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Roberts, SL

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Original Article.

The role of p38alpha in Schwann cells in regulating peripheral nerve myelination and repair.

Sheridan L, Roberts, Xin-peng Dun, Gemma Dee, Bethany Gray, Thomas Mindos and David B. Parkinson.

Plymouth University Peninsula School of Medicine and Dentistry, John Bull Building, Derriford, Plymouth, Devon PL6 8BU, United Kingdom.

Address for correspondence and reprint requests: Professor David Parkinson, Plymouth University Peninsula School of Medicine and Dentistry, John Bull Building, Derriford, Plymouth, Devon PL6 8BU, United Kingdom.

e-mail: david.parkinson@plymouth.ac.uk

Telephone: 0044 (0)1752 431037

Fax: 0044 (0)1752 517846

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Abstract.

Myelination in the peripheral nervous system (PNS) is controlled by both positive and negative regulators within Schwann cells to ensure timely onset and correct myelin thickness for saltatory conduction by neurons. Transcription factors such as Sox10, Oct6 and Krox20 form a positive regulatory network, whereas negative regulators such as cJun and Sox2 oppose myelination in Schwann cells. The role of the p38 mitogen activated protein kinase (MAPK) pathway has been studied in PNS myelination, but its precise function remains unclear, with both positive and negative effects of p38 activity reported upon both myelination and processes of nerve repair. To clarify the role of p38 MAPK in the PNS, we have analysed mice with a Schwann cell-specific ablation of the major p38 isoform, p38alpha. In line with previous findings of an inhibitory role for p38 MAPK, we observe acceleration of post-natal myelination in p38alpha null nerves, a delay in myelin down-regulation following injury, together with a small increase in levels of re-myelination following injury. Finally we explored roles for p38alpha in controlling axonal regeneration and functional repair following PNS injury and observe that loss of p38 function on Schwann cells does not appear to affect these processes as previously reported. These studies therefore provide further proof for a role of p38 MAPK signalling in the control of myelination by Schwann cells in the PNS, but do not show an apparent role for signalling by this MAP kinase in Schwann cells controlling other elements of Wallerian degeneration and functional repair following injury.

Introduction.

Schwann cells are the myelinating glia of the peripheral nervous system and the insulating myelin they provide allows the saltatory conduction of large diameter axons. The correct control of both the initiation and extent of myelination involves a number of positive and negative regulators and signalling pathways that act to preserve peripheral nerve function and homeostasis. Positive regulators that have been identified include the transcription factors Krox20, Oct6, Sox10, NFATC4 and YY1 (Topilko *et al.* 1994, Parkinson *et al.* 2004, Jaegle *et al.* 1996, He *et al.* 2010, Kao *et al.* 2009, Finzsch *et al.* 2010, Svaren & Meijer 2008), whereas negative regulation of myelination is controlled by transcription factors such as cJun, Sox2 and Pax3 (Parkinson *et al.* 2008, Le *et al.* 2005, Jessen & Mirsky 2008, Doddrell *et al.* 2012). As well as through transcriptional control, regulation of mitogen activated protein kinase (MAPK) signalling in Schwann cells has been shown to both positively and negatively regulate the control of myelination. Analysis of ERK1/ 2 MAP kinase mutants in Schwann cells has been shown that signalling through this pathway is required for normal myelination by Schwann cells (Ishii *et al.* 2013, Newbern *et al.* 2011, Sheean *et al.* 2014). Conversely, activation of ERK1/2 signalling using a tamoxifen-regulated Raf-TR molecule in Schwann cells is sufficient to trigger de-myelination of Schwann cells in the absence of axonal damage (Harrisingh *et al.* 2004, Napoli *et al.* 2012). For signalling through the PI-3 kinase pathway, both *in vitro* and *in vivo* studies have shown both a requirement for signalling through this pathway and that raised PI-3 kinase signalling in Schwann cells leads to increased myelin thickness in the PNS (Maurel & Salzer 2000, Ogata *et al.* 2004, Cotter *et al.* 2010, Fledrich *et al.* 2014, Domenech-Estevez *et al.* 2016). In addition to this, the p38 MAPK signalling pathway has also been identified as a strong regulator of both

Schwann cell behaviour and myelination in a number of *in vitro* and *in vivo* studies utilising chemical inhibitors of p38 MAP kinase activity. Our own previous findings (Yang *et al.* 2012), using both p38 inhibitors *in vitro* and *in vivo* and using specific activation of p38 signalling *in vitro* have shown an inhibitory effect of p38 signalling in controlling Schwann cell myelination. However other studies, using Schwann cell/dorsal root ganglion (SC/DRG) neuron co-cultures, have shown that blocking p38 MAPK activity at the same time as inducing myelination in these culture inhibits Schwann cell myelination (Fragoso *et al.* 2003, Hossain *et al.* 2012). In other experiments using the SD-169 p38 inhibitor, *in vivo* experiments have identified positive effects of global p38 inhibition upon axonal regeneration and maturation following PNS injury (Myers *et al.* 2003).

There are four isoforms of p38 MAPK, α, β, γ and δ (Nebreda & Porras 2000), and although chemical inhibitors such as SB202190, SB203580 are specific for α and β isoforms, there is still an issue with 'off-target' effects of such compounds (Davies *et al.* 2000, Bain *et al.* 2007) and that, in SC/DRG co-cultures, which may not fully mimic processes of myelination in vivo, the effects of p38 inhibition on Schwann cells cannot be completely differentiated from those on the DRG neurons. In order to try and address these issues, Kato et al used a transgenic mouse model with a mutation in the p38α docking site, reported to reduce p38 MAP kinase activity. Analysis of these mice is, however, complicated by the fact that the mutation is present in all cell types within the nerve and, as complete p38α loss is embryonic lethal, only heterozygous mice with reduced p38α activity could be analysed in these experiments (Kato *et al.* 2013, Adams *et al.* 2000, Allen *et al.* 2000, Mudgett *et al.* 2000).

In order to fully characterise the role of p38 MAPK activity within Schwann cells in controlling myelination and PNS repair, we have generated and analysed a Schwann cell-specific null for the major p38 isoform, p38α. In line with our previous findings, that p38 MAPK activity is a negative regulator of myelination (Yang et al. 2012), we observed both an earlier onset of myelination in the PNS and a delayed loss of myelin in Schwann cells following injury and loss of axonal contact; we also observe a slight, but non-significant, increase in myelin thickness of regenerated axons following a crush injury.

In contrast to previous studies on axonal regeneration and the immune response following injury, we find no changes in the rates of axonal regeneration and functional repair following injury in nerves containing p38α null Schwann cells. Similarly, we find no changes in the proliferation of Schwann cells or numbers of macrophages recruited to the nerve following injury. Thus, although we can confirm a negative role for p38 MAPK activity in regulating myelination, these findings suggest that previously observed effects upon the immune response, axonal regeneration and repair following injury appear to be either due to the effects of p38 inhibition on other cell types within the nerve, or are perhaps due to the non-specificity of the p38 MAPK chemical inhibitors used in these previous studies.

Materials and Methods.

Transgenic mice.

All animal experiments were carried out in strict accordance with the UK Home Office regulations under the Animals (Scientific Procedures) Act of 1986. All experiments have been approved by the Plymouth University Animal Welfare and Ethical Review Board. Mice with a conditional p38 alpha (α) allele, p38$\alpha^{fl/fl}$, have been previously described; CRE mediated recombination deletes exons 2 and 3 of the coding sequence generating p38α null cells (Heinrichsdorff *et al.* 2008). p38$\alpha^{fl/fl}$ animals were crossed with mice carrying the mP_0TOTA-CRE (P0-CRE) transgene (Feltri *et al.* 1999) to generate p38$\alpha^{fl/fl}$ CRE- controls and p38$\alpha^{fl/fl}$ CRE+ Schwann cell-specific p38α null animals; both male and female animals were used in this study in approximately equal numbers with appropriate littermate age and sex-matched controls. Animals were genotyped as previously described (Truett *et al.* 2000, Feltri et al. 1999, Heinrichsdorff et al. 2008). Mice were housed in a 12 hour light/dark cycle with *ad libitum* access to food and water. p38$\alpha^{fl/fl}$ CRE- controls and p38$\alpha^{fl/fl}$ CRE+ null animals were born with the expected Mendelian ratios and no apparent physiological differences were observed between the two groups of animals.

Sciatic nerve injury and assessment of functional recovery.

Sciatic nerve injury, cut or crush, was carried out in adult animals under isoflurane anaesthesia as previously described (Dun & Parkinson 2015) and nerve samples taken at various timepoints following injury; contralateral uninjured nerves were used as controls. The mouse static sciatic index (SSI) measurement (Baptista *et al.* 2007) was used to assess recovery of sensory-motor co-ordination in animals at timepoints

following sciatic nerve injury. The evaluation of SSI values was made by an individual blinded to the genotype of the animal under test.

Electron Microscopy.

Samples for low vacuum (LV) scanning or transmission electron microscopy (SEM/TEM) were fixed in glutaraldehyde and embedded in resin blocks. For LV-SEM sample block surfaces were polished and analysed using a JEOL 6610 LV-SEM machine. For TEM, ultrathin sections were prepared, stained and visualised using a JEOL 1400 microscope. Myelin thickness or G ratio (myelin thickness/myelin + axon thickness) measurements were made from at least 200 myelinated fibres from the same area of sciatic nerve per animal at stated timepoints. Unless otherwise stated, n=3 mice of each genotype were used for each measurement of myelin thickness/ G ratio at each developmental age or timepoint following injury. Measurements of axon and myelinated fibre (axon + myelin) diameter were made using ImageJ software.

Immunocytochemistry, wholemount staining, Schwann cell culture and western blotting.

Nerve samples were fixed in 4% paraformaldehyde/PBS overnight, cryoprotected in 30% sucrose/PBS overnight at 4°C, and embedded in Optimal Cutting Temperature (OCT) compound and frozen for cryosectioning. Sections for immunostaining (10µm thickness) were cut from the same area of nerve for each sample using a Leica CM1860 UV cryostat. Sections were washed in PBS and blocked in either antibody diluting solution (Parkinson et al. 2004) or 10% goat serum/PBS, supplemented with 0.2% Triton X-100 before addition of primary antibody. A two or three layer labelling system was used, either directly fluorophore conjugated secondary antibody or a biotinylated secondary antibody followed by a streptavidin fluorophore conjugate.

Samples were counterstained with Hoechst stain to reveal nuclei. Wholemount staining of nerve samples after injury was performed as previously described (Dun & Parkinson 2015). The following primary antibodies were used for immunofluorescence staining at the indicated dilutions: Neurofilament (NF-200) heavy 200 kDa (1/500, Abcam ab4680), Iba1 (1/300, WAKO 019-19741), Ki67 (1/200, Abcam ab15580) and Sox10 (1/100, R & D systems AF2864)

Schwann cells were prepared from post-natal day 3-5 rats using serum purification and maintained in defined medium prior to lysing for western blot (Brockes *et al.* 1979, Jessen *et al.* 1994, Parkinson et al. 2004).For western blotting of nerve samples, control and injured nerves were snap frozen in liquid nitrogen and stored at -80^{o}C before extraction. Sample extraction, protein gel electrophoresis and analysis were all as previously described (Parkinson et al. 2004, Parkinson *et al.* 2003). All western blot analysis was performed three times for each analysis and representative blots presented. The following primary antibodies were used for western blotting: Protein zero, MPZ, (1/1000, Sigma Aldrich SAB2500665), β-Tubulin (1/2000, Santa Cruz Biotechnology sc-134229). Antibodies to p38 alpha (#9218), p38 beta (#2339) and phospho-p38 (#4631), were all used at 1/500 were from Cell Signaling Technology.

Statistical methods.

All data shown is expressed as the mean ± Standard Error of the Mean (SEM). The statistical significance of any observed differences was assessed using the student's t-test between the two groups of data. P values of <0.05 are indicated with an asterisk (*) on graphs. Where graphs are not labelled with an asterisk, any differences between groups of data were non-significant.

Due to the small sample sizes (n<5 for all comparisons), assumptions of how well normality and equal variances fit the data could not be reliably assessed. Sample size was not predetermined by statistical methods and randomisation was not applied. For functional testing by SSI, the evaluation of values was made by an individual blinded to the genotype of the animal. No samples or data were excluded from the analysis. The n number for each experiment has been stated in all Figure legends.

Results.

Characterisation of peripheral nerves with Schwann cell-specific deletion of p38α

Our previous work and that of other groups to examine the role of p38 MAPK signalling in Schwann cells has relied upon the use of either chemical inhibitors, such as SB202190 or PD169316, combined with *in vitro* assays of myelination, or the use of genetically modified mice that have globally reduced, but not absent, p38 MAPK activity in all cells of the PNS. These studies have seemingly identified both positive and negative control of myelination by p38 activity, plus effects upon axonal regeneration and remyelination (Yang et al. 2012, Myers et al. 2003, Kato et al. 2013, Hossain et al. 2012, Fragoso et al. 2003). Among the four isoforms of the p38 MAPK proteins, the p38α isoform is the major isoform expressed in rat Schwann cells and sciatic nerves (Figure 4A and (Hossain et al. 2012)). To determine the Schwann cell specific function of p38 MAPK in vivo, we chose to specifically delete the p38α protein in Schwann cells and examine the effects upon peripheral nerve myelination and repair following injury. Deletion of the p38α coding sequence was accomplished using the mP_0TOTA-CRE line which induces Schwann cell-specific recombination in the PNS on or around embryonic day 13.5 in mouse development (Feltri et al. 1999).

We first examined myelination in the mouse sciatic nerve at early post-natal timepoints. For samples of post-natal day (P) 2 nerve, we first identified Schwann cells that had sorted and were in a clear 1:1 relationship with an axon and then counted the proportion of these Schwann cells that had produced compact myelin. Samples from post-natal day (P) 2 from p38α null pups showed significantly higher percentage of Schwann cells that had produced compact myelin than their control

littermates (Figure 1 A-C); measurements of myelin also showed a small but non-significant increase in myelin thickness at this age (Figure 1D).

At P6, using both low vacuum scanning electron microscopy and transmission electron microscopy, we observed a shift in axons to having thicker myelin (Figure 2 A-E), in support of our previous findings that p38 MAPK activity in Schwann cells is an inhibitor of myelination (Yang et al. 2012). For the percentage of axons with thicker myelin at this age (i.e. myelin thickness between 0.7-0.8 μm), we observed a significant ($P<0.05$) increase in the p38α null nerves as compared to controls (Figure 2E).

In our *in vivo* p38α null nerves, an increase in the myelin protein P-zero (MPZ) was also observed at P6 and the expected reduction of p38α expression confirmed by western blotting; we presume that the small residual p38α levels are from other cell types within the nerve. No change in the level of the Sox10 protein was observed in vivo (Figure 2F), in contrast to the reduction of Sox10 and block in myelination observed *in vitro* in Schwann cell/DRG neuron co-cultures with the p38 inhibitor PD169316 (Hossain et al. 2012, Fragoso et al. 2003). No apparent changes were observed in Schwann cell number, as assessed by nuclei counts of cryostat sections or any abnormalities in axon sorting, axon number or axon diameter in either P2 or P6 p38α null pups or adult animals as compared to controls (data not shown).

Following on from this finding, we then analysed control and p38α null nerves at both P21 and P90 timepoints to check for whether we would observe increased levels of myelination at these timepoints. At both P21 and P90 we observed consistent, but small, increases in myelin thickness in the p38α null animals and a shift towards lower G ratio, however these changes were not significant at either P21 or P90

timepoints (at P90, G ratio for p38α nerves was 0.60±0.07 compared to 0.61±0.02 in control animals; Figure 3 A-F). We also observed no significant differences in numbers of myelinated fibres at P21 and P90 between control and p38α null animals (data not shown).

p38α loss leads to slower myelin breakdown in Schwann cells following injury.

Our previous work has shown that the p38 inhibitors SB202190 *in vitro* and SB203580 *in vivo*, block the down-regulation of myelinating Schwann cell markers expression following injury (Yang et al. 2012). We next tested whether loss of Schwann cell-expressed p38α had similar effects *in vivo*. Following nerve injury, we measured the loss of myelin protein expression by western blot. As previously described, nerve injury induces activation and phosphorylation of p38 (Myers et al. 2003, Yang et al. 2012). Using a phospho-specific p38 antibody, which detects all phosphorylated isoforms of p38, we observed loss of signal for phospho-p38, indicating both the efficient loss of p38α in Schwann cells within the nerve and that p38α is the major isoform expressed and confirming that, within the nerve, Schwann cells activate the p38 MAPK pathway following injury (Yang et al. 2012, Myers et al. 2003). Activation of ERK1/2 that occurs in Schwann cells following injury (Harrisingh et al. 2004) was unchanged in p38α null nerves (Figure 4B). Expression of the myelin proteins myelin basic protein (MBP) and P-zero were unchanged in adult p38α null animals as compared to controls in intact nerve, but at 7 days after injury we observed higher levels of P-zero and MBP protein in the distal stumps of p38α null animals, indicating a slower process of demyelination in Schwann cells lacking p38α (Figure 4C). Western blotting showed that loss of p38α does not cause any

compensatory changes in levels of the p38β isoform within the intact or injured nerve (Figure 4D). In line with our previous findings in vitro for regulation of cJun by the p38 MAPK pathway (Yang et al. 2012), we also observed some reduction in cJun expression, but not Sox2, in p38α null nerves following injury (Figure 4E).

Although we observed a slower loss of myelin protein expression, axonal breakdown distal to the injury site appeared unchanged in p38α null nerves as compared to controls (Figure 4 F, G; 4d post-crush injury). In nerve transection experiments, in which a 0.5cm segment of nerve was removed between proximal and distal stump to prevent regeneration, complete loss of axons and myelin was observed in both control and p38α null nerves at 14d post-injury (Figure 4 H-K). These experiments indicate that although myelin breakdown is slower in p38α null nerves at earlier timepoints (7 days), the processes of Wallerian degeneration at later timepoints appear to have completed in both the p38α null and control nerves at 14 days post-injury.

Effects of p38α loss upon Schwann cell proliferation and macrophage recruitment following injury.

Activation of the p38 MAPK pathway is associated with many different cell behaviours, including proliferation, survival and the control of the inflammatory response (Zarubin & Han 2005). Following PNS injury, there is a proliferation of Schwann cells distal to the injury site as well as the breakdown of the blood-nerve barrier and entry of macrophages into the distal nerve (Chen *et al.* 2007). In order to determine the effects of the loss of p38 MAPK in Schwann cells in these events, we next measured both Schwann cell proliferation by Sox10/Ki67 double labelling and

macrophage numbers by Iba1 immunolabelling at 7 days post transection injury in control and p38α distal null nerves.

Double immunolabelling of nerve sections from both control and p38α null nerves with Sox10 and Ki67 antibodies showed that approximately 80% of proliferating Ki67 positive cells in these sections were Sox10-positive Schwann cells (Figure 5 B-D and data not shown). No significant differences in numbers of Sox10 positive/Ki67 positive or Sox10 negative/Ki67 positive cells were observed between control and p38α null nerves. Total numbers of Sox10/Ki67 positive cells per section were counted and showed that loss of p38α caused no significant change (p=0.80) in Schwann cell proliferation at this timepoint following injury (Figure 5A). Counts of Iba1-positive macrophages in distal p38α nerves also showed no significant differences to those seen in control nerves (p=0.44) (Figure 5E, F).

Loss of p38α in Schwann cells has no effect upon axonal regeneration and functional repair.

The use of mouse models with a global reduction of p38 MAPK activity and treatment of animals with p38 inhibitors has yielded very different results for the roles of p38 MAPK in axonal regeneration and functional repair (Myers et al. 2003, Kato et al. 2013). Use of the sevenmaker mouse mutant, with a proposed reduced p38α activity in all cells of the nerve, showed a reduced rate of functional recovery, which was proposed to be due to an abnormal cytokine response (Kato et al. 2013). In contrast, use of the p38 MAPK inhibitor SD-169 administered to mice following a

nerve crush injury increased rates of axonal re-growth and re-myelination (Myers et al. 2003).

Following PNS injury, Schwann cells distal to the site of injury re-program to a specialised repair-competent cell, or Büngner cell, which directs axonal regeneration, remyelination and allows functional repair (Jessen & Mirsky 2016, Arthur-Farraj *et al.* 2012, Parkinson et al. 2008). We next carried out a series of experiments to determine the role of p38α and how loss of p38 MAPK function in Schwann cells would influence rates of axonal regeneration and remyelination following a crush injury to the sciatic nerve. Following such an injury, mice regain full functional repair after 21 days as measured by the static sciatic index (SSI) measure of sensorimotor recovery (Arthur-Farraj et al. 2012, Baptista et al. 2007).

As previous work has identified an acceleration of axonal regrowth with p38 MAPK inhibition, using the chemical inhibitor SD-169 (Myers et al. 2003), we first used a wholemount staining protocol to measure the rate of axonal regeneration in control and p38α null animals following crush injury (Dun & Parkinson 2015). Neurofilament staining of the nerve at 5 and 7 days following crush injury showed that there was no significant difference in the rate of axonal growth in p38α null nerves as compared to control animals at 7days post-crush injury (Figure 6 A-C and data not shown). Measurements of SSI following nerve crush injury revealed no significant differences in recovery rate between control and p38α null animals at any of the timepoints tested up to 21 days (Figure 6D).

Measurements of myelin thickness and G ratio, however, as during development, showed a slight, but not significant, increase in the myelin thickness of remyelinated fibres in the distal sciatic nerve at 21 days post-crush injury (G ratio p38α repaired

nerves 0.67±0.06; control repaired nerves 0.77±0.02; P=0.17) (Figure 6E-G). In contrast to the previous study using SD-169 (Myers et al. 2003), however, we observed no significant changes in the average diameter of myelinated regenerated axons in control and p38α null nerves at this timepoint (Figure 6I).

Discussion

Schwann cells that myelinate the PNS possess the ability to reprogramme their function following injury to generate a specialised repair cell that allows axonal regeneration and functional repair (Kim *et al.* 2013, Jessen & Mirsky 2016). Research into the signalling mechanisms and transcriptional control that regulates both myelination during development and the cellular reprogramming of Schwann cells following injury has identified a number of positive and negative regulators of these processes, with genetic evidence for roles of many transcription factors such as Krox20, Sox10 and Oct6 in driving myelination and for cJun, Sox2 and Pax3 to negatively regulate myelination (Svaren & Meijer 2008, Doddrell et al. 2012); further research into the role of cJun identified it as crucial to the generation of the specialised repair or Büngner cells that drive functional repair following injury (Parkinson et al. 2008, Arthur-Farraj et al. 2012, Fontana *et al.* 2012, Jessen & Mirsky 2016).

Evidence to date for the role of the p38 MAP kinase pathway has been contradictory, with some findings showing a requirement for this pathway to drive myelination in Schwann cells (Fragoso et al. 2003, Hossain et al. 2012) and other studies showing that increased p38 activity inhibits myelination (Yang et al. 2012). These studies have been complicated by the use of either chemical inhibitors of the p38 pathway which are applied, often systemically or to *in vitro* mixed cell cultures (eg. DRG/Schwann cell co-cultures) and the requirement for p38 signalling in each cell type cannot be fully established (Hossain et al. 2012, Fragoso et al. 2003), in addition to the possible ‘off-target’ effects of such inhibitors (Davies et al. 2000, Godl *et al.* 2003, Bain et al. 2007).

Genetic approaches to reduce p38 activity, such as the sevenmaker or sem mouse, can only be analysed in the heterozygous state (due to embryonic lethality of p38α null animals) and again are non-cell type specific with loss of p38 MAPK activity within all cell types within the nerve (Kato et al. 2013). To date, no study has specifically ablated p38 MAPK activity in Schwann cells alone and determined the effects upon PNS myelination and repair *in vivo*.

In this study, using Schwann cell-specific p38α null animals, we have systematically measured the effects of p38 function in the regulation of myelination at different developmental stages and events following PNS injury, namely myelin breakdown, Schwann cell proliferation, macrophage infiltration, axonal regeneration and functional repair of the nerve. From these analyses, we can confirm that, *in vivo*, p38 MAPK does appear to negatively regulate both developmental myelination and myelin breakdown, but does not appear to regulate axonal regeneration or functional repair.

Previous work using *in vitro* SC/DRG co-culture system using p38 inhibitors found that both Schwann cell-axon alignment and consequent myelination, induced by ascorbic acid addition to the cultures, required p38 activity (Fragoso et al. 2003, Hossain et al. 2012). In our own experiments, in order to distinguish between the early events of laminin-induced Schwann cell-axon alignment and the later induction of myelination, we added p38 inhibitors at 3 days after ascorbate addition and observed an enhancement of myelination in these cultures (Yang et al. 2012). The P0-CRE line used in our work would be expected to remove the p38α gene at E13.5 in Schwann cells of the nerve (Feltri et al. 1999) and has been used to study the roles of several integrins in regulating laminin signalling, axonal sorting and

myelination during PNS development (Pellegatta *et al.* 2013, Nodari *et al.* 2007, Feltri *et al.* 2002). It would seem probable that any effects of p38 MAPK loss upon Schwann cell-axon interaction and radial sorting would have been seen in our *in vivo* experiments at the timepoints examined, but none were apparent. Instead, we only observed an enhancement of myelination at post-natal days 2 and 6 in the p38α null nerves, supporting our idea that p38 MAPK is functioning as a negative regulator of myelination.

Our finding of slightly increased remyelination following injury in p38α null nerves is in agreement with previous work using an *in vivo* regeneration chamber experiment in rat sciatic nerve using the SD-169 inhibitor (Myers et al. 2003), but our data identifies this effect as a Schwann cell-dependent role for p38 MAPK. However, in contrast to previous findings, we do not see effects of loss of p38 MAPK activity in Schwann cells upon the control of the rate of axonal regeneration or functional repair. The slight delay in myelin clearance from the distal nerve in p38α nulls does not seem to slow axonal regrowth or functional recovery in our experiments. Analysis of nerves at later timepoints following injury (14 days) shows that axonal breakdown and myelin loss are seemingly complete by this time in both control and p38α null nerves. Furthermore, we see no apparent changes in axonal fragmentation distal to the site of injury in p38α null nerves at earlier timepoints (4 days) post-injury. Thus although we see some delay in myelin clearance at 7 days post-injury, this early delay appears to correct in p38α null nerves and myelin debris, which otherwise may prevent axonal regeneration (Kang & Lichtman 2013, Zhang *et al.* 2013) does not hinder the functional recovery of p38α null animals. In addition, we see no apparent

effects of p38α ablation upon the events of both macrophage recruitment or Schwann cell proliferation following injury.

In conclusion, using Schwann cell-specific ablation of p38α in the peripheral nervous system we find that p38 MAPK activity does appear to play an inhibitory role in the control of myelination, both during development and remyelination following injury and the loss of myelin protein expression following injury. However, we find no effect of the loss of Schwann cell-derived p38 MAPK activity in the regrowth of axons, functional recovery or other elements of Wallerian degeneration such as macrophage entry or Schwann cell proliferation following injury. As this study has for the first time used a Schwann cell-specific ablation of p38 MAPK, this would suggest these effects upon axonal regrowth and remyelination are due either to the contribution of p38 activity from other cell types within the nerve, such as neurons, macrophages or nerve fibroblasts, or to 'off target' effects of the p38 inhibitors used in these previous studies.

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Figure legends

Figure 1: Increased myelination in the nerves of p38α null mice at post-natal day (P) 2. Transmission electron micrographs of sciatic nerve sections from control (A) and p38α null (B) P2 mouse pups. Arrows in panel A indicate Schwann cells in a 1:1 relationship with an axon and arrowheads indicate Schwann cells that have produced compact myelin. Scale bar 5μm. C. Graph showing a significant increase (P=0.02) in the percentage of axons myelinated in the nerves of p38α P2 animals compared to controls. D. Distribution graph showing a small shift in the percentage of axons with thicker myelin in p38α null animals compared to controls

Figure 2: Comparison of myelination in the nerves of p38α null and control mice at P6. A, B. Low vacuum scanning electron microscopy images of sciatic nerves from control (A) and p38α null (B) P6 mouse pups. Scale bars 50μm. C, D. Transmission electron micrographs of sciatic nerve samples from control (C) and p38α null (D) P6 mouse pups. Scale bars 5μm E. Distribution graph showing a shift in percentage of axons having thicker myelin in p38α null nerves compared to controls. Comparison between control and p38α null nerves with myelin thickness between 0.7-0.8μm shows significant increase ($P<0.05$) in p38α null nerves. F. Western blot of control and p38α null P6 mouse nerve samples (n=3 pooled nerve samples, 1 pool=6 sciatic nerves from 3 mice for each genotype; experiments performed in triplicate and representative blot shown). Values given below MPZ blot samples represent normalised expression of p38α null samples against the control. β-tubulin was used as loading control.

Figure 3: Measurements of myelin thickness in P21 and P90 in control and p38α null animals. A, B. Transmission electron micrographs of P21 control (A) and p38α null (B) mouse nerves. Scale bar 5μm C. Distribution graph showing percentage of axons and myelin thickness in control and p38α null P21 nerves. D. Scatter plot of G ratio versus axon diameter for control and p38α null P21 nerves. E. Distribution graph showing percentage of axons and myelin thickness in control and p38α null P90 nerves. F. Scatter plot of G ratio versus axon diameter for control and p38α null P90 nerves. For all data, n=3 for each mouse genotype.

Figure 4: Western blot analysis of control and p38α null nerves before and following injury. A. Expression of p38α and p38β isoforms in cultured rat Schwann cells and samples of post-natal day 2 (P2), P14 and adult (Ad) rat sciatic nerve. Kidney tissue (K) was used as a positive control for p38α and p38β protein expression. B. Reduction of phospho-p38, but not phospho-ERK1/2 at 3 days (d) following injury in control and p38α null nerves. C. Blot showing slower breakdown of P-zero (MPZ) and myelin basic protein (MBP) in the distal stumps of p38α null nerves in comparison to controls at 7d following transection injury. Values for MPZ and MBP for p38α null represent normalised expression against the control wild-type animal. D. Western blot of levels of p38α and p38β in control and p38α null intact and injured nerves (7d following nerve transection). E. Western blotting for cJun and Sox2 levels in control and p38α null intact and injured nerves (7d following nerve transection). For western blotting, n=1 mouse for each sample for both genotypes; experiments performed in triplicate and representative blot shown. β-tubulin was used as loading control.

F, G. Wholemount immunolabelling of distal sciatic nerve at 4d following crush injury. Neurofilament staining shows equally fragmented axons in control (F) and p38α null (G) nerves. Scale bar 100μm. H-K. Semi-thin sections of control (H, I) and p38α null (J, K) nerves at 14d following sciatic nerve transection injury. Images are shown for both distal sciatic (H, I) and tibial (J, K) nerves showing axonal loss and myelin breakdown at this timepoint in both control and p38α null nerves. Scale bar 75 μm.

Figure 5: Schwann cell proliferation and macrophage recruitment post transection injury in control and p38α null nerves. A. Counts of proliferating Sox10/Ki67 positive cells per section in distal sciatic nerve at 7 days post-injury. B-D. Immunofluorescence staining of control nerve sections with Ki67 (C) and Sox10 (D) antibodies. Arrows in C and D indicate Sox10/Ki67 double positive Schwann cells. Arrowhead in C and D indicates Sox10 negative/Ki67 positive cell. Cells were counterstained with Hoechst (Ho) to show nuclei (B). Scale bar 25μm E. Labelling of control nerve section with Iba1 antibody, arrows indicate Iba1 positive macrophages. Nuclei are counterstained with Hoechst dye (Ho). Scale bar 50 μm F. Counts of Iba1 positive cells per section in control and p38α null distal sciatic nerves 7d post-injury. For all data, n=3 for each mouse genotype.

Figure 6: Axon regrowth and functional recovery in control and p38α null nerves. A, B. Representative wholemount images of control (A) and p38α null (B) nerves at 7d following crush injury immunolabelled with neurofilament antibody to reveal regrowing axons. Arrows in panels A and B to the left show the position of the crush

site. Scale bar A and B 1mm. A' and B' show higher magnifications of boxed areas in panels A and B of control (A') and p38α null (B') nerves with the furthest regrowing axons marked by an arrowhead. Scale bar A' and B' 100μm. C. Graph of distance of furthest axon from crush site in control and p38α null nerves at 7d post-injury. D. Measurements of functional recovery by SSI analysis in control and p38α null mice up to 21d after crush injury. E, F. TEM images of distal sciatic nerve from control (E) and p38α null (F) animals at 21d post-crush injury. G. Scatter plot of G ratio vs axon size at 21d post-crush injury. H Average G ratios for control and p38α null nerves at 21d post-crush injury showing a small, but non-significant, reduction in G ratio in repaired p38α null nerves. I. Average axon diameter of myelinated fibres in distal sciatic nerve of repaired control and p38α null nerves at 21d post-crush injury. For all data, n=3 for each mouse genotype.

List of Abbreviations.

Oct6: Octamer-Binding Transcription Factor 6

YY1: Ying Yang 1

NFATc4: Nuclear Factor of Activated T-cells c4

ERK1/2: Extracellular signal-regulated kinases ½

DRG: Dorsal Root Ganglion

PBS: Phosphate Buffered Saline

MAPK: Mitogen Activated Protein Kinase

MPZ: Myelin Protein Zero

MBP: Myelin Basic Protein.

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Figure 1

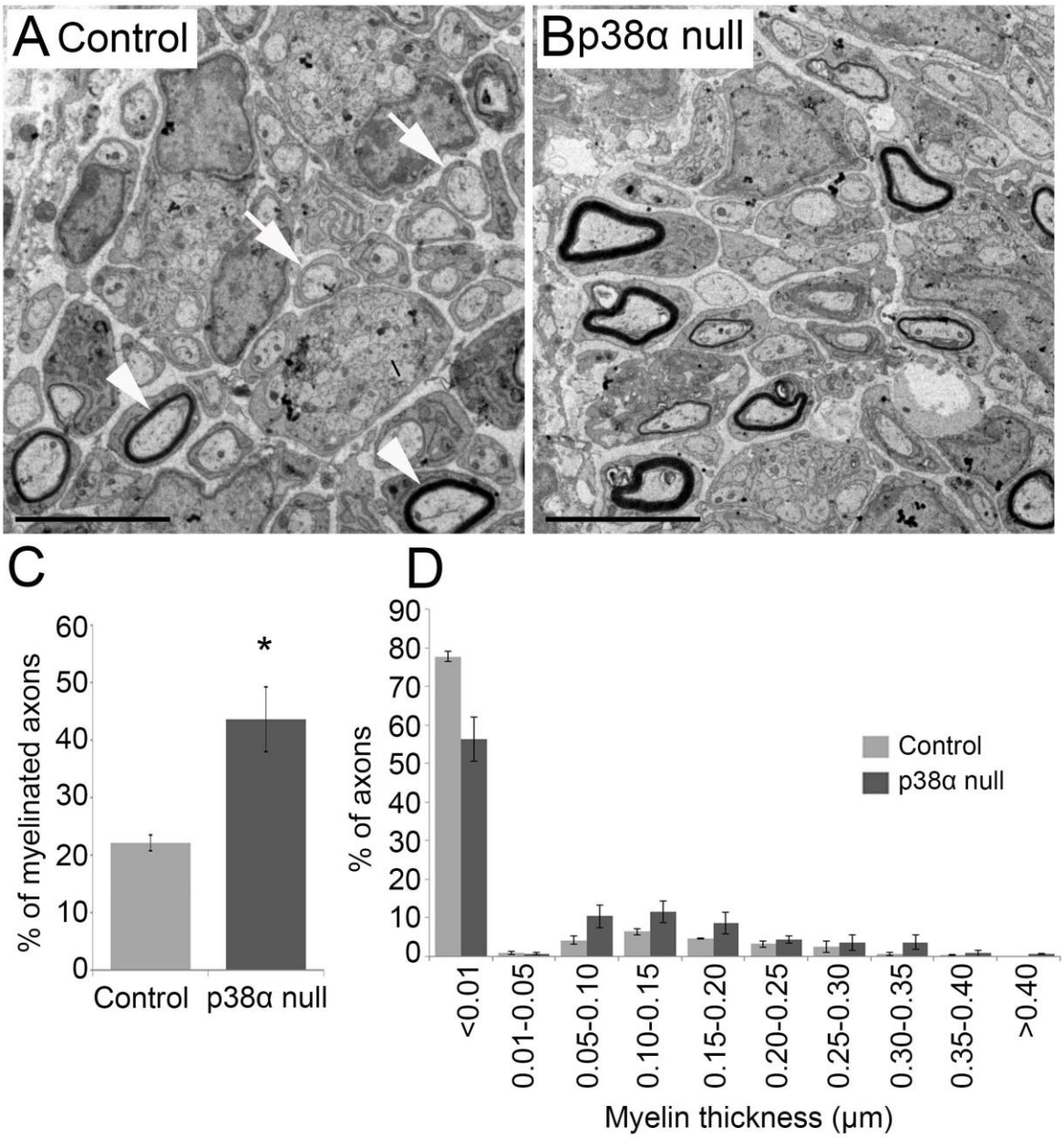

A Control
B p38α null
C
% of myelinated axons
60
50
40
30
20
10
0
*
Control
p38α null
D
% of axons
90
80
70
60
50
40
30
20
10
0
Control
p38α null
<0.01
0.01-0.05
0.05-0.10
0.10-0.15
0.15-0.20
0.20-0.25
0.25-0.30
0.30-0.35
0.35-0.40
>0.40
Myelin thickness (μm)

Figure 2

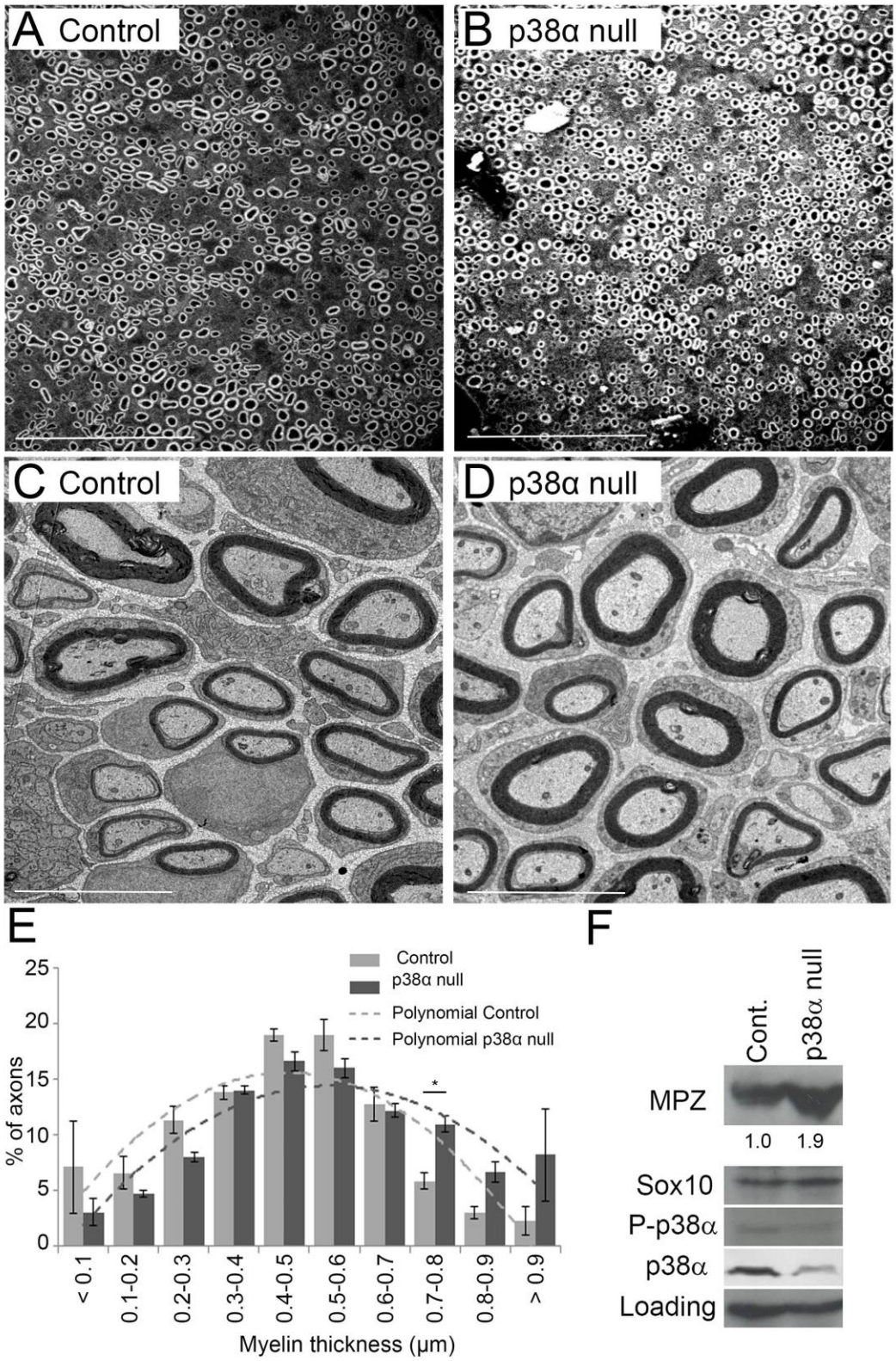
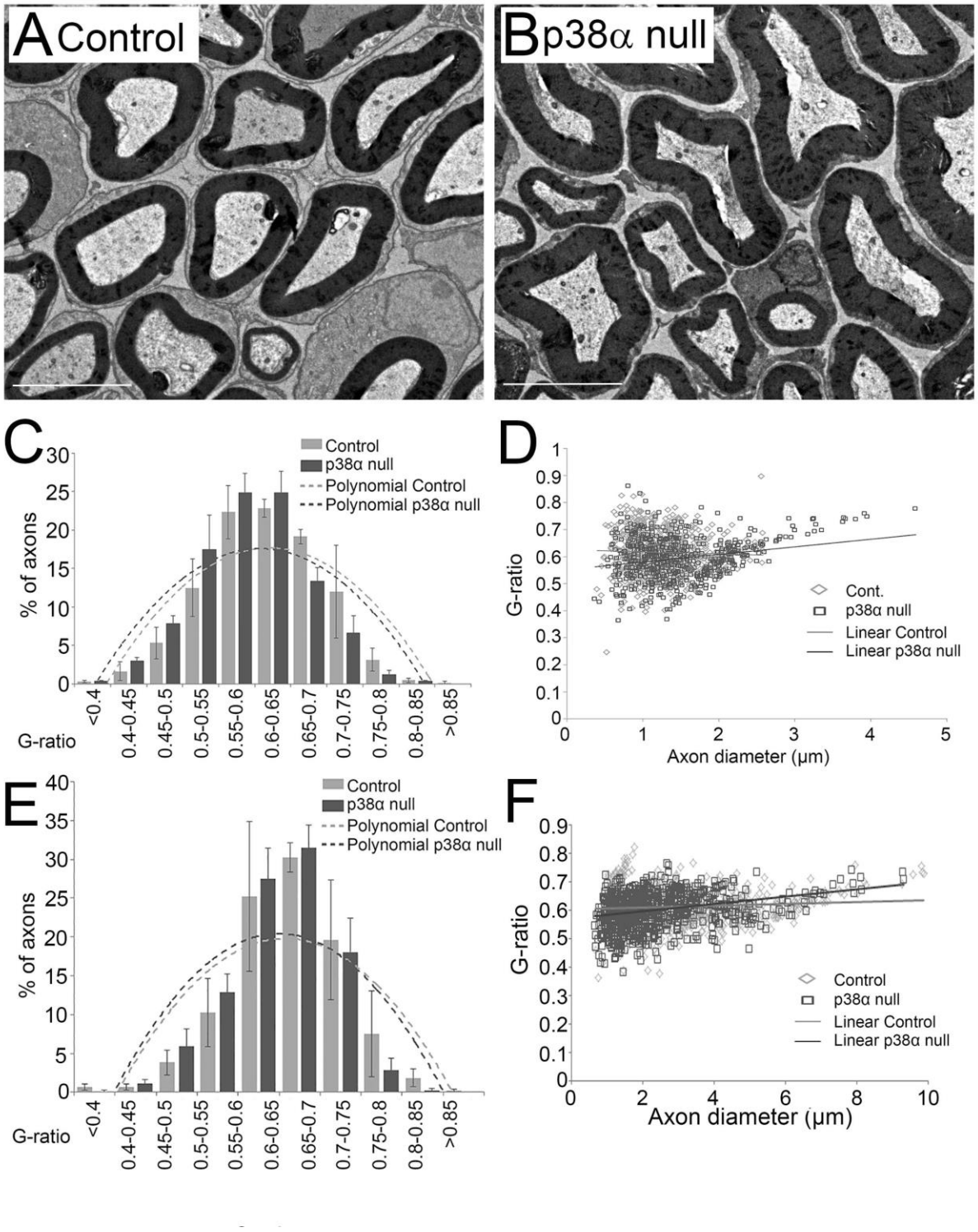

A Control
B p38α null
C Control
D p38α null
E
Control
p38α null
Polynomial Control
Polynomial p38α null
% of axons
25
20
15
10
5
0
*
< 0.1
0.1-0.2
0.2-0.3
0.3-0.4
0.4-0.5
0.5-0.6
0.6-0.7
0.7-0.8
0.8-0.9
> 0.9
Myelin thickness (μm)
F
Cont.
p38α null
MPZ
1.0
1.9
Sox10
P-p38α
p38α
Loading

Figure 3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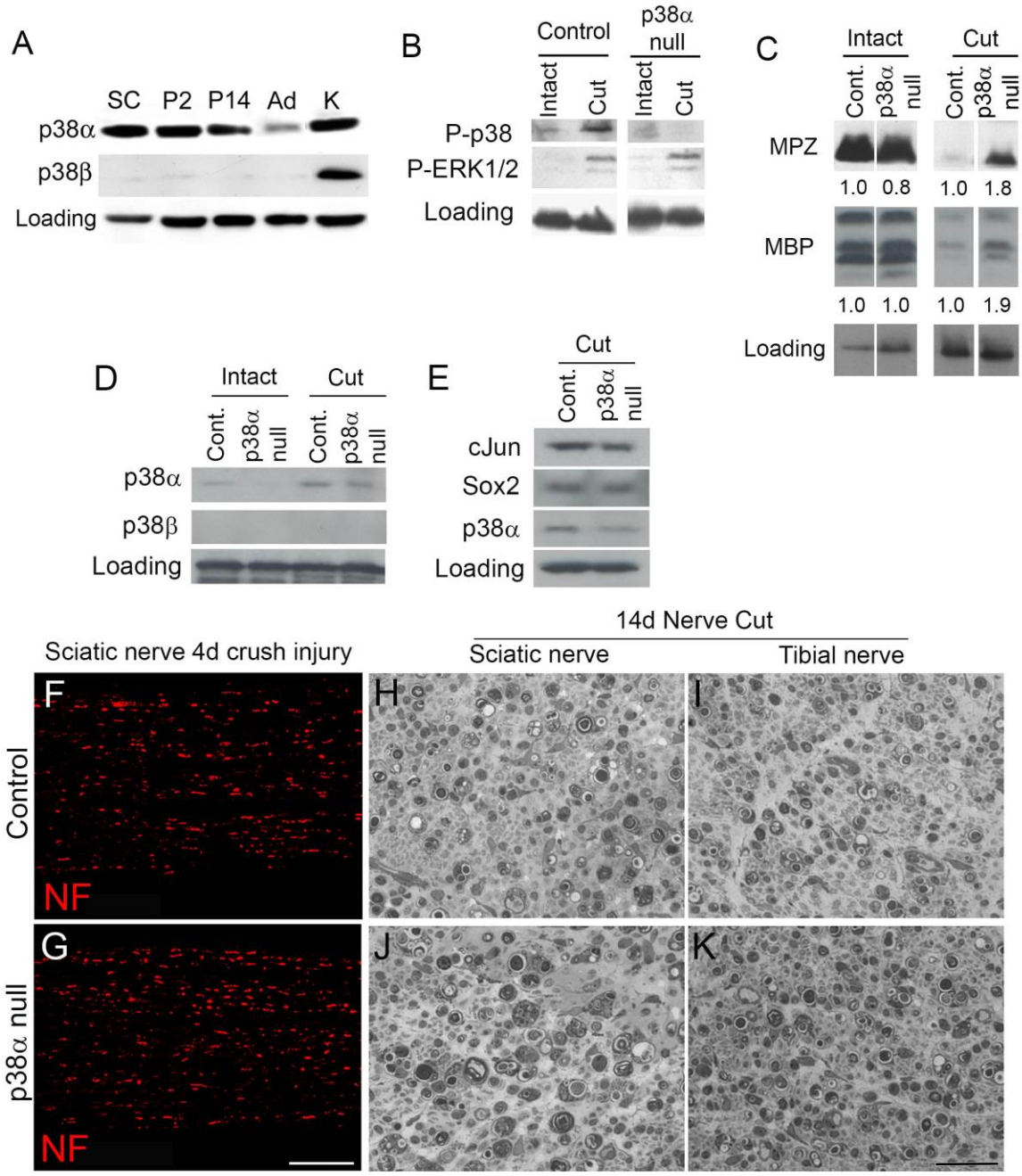
B p38α null
C
Control
p38α null
Polynomial Control
Polynomial p38α null
% of axons
G-ratio
<0.4
0.4-0.45
0.45-0.5
0.5-0.55
0.55-0.6
0.6-0.65
0.65-0.7
0.7-0.75
0.75-0.8
0.8-0.85
>0.85
D
G-ratio
Axon diameter (μm)
Cont.
p38α null
Linear Control
Linear p38α null
E
Control
p38α null
Polynomial Control
Polynomial p38α null
% of axons
G-ratio
F
G-ratio
Axon diameter (μm)
Control
p38α null
Linear Control
Linear p38α null

Figure 4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SC P2 P14 Ad K
p38α
p38β
Loading
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Control
p38α null
Intact Cut Intact Cut
P-p38
P-ERK1/2
Loading
C
Intact Cut
Cont. p38α null Cont. p38α null
MPZ
1.0 0.8 1.0 1.8
MBP
1.0 1.0 1.0 1.9
Loading
D
Intact Cut
Cont. p38α null Cont. p38α null
p38α
p38β
Loading
E
Cut
Cont. p38α null
cJun
Sox2
p38α
Loading
Sciatic nerve 4d crush injury
14d Nerve Cut
Sciatic nerve
Tibial nerve
Control
p38α null
F
G
H
I
J
K
NF
NF

Figure 5

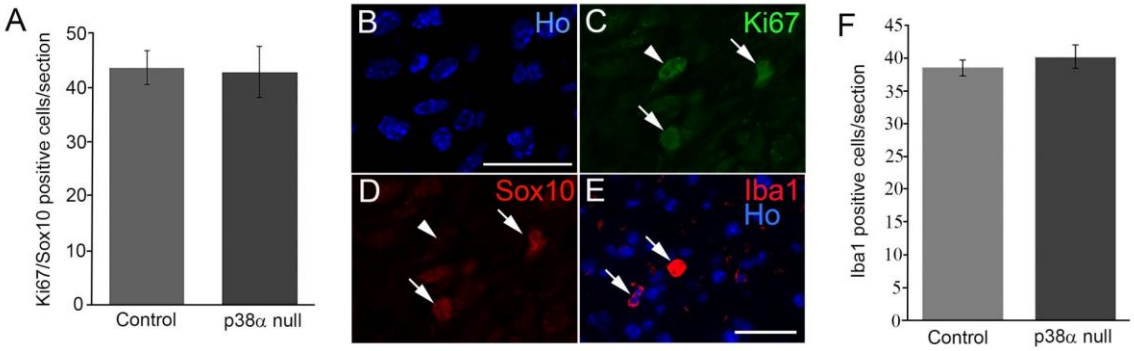
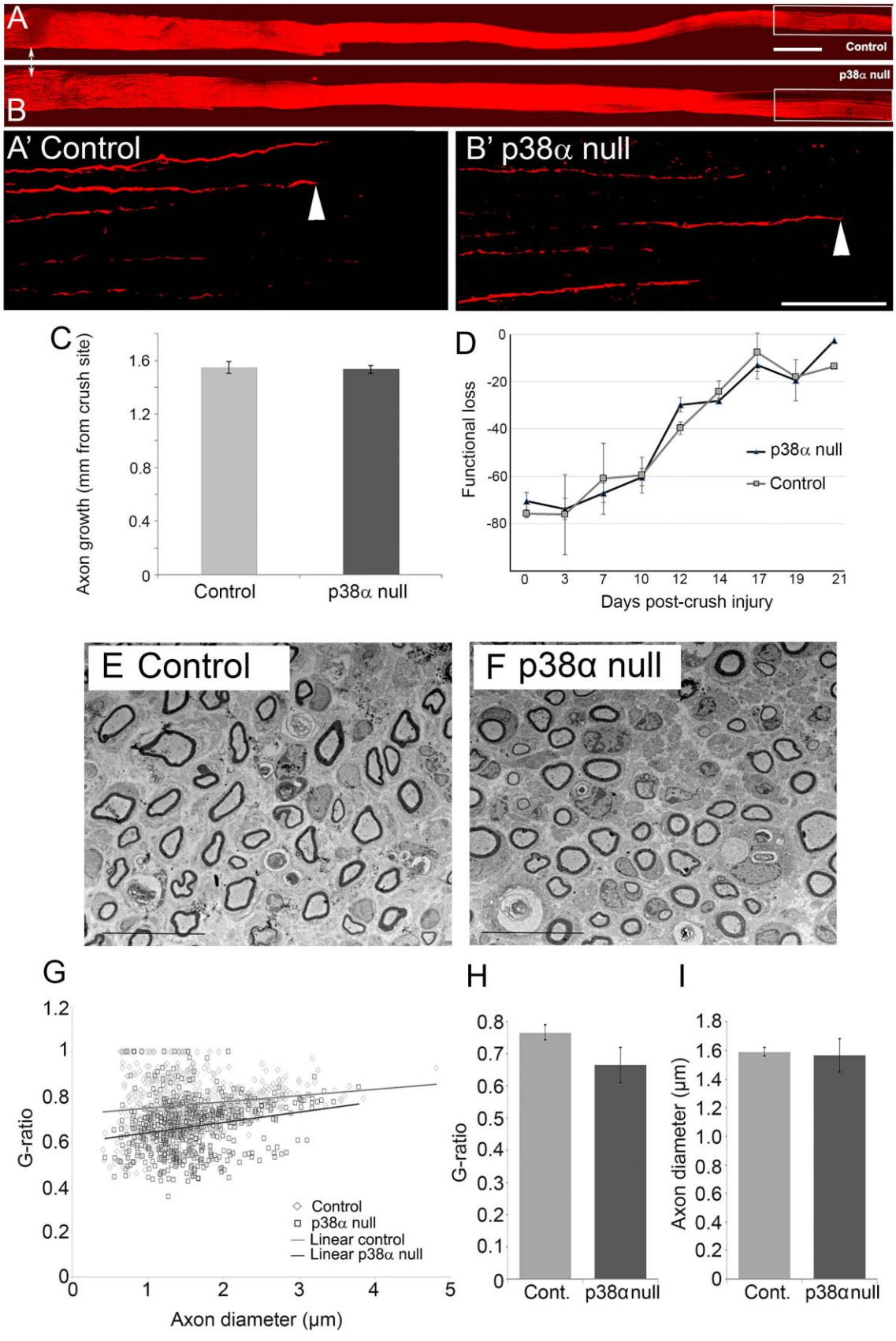

A
Ki67/Sox10 positive cells/section
50
40
30
20
10
0
Control
p38α null
B
Ho
C
Ki67
D
Sox10
E
Iba1
Ho
F
Iba1 positive cells/section
45
40
35
30
25
20
15
10
5
0
Control
p38α null

Figure 6


A
Control
p38α null
B
A' Control
B' p38α null
C
Axon growth (mm from crush site)
1.6
1.2
0.8
0.4
0
Control
p38α null
D
Functional loss
0
-20
-40
-60
-80
p38α null
Control
0
3
7
10
12
14
17
19
21
Days post-crush injury
E Control
F p38α null
G
G-ratio
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
Axon diameter (μm)
Control
p38α null
Linear control
Linear p38α null
H
G-ratio
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Cont.
p38αnull
I
Axon diameter (μm)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Cont.
p38αnull