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Supplemental Information

Macrophage-Derived Slit3 Controls Cell Migration and Axon Pathfinding in the Peripheral Nerve Bridge

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Supplemental Information

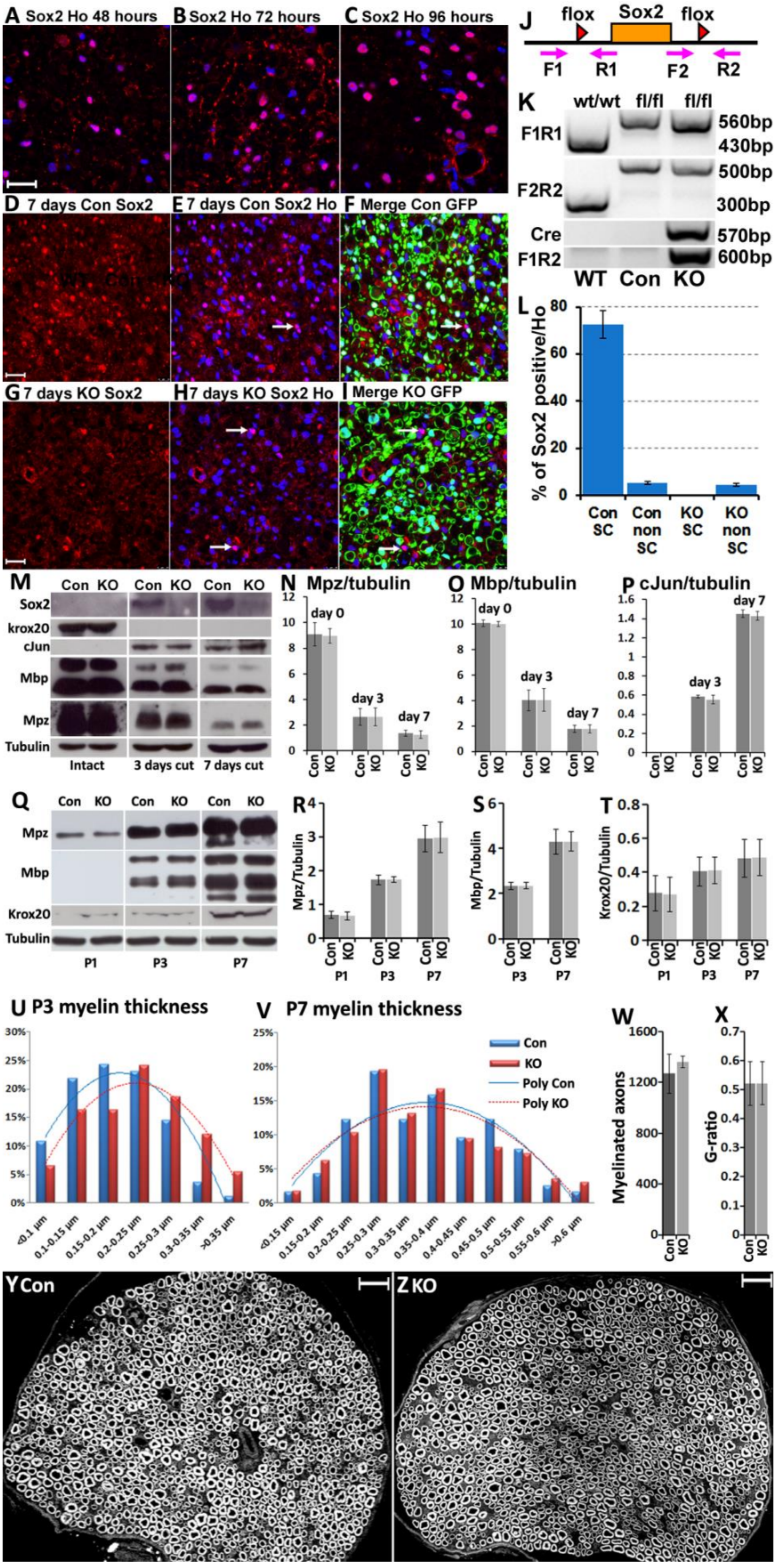

Figure S1 (Related to Figure 1): Normal peripheral nerve development and Schwann cell dedifferentiation in Sox2 KO mice. A-C) Sox2 (red) up-regulation in mouse (C57/BL6) distal sciatic nerve at 48, 72 and 96 hours following transection injury. D-F) Sox2 up-regulation in the distal sciatic nerve of control mice 7 days (d) after transection injury, Schwann cells are GFP+, arrows show Sox2 up-regulation in GFP- cells (non-Schwann cells). G-I) The lack of Sox2 expression in Schwann cells of Sox2 KO mice 7d after transection injury, arrows show expression of Sox2 only in GFP- (ie non-Schwann cells) within the nerve. Panels (A-I) show sections counterstained with Hoechst (Ho) to stain nuclei of cells. J-K) Primer design and PCR identification of recombination in Sox2$^{fl/fl}$ CRE+ (Sox2 KO) mice. Sox2 DNA gene deletion was confirmed with sciatic nerve samples using the forward (F1) and reverse (R2) primers which show a 600bp band on the gel (F1R2) for the recombined allele. L) The percentage of Sox2 expression in GFP+ (Schwann) and GFP- (non-Schwann) cells in the distal sciatic nerve of control and Sox2 KO mice at 7d post-transection, n=3. M) Western blots comparing the protein levels of Sox2, Krox20, cJun, Mpz and Mbp in the distal nerve stump of control and Sox2 KO mice at 3d and 7d after transection injury. Sox2 is undetectable in the distal nerve stump of Sox2 KO mice. N-P) Quantification of Mpz, Mbp and cJun proteins levels from (M), n=3. Q) Western blots comparing the protein levels of Mpz, Mbp and Krox20 between control and Sox2 KO mouse nerves at postnatal (P) day 1, P3 and P7. R-T) Quantification of Mpz, Mbp and Krox20 proteins levels at P1, P3 and P7 in control and Sox2 KO mice, n=3. U, V) Myelin thickness in control and Sox2 KO mice at P3 and P7, sciatic nerve samples were analysed by transmission electron microscopy, n=3. W,X) Numbers of myelinated axons (W) and G-ratio (X) in control and Sox2 KO mice in P60 sciatic nerve, analysed by low vacuum scanning electron microscopy (LV-SEM), n=3. Y-Z) Sciatic nerve LV-SEM images of control and Sox2 KO mice at P60. Scale bar in (A-I) 20µm and in (Y-Z) 40µm.

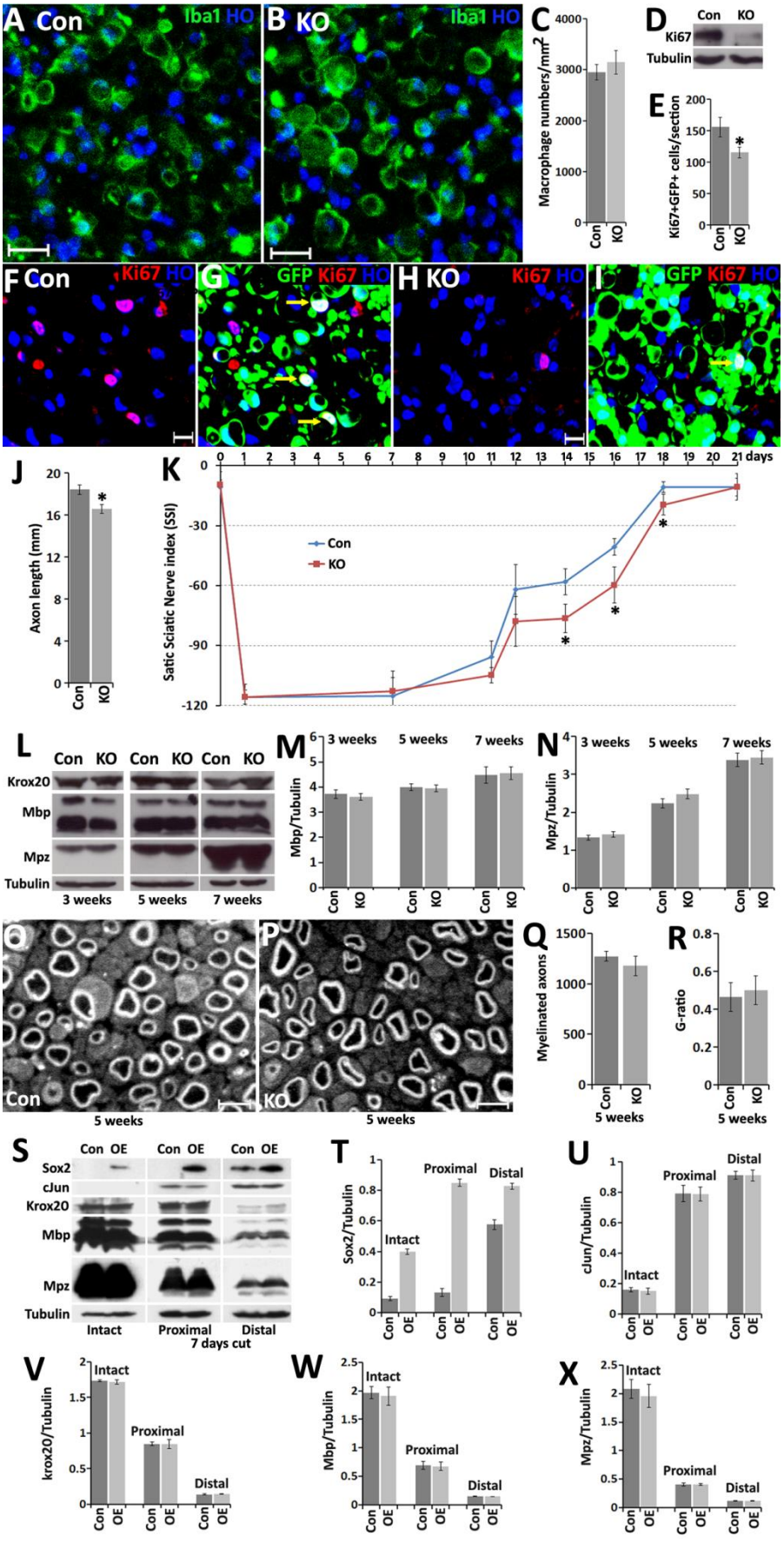

Figure S2 (Related to Figure 1): Macrophage recruitment, functional recovery, Schwann cell proliferation and remyelination in Sox2 KO mice. A-C) Iba1 staining of macrophages in the distal nerve stump of control (A) and Sox2 KO (B) mice 7d after transection injury. C) No significant difference (P=0.15, n=3) on macrophage recruitment between control and Sox2 KO mice was observed. D) Western blots comparing Ki67 protein levels in the distal nerve stump (5mm from the cut site) of control and Sox2 KO mice 7d after transection injury. E) Numbers of Ki67+/GFP+ Schwann cells in control (GFP) and Sox2 KO (GFP) mice, $p<0.05$, n=3. F-I) Ki67 staining in the distal nerve stump of control (GFP) and Sox2 KO (GFP) mice at 7d after nerve transection injury. Yellow arrows indicate GFP+/Ki67+ proliferating Schwann cells. J) The distance of leading axons from the crush point at 7d after crush injury on control and Sox2 KO mice, $p<0.05$, n=4. K) Static sciatic functional index (SSI) of control and Sox2 KO mice after crush injury, a significant difference of SSI between control and Sox2 KO mice was observed at 14d, 16d and 18d post-injury, $p<0.05$, n=4. L) Western blots comparing Krox20, Mbp and Mpz in the distal nerve stump of control and Sox2 KO mice at 3, 5 and 7 weeks following crush injury. M-N) Quantification of Mpz and Mbp protein levels from (L), n=3. O-P) Sciatic nerve LV-SEM images of control and Sox2 KO mice 5 weeks after crush injury. Q-R) Numbers of myelinated axons and G-ratio of control and Sox2 KO mice 5 weeks after crush injury, sciatic nerve samples were analysed by LV-SEM, n=3. Scale bar in (A) and (B) 20μm, in (F) and (H) 10μm, in (O) and (P) 5μm. S) Western blots comparing the protein levels of Sox2, Krox20, cJun, Mpz and Mbp in the intact, proximal and distal sciatic nerve stump of control and Sox2 OE mice at 7d after transection injury, Sox2 is highly expressed in Sox2 OE mice. T-X) Quantification of Sox2, Krox20, Mpz, Mbp and cJun proteins levels from (S), n=3.

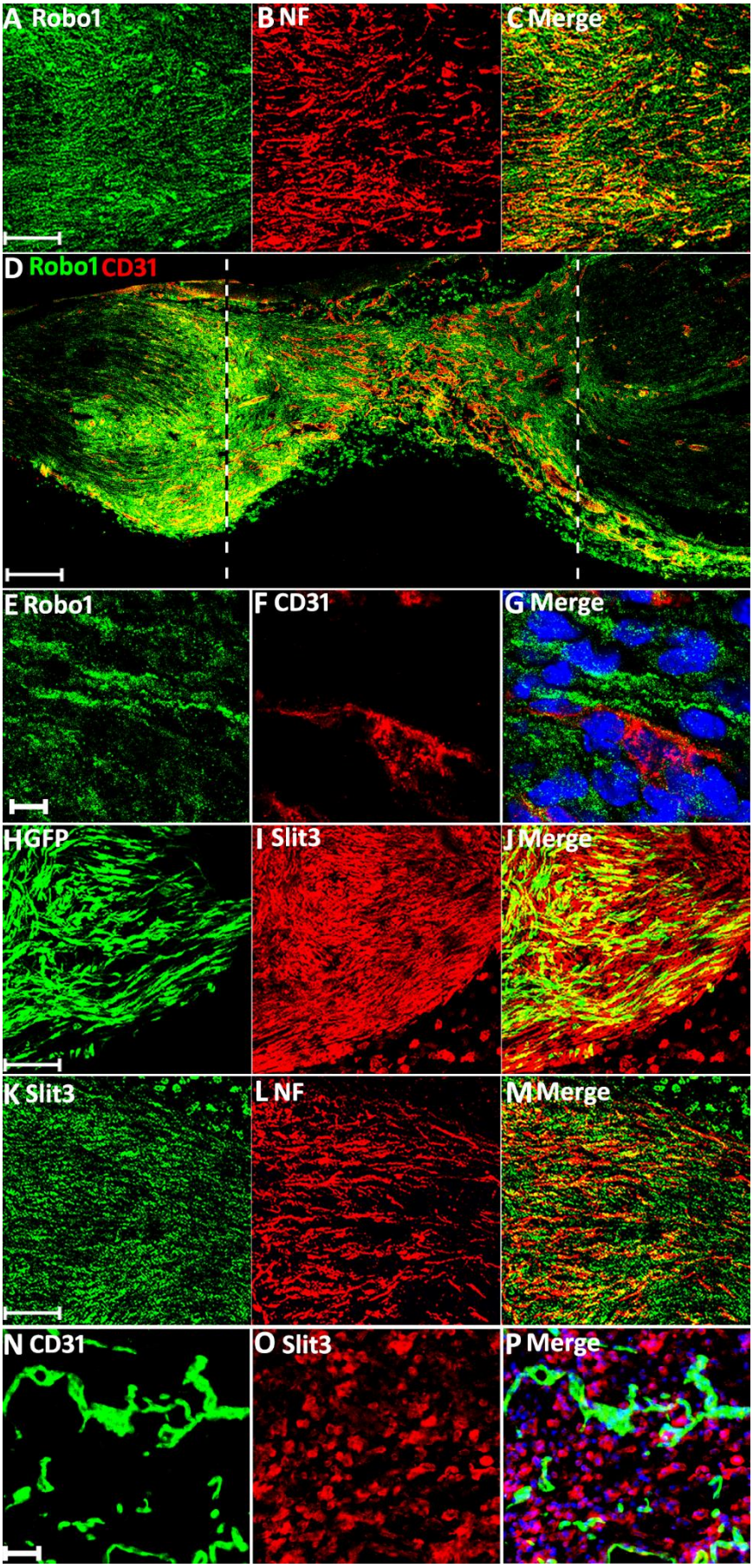

Figure S3 (Related to Figure 3 and 4): Expression of Robo1 and Slit3 in the mouse sciatic nerve bridge. A-C) Double staining of Robo1 with neurofilament heavy chain (NF) on a longitudinal bridge section at 7d post-transection showing that regenerating axons in the proximal nerve stump express Robo1. D) Double staining of Robo1 with CD31 on a longitudinal nerve bridge section at 7d post-transection showing that endothelial cells inside the nerve bridge express low levels of Robo1. E-G) High magnification image from D showing that endothelial cells (red) in the nerve bridge express very low levels of Robo1. Hoechst staining (blue) shows nuclei of cells. H-J) Staining of Slit3 in the 7d nerve bridge of PLP-GFP mice showing that Slit3 is expressed in migrating Schwann cells. K-M) Double staining of Slit3 with neurofilament (NF) showing that regenerating axons in the proximal nerve stump express low levels of Slit3. N-P) Double staining of Slit3 with CD31 showing that Slit3 is not expressed in the blood vessels of the nerve bridge. Nerve section in (N-P) was taken from the outermost layer of the nerve bridge in order to stain blood vessels. Slit3 positive cells in (O) are macrophages. Scale bar in (A, H, K) 50µm, in (D) 150µm, in (E) 7µm and in (N) 20µm. Several Z-series were captured on a Zeiss LSM510 confocal microscope in (D), covering the entire field of interest. The individual series were then flattened into a single image for each location and combined into one image using Adobe Photoshop software (Adobe Systems).

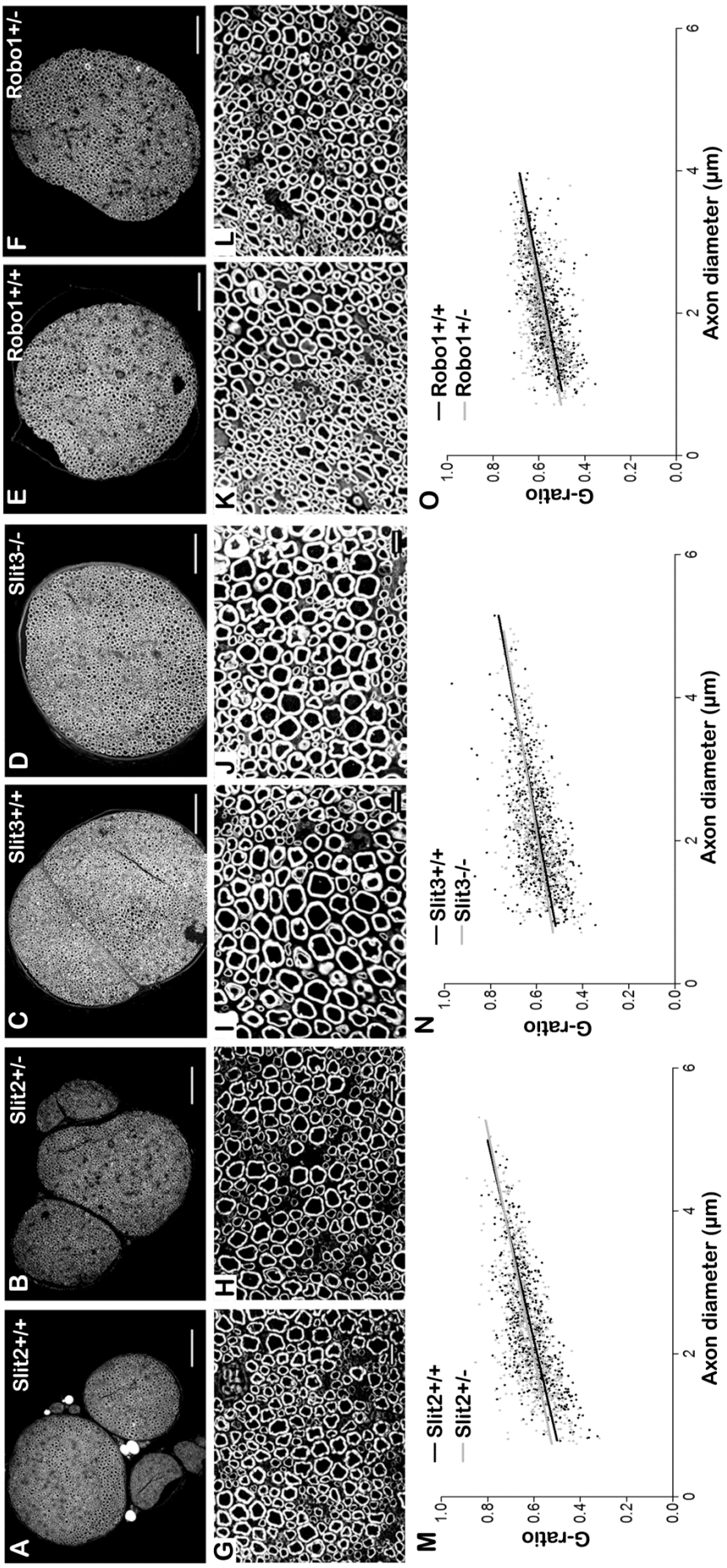

Figure S4 (Related to Figure 5): Normal sciatic nerve structure in adult Slit2+/-, Slit3-/- and Robo1+/- mice. A-F) Low vacuum scanning electron microscopy images of sciatic nerves from 60d old control, Slit2+/-, Slit3-/- and Robo1+/- mice. Scale bar in (A-F) 100µm. G-L) Higher magnification images for (A-F). Scale bar in (G-L) 10µm. M-O) Scatter plot of G-ratio against axon diameter from control, Slit2+/-, Slit3-/- and Robo1+/- sciatic nerve, n=3.

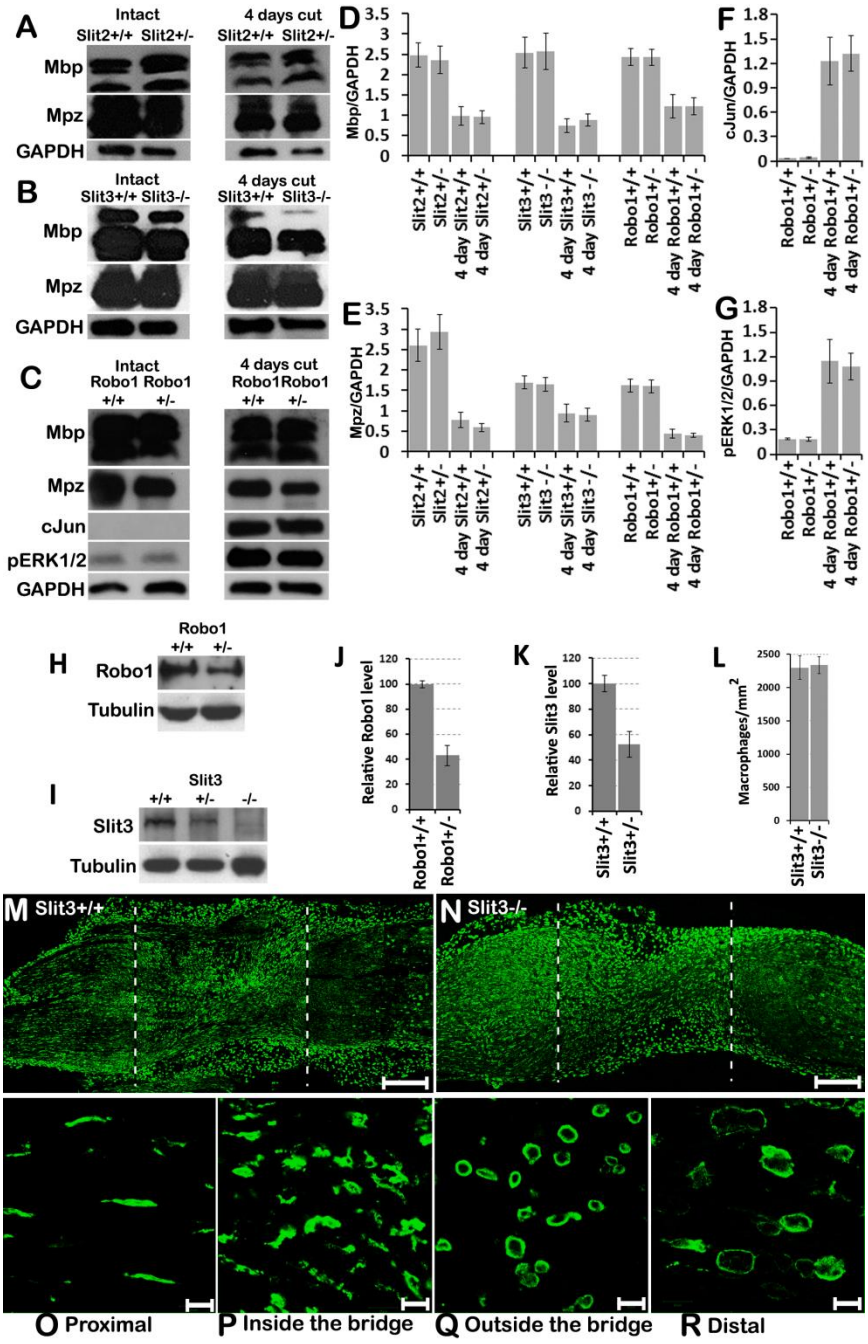

Figure S5 (Related to Figure 5): Normal dedifferentiation following injury in Slit2+/-, Slit3-/- and Robo1+/- mice. A-C) Western blot images for Mbp, Mpz, cJun and pERK1/2 to compare their expression levels in intact adult sciatic nerve (two months old) and in the distal nerve stump 4 days after transection injury (4 day cut) between control and Slit2+/- (A), Slit3-/- (B) and Robo1+/- (C) mice. D-E) Quantification of Mbp (D) and Mpz (E) levels in control, Slit2+/-, Slit3-/- and Robo1+/- mice, n=3. F-G) Quantification of cJun (F) and pERK1/2 (G) levels in control and Robo1+/- mice in intact nerves and 4 days after injury. No significant difference was found between control, Slit2+/-, Slit3-/- mice and Robo1+/- mice, n=3. H) Robo1 protein levels in the sciatic nerve of two month old Robo1+/+ and Robo1+/- mice. I) Slit3 protein levels in the sciatic nerve of two month old Slit3+/+, Slit3+/- and Slit3-/- mice. J) Quantification of Robo1 protein levels in (H), n=3. K) Quantification of Slit3 protein levels in (I), n=3. L) Macrophage recruitment in the outermost layer of the nerve bridge of control and Slit3-/- mice 7 days following transection injury, n=3. M-N) Macrophage staining by the CD206 antibody in longitudinal sections of control (M) and Slit3-/- (N) nerves at 7 days following transection injury. Scale bar 200µm. O-R) Differing macrophage morphology and size in the proximal nerve stump, inside the nerve bridge, outermost layer of the nerve bridge and inside the distal nerve stump. Scale bar 20µm. Several Z-series were captured on a Zeiss LSM510 confocal microscope in (M) and (N), covering the entire field of interest. The individual series were then flattened into a single image for each location and combined into one image using Adobe Photoshop software (Adobe Systems).

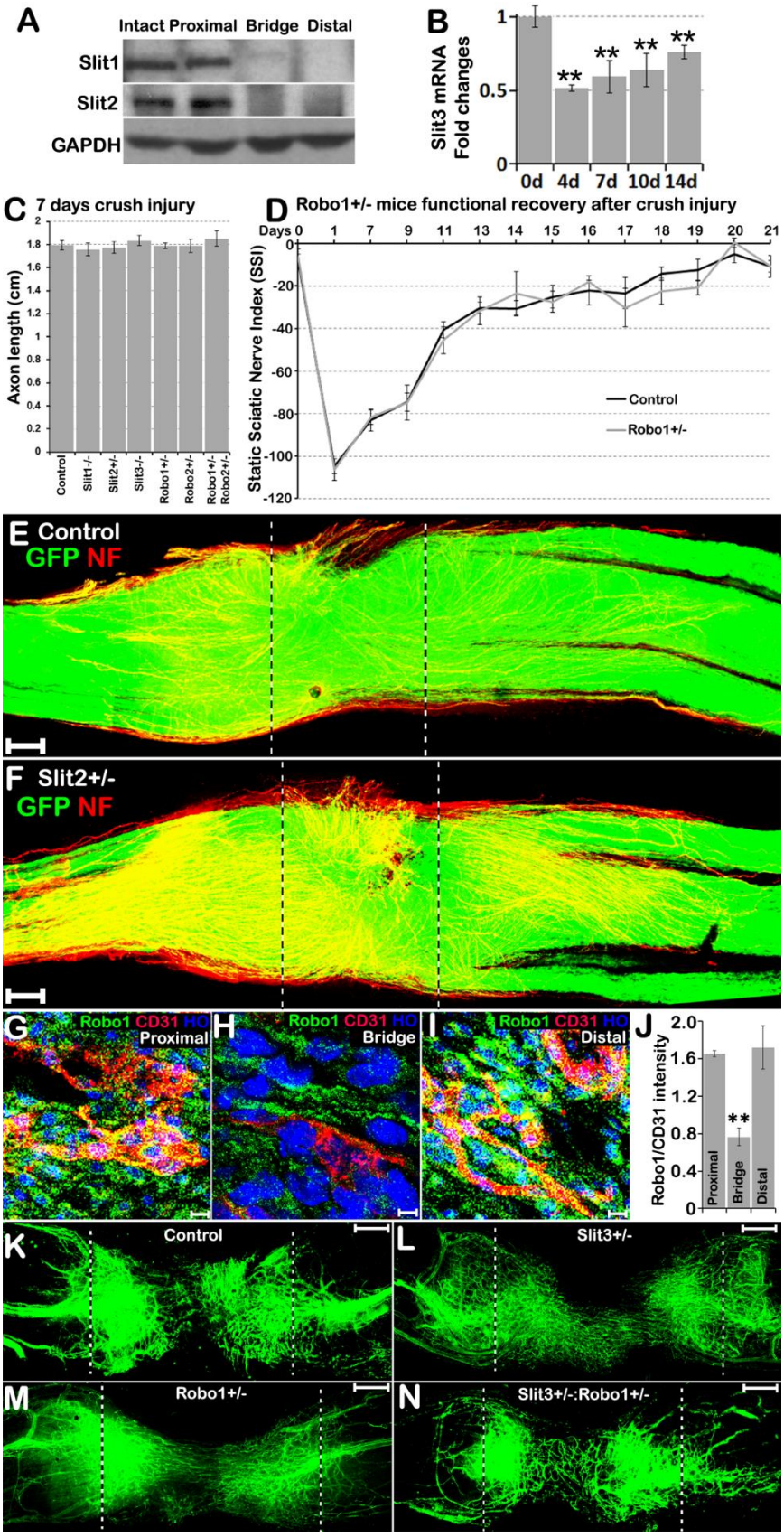

Figure S6 (Related to Figure 5 and 6): Slit1-3 expression in the distal sciatic nerve stump at 7 days after transection injury, normal speed of axon re-growth, functional recovery and blood vessel regeneration in Slit and Robo gene mutant mice. A) Western blot showing that Slit1 and Slit2 are expressed in intact sciatic nerve and the proximal nerve stump but not in the nerve bridge and the distal nerve stump at 7 days after transection injury. B) Fold changes of Slit3 mRNA in the distal nerve stump. Slit3 mRNA is down-regulated at 4, 7, 10 and 14 days post-injury compared to intact (0d) nerve (n=3). Double asterisks indicate significant ($p<0.01$) fold changes of Slit3 mRNA levels compared with intact nerve. C) Normal distance of axon regrowth in in Slit1-3 and Robo1-2 gene mutant mice at 7 days after crush injury. The distance of leading axons from the crush site was measured after whole mount nerve neurofilament antibody staining, n=4. D) Normal functional recovery by SSI measurement in Robo1+/- mice, n=4. E-F). Normal Schwann cell migration and axon regeneration in control (GFP) and Slit2+/- (GFP) mice 14 days after transection injury. G-I) Robo1 and CD31 double staining showing Robo1 expression in endothelial cells of the proximal nerve stump (G), the nerve bridge (H) and the distal nerve stump (I). J) Comparison of Robo1 expression levels in endothelial cells in the proximal nerve stump, the nerve bridge and the distal nerve stump. CD31+ endothelial cells (red) in the nerve bridge express very low levels of Robo1. Robo1 and CD31 fluorescence intensity was measured from three independent nerve samples. ** indicates significant differences ($p<0.01$) compared with proximal and distal nerve stumps, n=3. K-N) Normal pattern of endothelial cell migration and blood vessel regeneration in Slit3+/-, Robo1+/- and Slit3+/-:Robo1+/- mice compared to control littermates. The whole nerve was stained with CD31 antibody to reveal endothelial cell migration and blood vessel regeneration in the nerve bridge at 6d following transection injury. Proximal nerve stump is on the left and distal nerve stump is on the right. The nerve bridge is indicated between two dashed lines. Scale bar in (E-F) and (K-N) 200µm, in (G-I) 7µm. Several Z-series were captured on a Zeiss LSM510 confocal microscope in (E-F) and (K-N), covering the entire field of interest. The individual series were then flattened into a single image for each location and combined into one image using Adobe Photoshop software (Adobe Systems).

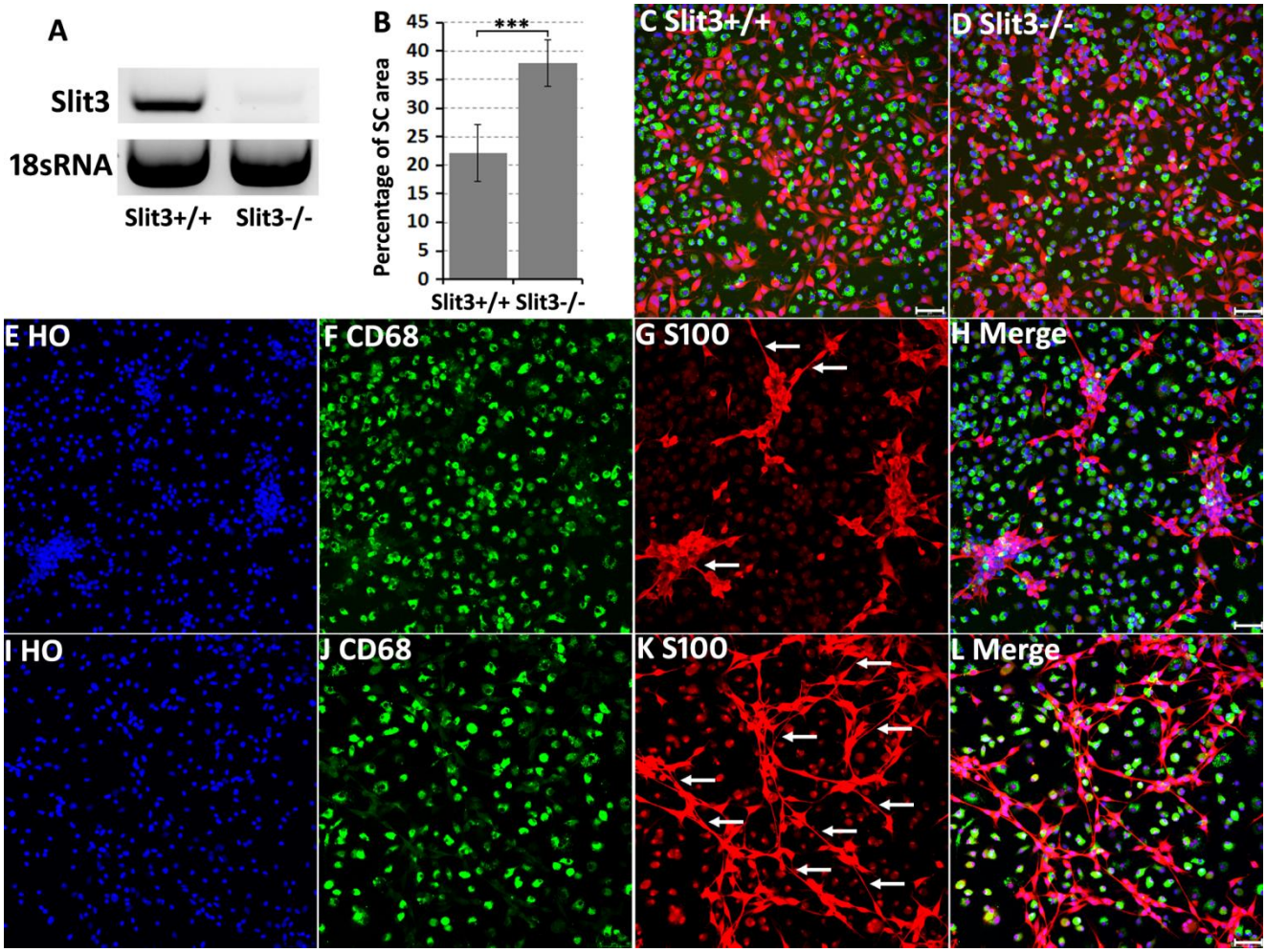

Figure S7 (Related to Figure 6): Schwann cell sorting in Schwann cell (red S100+) **and macrophage** (green, CD68) **co-cultures.** A) Slit3 mRNA is expressed in Slit3+/+ bone marrow macrophages but not Slit3-/- bone marrow macrophages. B) The percentage of Schwann cell covered area on coverslips in the co-culture. n=4, *** ($p<0.001$). The area of Schwann cells on the co-culture coverslips is quantified in ImageJ. C) Rat Schwann cell and Slit3+/+ bone marrow macrophages show a random distribution after 2 hours of cell seeding with 5:3 ratio (Schwann cell:macrophage). D) Rat Schwann cell and Slit3-/- bone marrow macrophages show a random distribution after 2 hours of cell seeding with 5:3 ratio (Schwann cell:macrophage). E-H) After 24 hours of Schwann cell and Slit3+/+ macrophage co-culture, Schwann cells have been sorted together and occupied less space than macrophages. Schwann cells often grow on top of each other and have lost their typical bi-polar processes (indicated by arrows G). I-L) After 24 hours of Schwann cell and Slit3-/- macrophage co-culture, Schwann cell sorting is inhibited and Schwann cells occupy relatively more space than Schwann cells in the Slit3+/+ macrophage co-cultures. The bi-polar processes (indicated by arrows in K) of Schwann cells are still clearly visible after S100 antibody staining in co-culture with Slit3-/- macrophages (K). Scale bar in (C-L) 50μm.