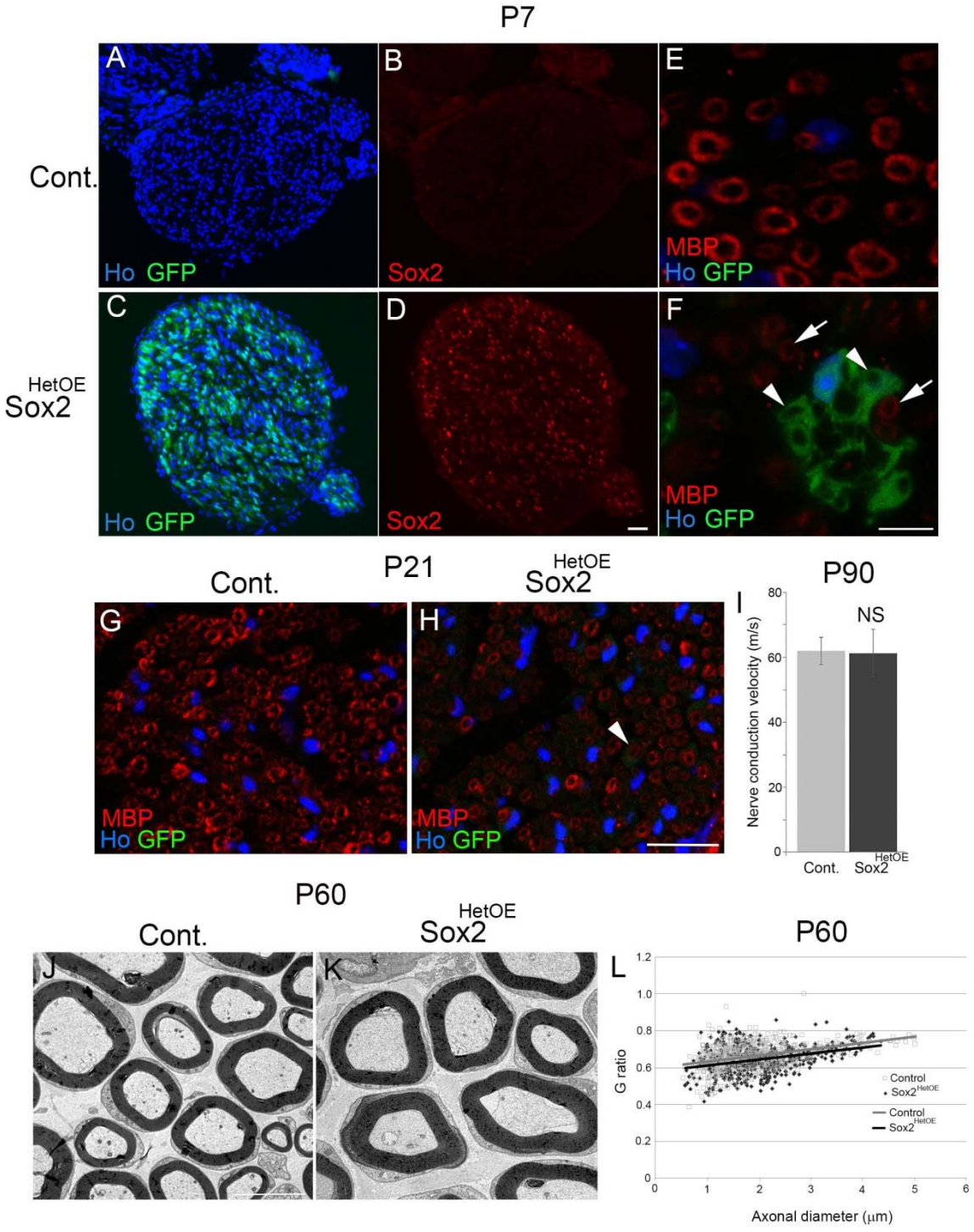

Figure S1: Analysis of Sox2 overexpressing nerves at P7, P21 and P60, showing post-natal decrease in transgene expression and normalisation of myelination at P60. A-D. Immunolabelling of control (A, B) and Sox2^{HetOE} (C, D) sciatic nerves with Sox2

antibody showing both GFP and Sox2 expression in the Sox2^{HetOE} nerve sections. Scale bar 20μm. E, F. Immunolabelling of P7 control (E) and Sox2^{HetOE} (F) nerves showing myelin basic protein (MBP) expression. GFP expressing Schwann cells (arrowheads) show no MBP expression, whereas GFP negative cells (arrows) in the nerve are MBP positive. Scale bar 5μm. G, H. Immunolabelling of P21 control (G) and Sox2^{HetOE} (H) nerves showing MBP expression; a single weakly GFP-positive cell is indicated (arrowhead). Scale bar 25μm. I. Measurement of nerve conduction velocity in P90 control and Sox2^{HetOE} nerves. Two sided two sample Student's t test; data from n=3 mice of each genotype. J, K. Transmission electron microscopy (TEM) pictures of P60 control (J) and Sox2^{HetOE} (K) nerves. Scale bar 5μm. L. Scatter plot of G ratio versus axonal diameter for P60 control and Sox2^{HetOE} nerves.

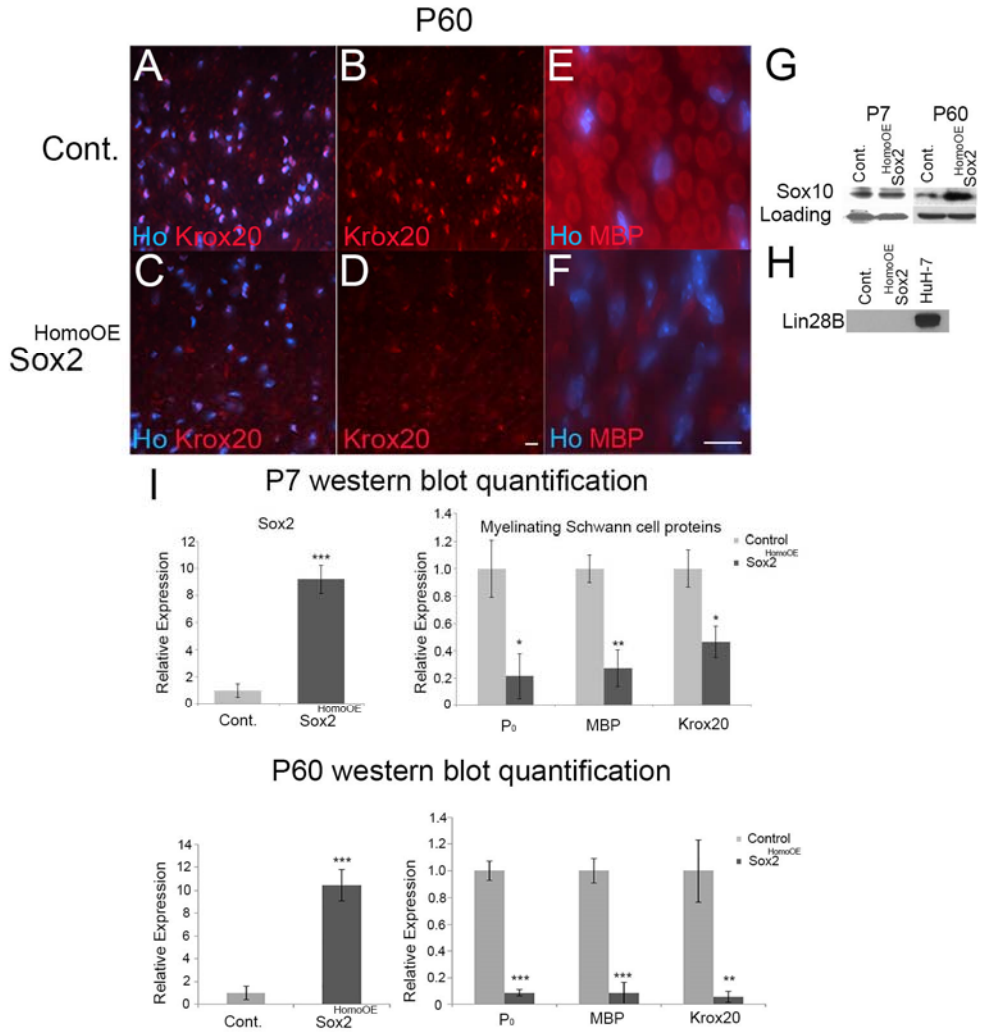

Figure S2: Analysis of P60 Sox2^{HomoOE} nerves. A-D. Immunolabelling of P60 control (A, B) and Sox2^{HomoOE} (C, D) nerves with Krox20 antibody revealed fewer Krox20 positive nuclei in Sox2^{HomoOE} sections; sections are counterstained with Hoechst dye (Ho) to reveal nuclei. Scale bar 20μm. E, F. Immunolabelling with myelin basic protein (MBP) antibody on P60 control (E) and Sox2^{HomoOE} (F) nerves. Scale bar 20μm. G. Western blots of control and Sox2^{HomoOE} P7 and P60 nerves with Sox10 antibody. H. Western blot of P60 control and Sox2^{HomoOE} nerves with Lin28B antibody. Cell extract from the hepatoma cell line HuH-7 was used as a positive

control for Lin28B protein expression. I. Quantification of western blots from Figure 3K for control (Cont.) and $Sox2^{HomoOE}$ nerves. Two sided two sample Student's t test; data from 3 independent blots.

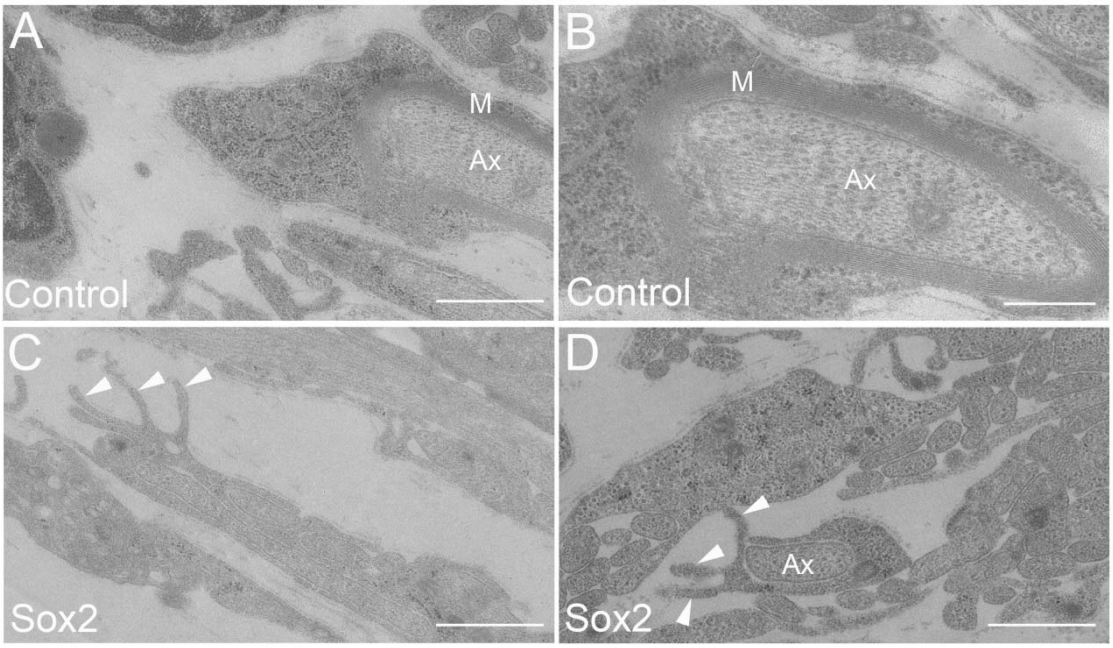

Figure S3: TEM images of Schwann cell/DRG neuron co-cultures. A, B. Control GFP infected Schwann cells show a normal contact with axons and myelin formation at 21 days post-ascorbic acid treatment (Panel B enlargement of Panel A). By contrast, Sox2 infected Schwann cells (C, D) do not complete axon ensheathment and still protrude cytoplasmic processes in the area at this timepoint (arrowheads in panels C and D). M: myelin; Ax: Axon. Scale bars A, C and D: 1μm; B: 500nm.

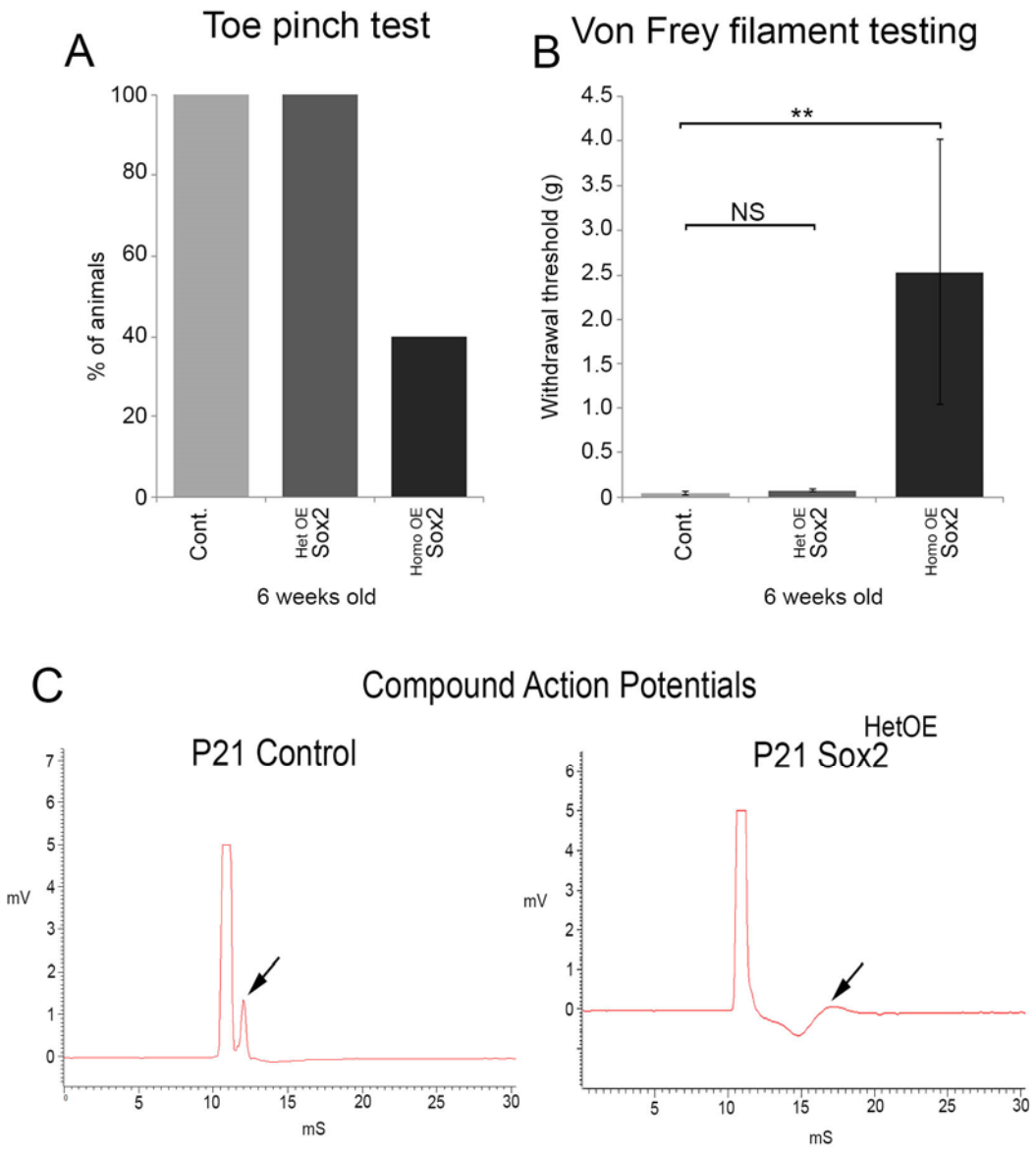

Figure S4: Sensory function is reduced in Sox2^{HomoOE} animals. A. Graph showing percentage of control (n=7), Sox2^{HetOE} (n=3) and Sox2^{HomoOE} (n=5) animals that responded to toe pinch testing at 6 weeks of age. B. Graph showing withdrawal threshold (g) for control (n=11), Sox2^{HetOE} (n=5) and Sox2^{HomoOE} (n=5) at 6 weeks of age to Von Frey filament sensory testing. Two sided two sample Student's t test. C. Example traces showing electrophysiological measurement of compound action potentials in P21 control and Sox2^{HetOE} sciatic nerves. Arrow indicates compound action potential peak.

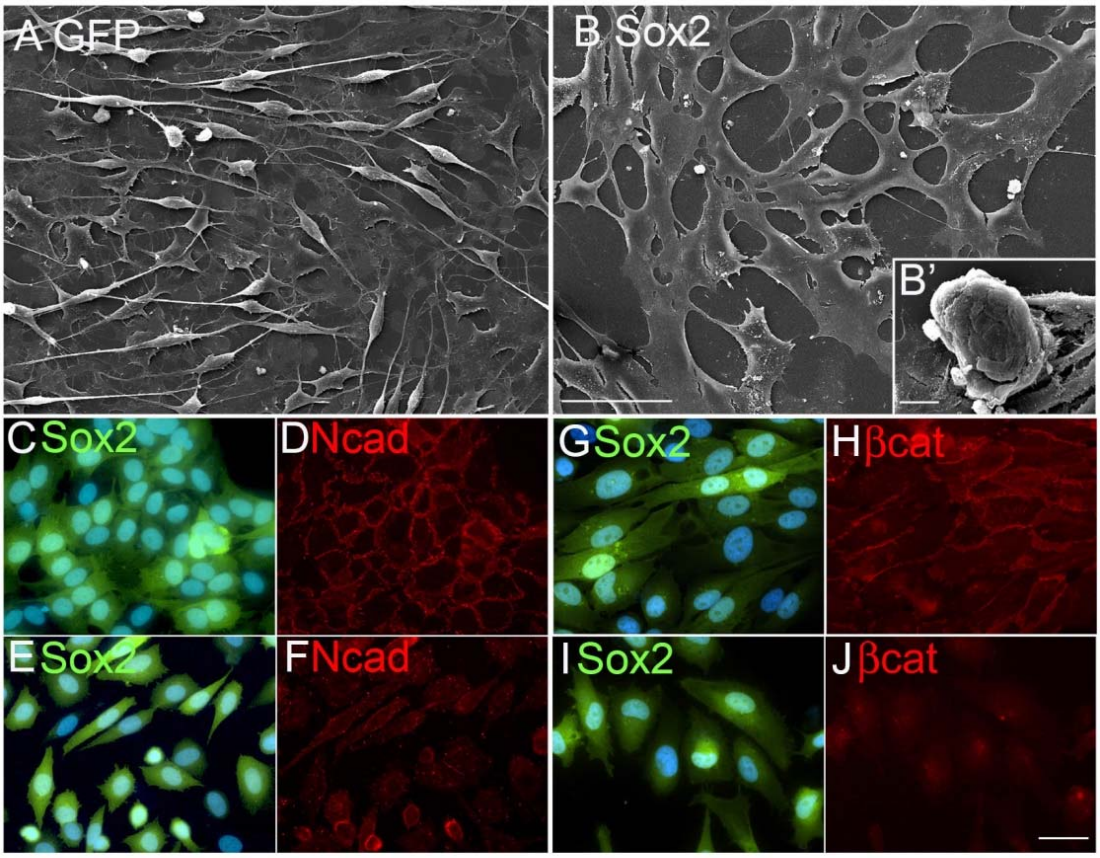

Figure S5: Sox2 expression causes cell clustering and relocalisation of N-cadherin and β-catenin *in vitro* to cell-cell contacts in Schwann cells. A, B. Scanning electron microscopy analysis of control GFP (A) and GFP/Sox2 (B) infected rat Schwann cells 48h after adenovirus addition. Sox2 expression causes such strong cell-cell adhesion that Schwann cells can form three dimensional clumps that begin to detach from the glass coverslip (B'). Scale bar for A, B 25μm; B' 10μm. C-J. Sox2 expression causes a calcium-dependent relocalisation of N-cadherin and β-catenin

to cell-cell contacts in Schwann cells. Immunocytochemistry of Schwann cells infected with GFP/Sox2 expressing adenovirus with antibodies against N-cadherin (C-F) and β-catenin (G-J). Cells in C, D, G and H were cultured under normal calcium concentration whereas cells in panels E, F, I and J were cultured in low calcium conditions. Note loss of both cell-cell clustering and membrane localisation of N-cadherin and β-catenin under low calcium conditions. Scale bar C-J 20μm. K. Western blot analysis of cell lysates from rat Schwann cells infected with GFP control (GFP) or GFP/Sox2 (Sox2) expressing adenoviruses at 24, 48 and 72 hours after infection. Cont. control uninfected rat Schwann cells. Representative blot shown from 3 independent experiments.

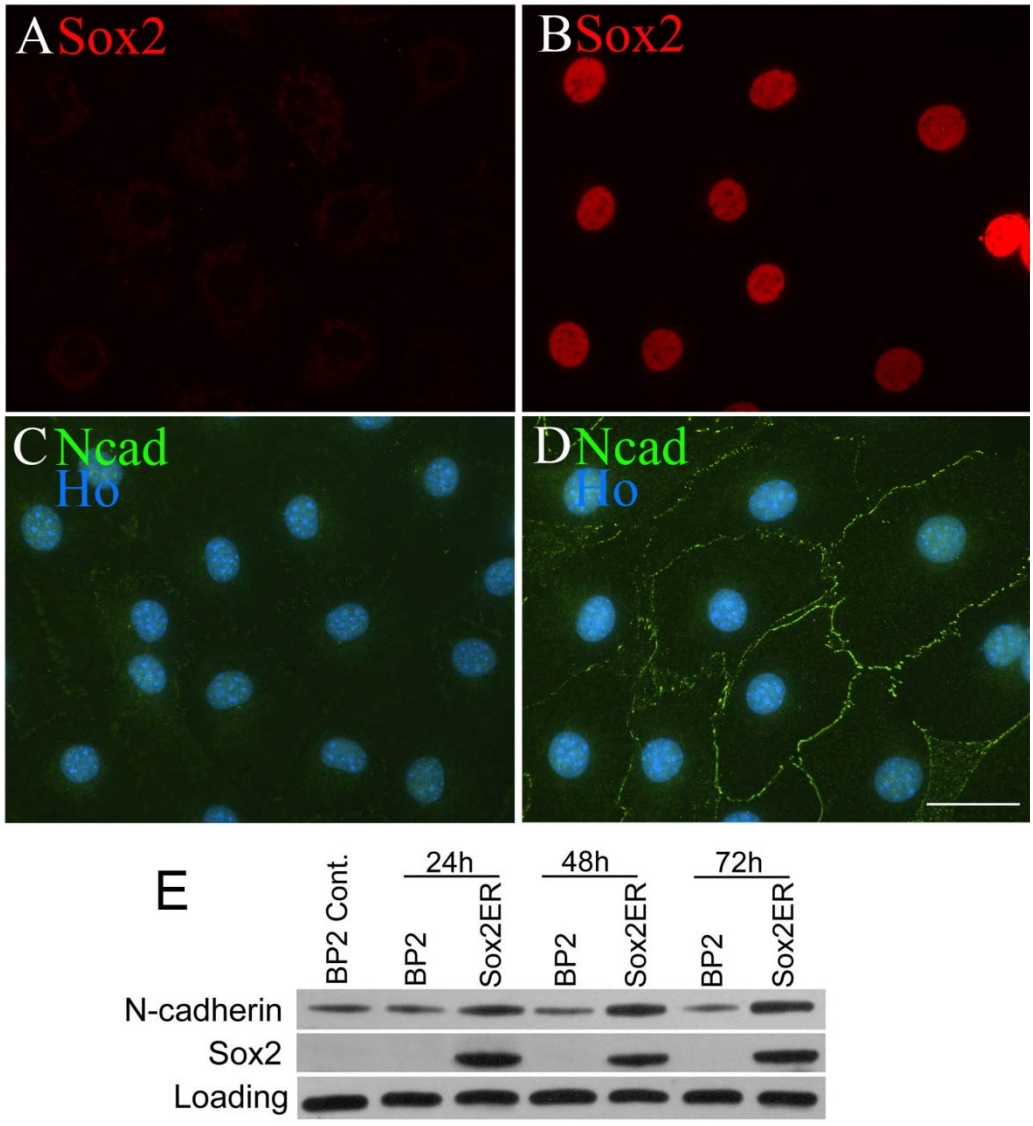

Figure S6: Sox2 expression causes N-cadherin relocalisation in 3T3 fibroblast cells. A-D Immunolabelling of 3T3 cells infected with empty control retroviral vector (A, C) and retrovirus expressing Sox2ER^{TM} protein (B, D), labelled with antibodies against Sox2 (A, B) and N-cadherin (Ncad, C, D). Cells were treated for 48h with 10^{-6}M 4-hydroxytamoxifen (4-OHT) before fixing. Nuclei are labelled with Hoechst dye (Ho) in panels C and D. Scale bar 20μm. E. Western blot showing levels of N-cadherin protein in control empty vector (BP2) and Sox2ER^{TM} expressing 3T3 cells. Shown

are untreated empty vector cells (BP2 Cont.) and Control (BP2) and Sox2ER^{TM} expressing cells at 24, 48 and 72h after addition of 4-OHT (10^{-6}M). Representative blot shown from 3 independent experiments.

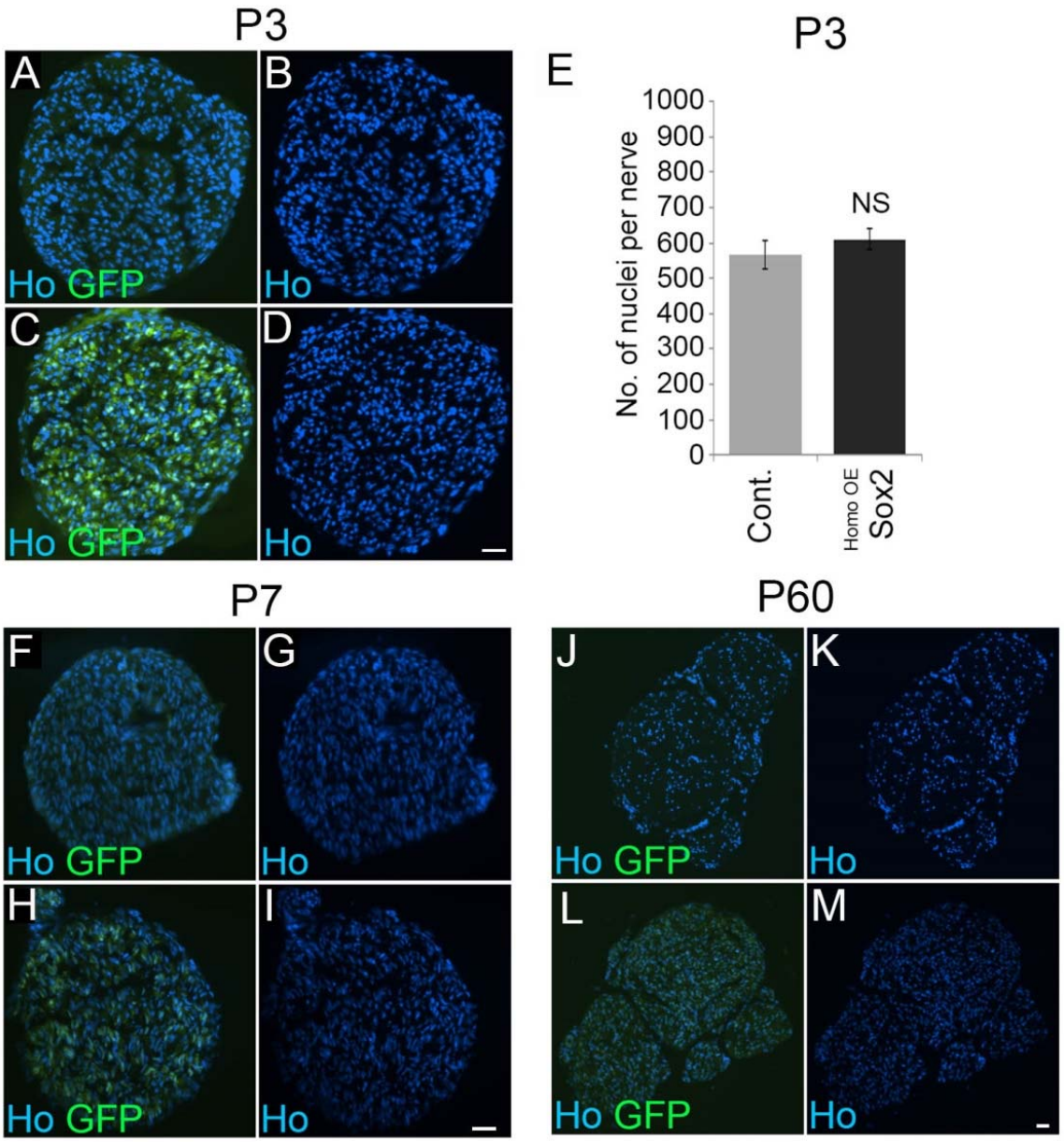

Figure S7: Measurement of cell nuclei number in control and Sox2^{HomoOE} nerves. Transverse sciatic nerve sections stained with Hoechst dye (Ho) to reveal GFP expression and numbers of nuclei from P3 control (A, B) P3 Sox2^{HomoOE} (C, D), P7 control (F, G) P7 Sox2^{HomoOE} (H, I), P60 control (J, K) and Sox2^{HomoOE} (L, M) nerves. Scale Bar 20μm. E. Counts of nuclei per nerve section from P3 control and Sox2^{HomoOE} sciatic nerves. Two sided two sample Student's t test; n=3 for each mouse genotype.

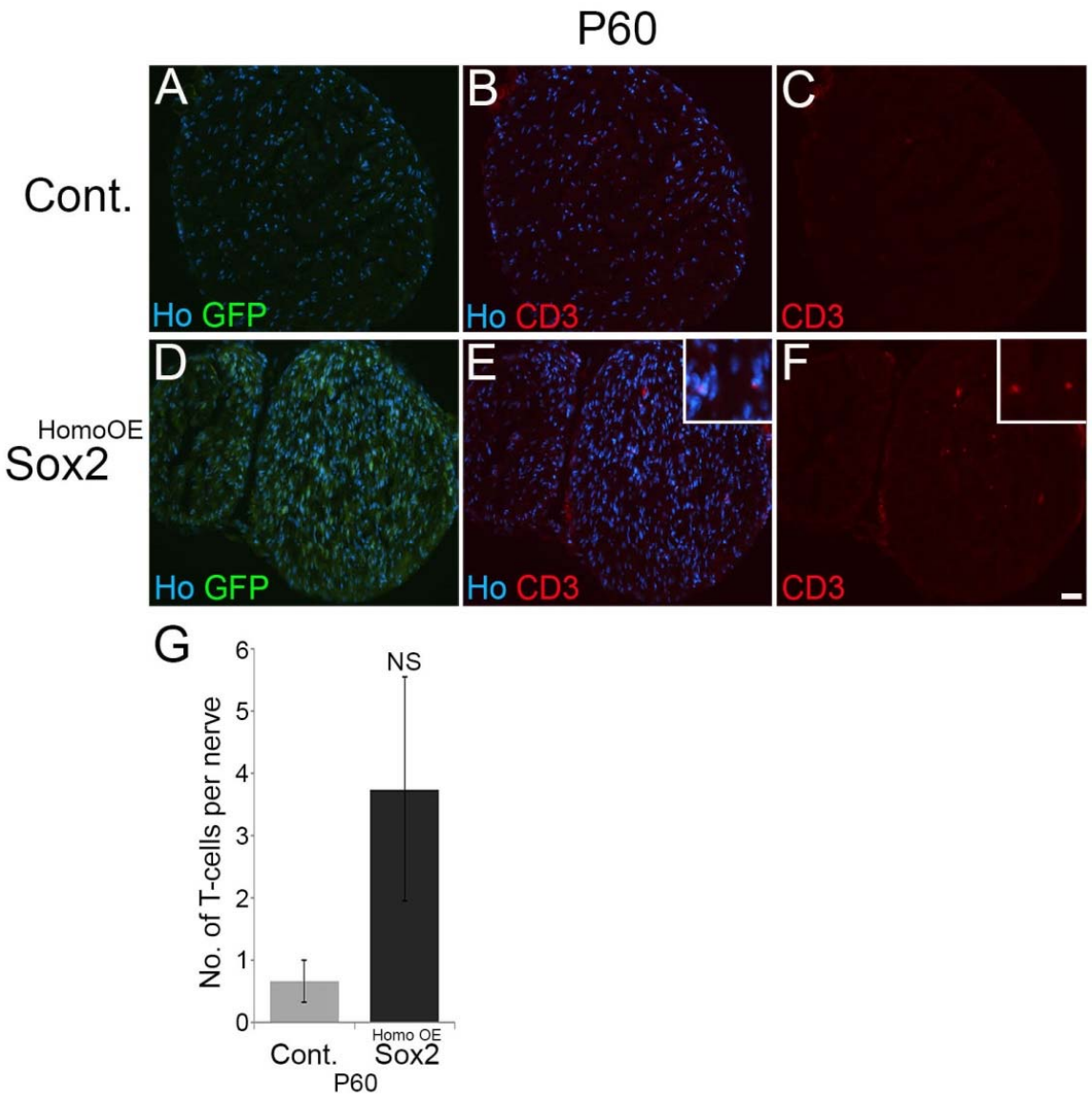

Figure S8: Raised numbers of T-lymphocytes in intact P60 Sox2^{HomoOE} nerves. Immunolabelling of P60 control (A-C) and Sox2^{HomoOE} (D-F) nerves with CD3 antibody to identify T-lymphocytes within the nerve. Scale bar 20μm. Inset shows higher magnification of CD3 positive T-cells in Sox2^{HomoOE} nerve G. Graph showing an increase, although not significant, in T-lymphocyte number in Sox2^{HomoOE} nerves. Two sided two sample Student's t test; n=3 for each mouse genotype.

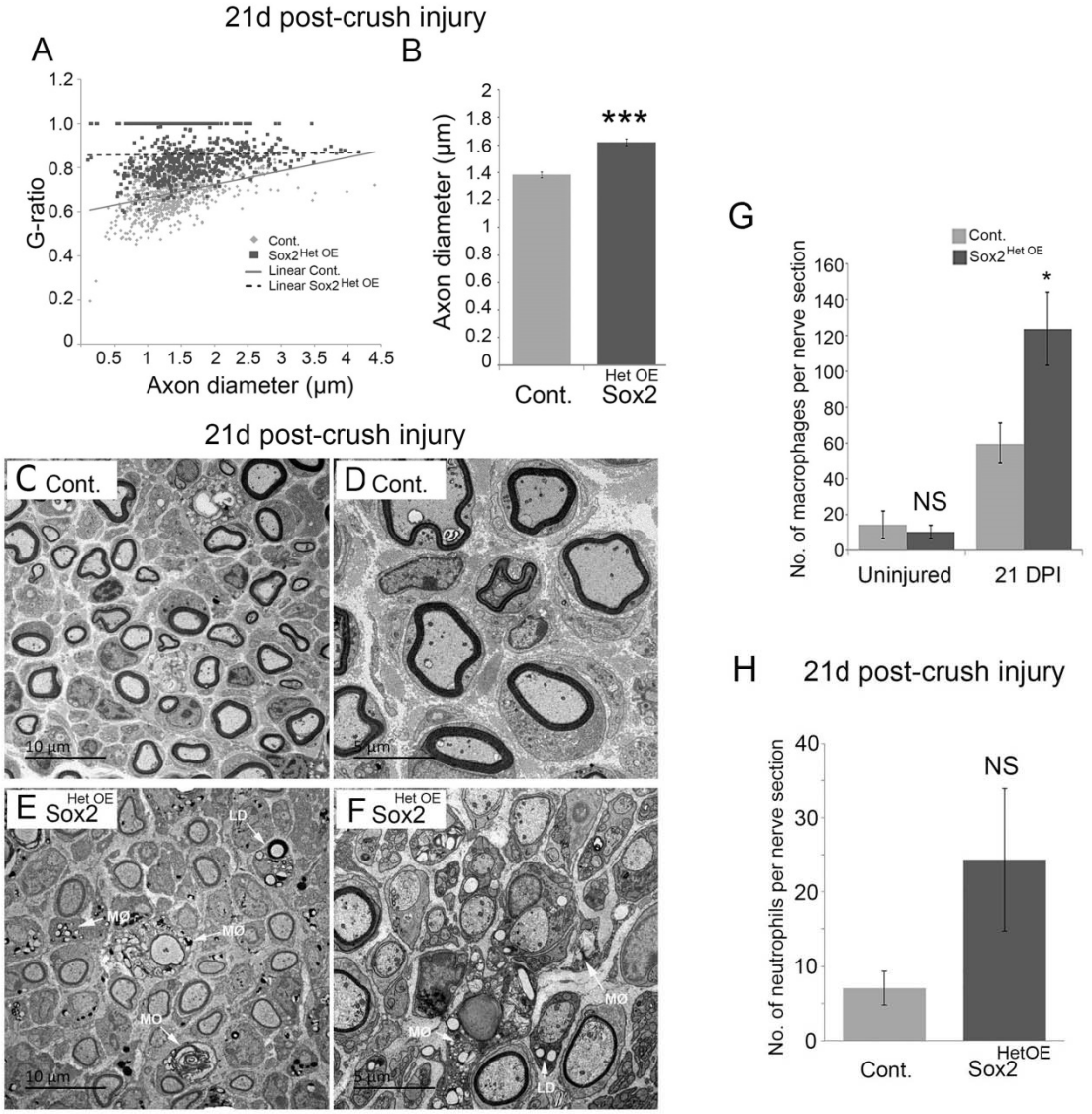

Figure S9: Morphology of control and Sox2^{HetOE} nerves following injury. A. Scatter plot of G-ratio versus axonal diameter in control and Sox2^{HetOE} distal sciatic nerves at 21 days post-crush injury. B. Graph showing measurements of axonal diameter in control and Sox2^{HetOE} nerves at 21d post-crush injury. C-F. TEM images of distal sciatic nerve from control (C, D) and Sox2^{HetOE} (E, F) nerves. Arrows indicate the increased presence of myelin ovoids (MO), macrophages (MØ) and lipid droplets (LD) at this timepoint in Sox2^{HetOE} nerves (E, F). Scale bars are as shown for each panel. G. Graph showing counts of F4/80/Iba1 double-positive macrophage numbers

in intact and injured (21 days post-crush injury) control and Sox2^{HetOE} nerves. H. Graph showing counts of NIMP-R14 positive neutrophils 21 days post-crush injury in control and Sox2^{HetOE} nerves. B, G, H. Two sided two sample Student's t test; n=3 for each mouse genotype.

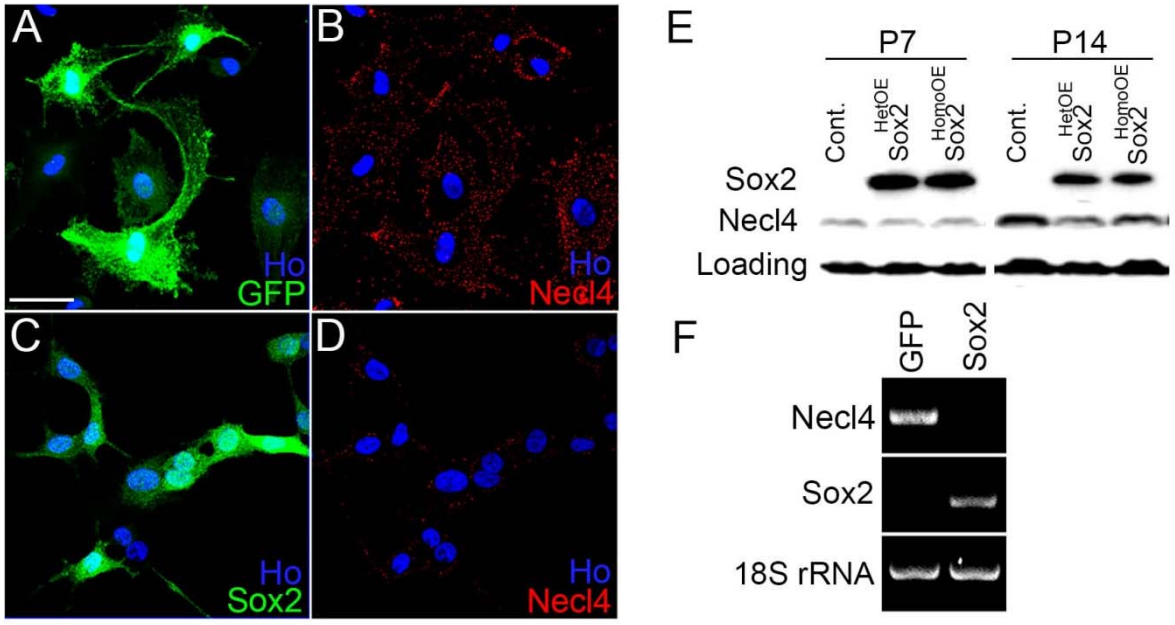

Figure S10: Regulation of Necl4 by Sox2. A-D. Necl4 immunolabelling of rat Schwann cells infected with GFP control (A, B) or Sox2 (C, D) expressing adenoviruses. Scale bar 20μm. E. Western blot of nerve samples from $Sox2^{HetOE}$ and $Sox2^{HomoOE}$ nerves at P7 and P14 timepoints. F. Semi-quantitative PCR of Necl4 and Sox2 mRNA levels in rat Schwann cells infected with GFP control and Sox2 expressing adenoviruses.

Supplementary Methods.

Sensory Testing

Sensory function testing was carried out on 6 week old mice and assessed using Von Frey Hairs and the response to toe pinching on a minimum of n=3 per genotype. For Von Frey filament hair testing, mice were placed into a plastic cage on a metal mesh floor and Von Frey filaments ranging from 0.008-10g were applied to the mid-plantar surface of the right hindpaw (when in contact with the cage lid) for 5-8 seconds. Filaments were applied in ascending order and the smallest filament to induce a foot withdrawal response was considered the threshold stimulus (Vogelaar et al., 2004). Three recordings were taken over three consecutive days.

For toe pinch testing: The distal part of the lateral three toes were pinched lightly using a pair of forceps. Hind limb withdrawal upon pinch was taken as a positive response. Three measurements were taken over three consecutive days (Arthur-Farraj et al., 2012).

Construction of Sox2ER^{TM} protein and retroviral infection.

For generation of the 4-hydroxytamoxifen-regulatable Sox2-estrogen receptor fusion protein, (Sox2ER^{TM}), the Sox2 cDNA was amplified by PCR using sequence specific primers and cloned into the pBP3:hbER^{TM} vector (Garner et al., 2002). Inclusion of a BamH1 restriction enzyme site, within the primers, at the 5' end of the cDNA and an EcoR1 site at the 3' end allowed the in-frame cloning of the Sox2 cDNA with the estrogen receptor sequence to make the Sox2ER^{TM} fusion protein. The correct sequence of the Sox2 cDNA and in-frame fusion to the estrogen receptor cDNA was verified by DNA sequencing.

Retrovirus was prepared as previously described and Swiss 3T3 fibroblasts infected and selected in puromycin for experiments (Morgenstern and Land, 1991). Swiss 3T3 cells were grown and selected in DMEM (low glucose) plus 10% donor calf serum containing 2.5 μg/ml puromycin. For immunocytochemistry and western blotting experiments, as for Schwann cells, cells were transferred into defined medium (DM) at time zero. 4-Hydroxytamoxifen (4-OHT, 10^{-6}M) or ethanol control were added at time zero and cells fixed or lysed 48 or 72 hours later.

Culture of Schwann cells and Adenoviral infection:

Schwann cells were prepared from the sciatic nerve and brachial plexus of post-natal day 3 rats as previously described (Brockes et al., 1979). Control GFP and GFP/Sox2 adenoviruses have been previously described (Le et al., 2005, Parrinello et al., 2010). For RNA work, cells were lysed 48h after infection for RNA preparation. Low calcium DM was prepared by substituting normal DMEM media for DMEM containing no calcium chloride.

RNA preparation and semi-quantitative PCR.

Total RNA was prepared from infected rat Schwann cells using a Qiagen RNeasy kit. cDNA. Total mRNA was extracted and first stand cDNA were synthesised with M-MLV reverse transcriptase (Promega) and random hexamer primers (Promega). The following primers were used for RT-PCR. Sox2 (forward 5' AGACGCTCATGAAGAAGGATAAG 3'; reverse 5' GAGCTGGTCATGGAGTTGTACTG 3'), Necl4 (forward 5' AGGGAGGGAGACACTCTGGTGCTG 3'; reverse 5' GTCATAGACCACAAGCACGTAGAG 3') and 18S rRNA (forward 5'

CCTCGAAAGAGTCCTGTA 3'; reverse 5' GGGAACGCGTGCATTTAT 3'). PCR products were electrophoresed on agarose gels for visualisation.

DRG/Schwann cell co-cultures and electron microscopy:

DRG were isolated from E13.5 FVB mice embryos and plated on 35 mm plastic dishes coated with rat collagen I (Cultrex). Five DRGs were plated in each dish in C medium (MEM, 2 mM L-glutamine (Gibco), 10% FBS, 4 mg/ml d-glucose (Sigma-Aldrich), 50 ng/ml NGF (Harlan Laboratories)) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco). In order to purify the neurons, on day 2 the C medium was replaced with NB medium (Neurobasal, 4 g/l d-glucose, 2 mM l-glutamine, 50 ng/ml NGF and B27 (Gibco)) supplemented with 10mm 5-Fluoro-2′-deoxyuridine (FdU) and 10mM Uridine (Sigma-Aldrich) and after two days the medium were replaced with NB without FdU and Uridine. The treatment was repeated three times in order to completely eliminate Schwann cells and fibroblasts from the culture. DRG neurons were then seeded with 200,000 infected or non-infected Schwann cells per DRG. Five days after the seeding 50 mg/ml of ascorbic acid (AA) (Sigma-Aldrich) were added to the fresh medium to induce myelination. The medium + AA was replaced every two days. After 14 or 21 days of AA treatment, the DRG were washed with phosphate buffer, fixed in 2% glutaraldeyde for 30 minutes at room temperature and then treated as previously described (Occhi et al., 2005) to prepare the samples for electron microscopy analysis.

Schwann cell culture and lentiviral transfection for SC/DRG co-cultures:

Primary Rat Schwann cells were dissected from P3 rats and cultured as described (Feltri et al., 1992). Cells were transduced with lentiviruses expressing Sox2-IRES-

GFP or IRES-GFP empty vector control. Transduction efficiency was assessed by counting GFP positive cells, 3 days after infection. The percentage of GFP positive cells was always greater than 70%. Schwann cells were then trypsinized, counted and seeded on purified DRG neurons.

Immunolabelling.

Antibodies against Necl4 were a gift from Prof. Patrice Maurel (Rutgers University, Newark, USA). Antibodies for the neutrophil marker NIMP-R14 were from Abcam (ab2557). Immunolabelling of GFP and GFP/Sox2 infected rat Schwann cells with antibodies against Necl4 was as described (Maurel et al., 2007).

Scanning electron microscopy:

Cultures of control GFP and GFP/Sox2 expressing Schwann cells were prepared for scanning electron microscopy as previously described (Doddrell et al., 2013).

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