

# The Characterisation of Pax3 Expressant Cells in Adult Peripheral Nerve

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## Abstract

Pax3 has numerous integral functions in embryonic tissue morphogenesis and knowledge of its complex function in cells of adult tissue continues to unfold. Across a variety of adult tissue lineages, the role of Pax3 is principally linked to maintenance of the tissue's resident stem/progenitor cell population. In adult peripheral nerves, Pax3 is reported to be expressed in nonmyelinating Schwann cells, however, little is known about the purpose of this expression. Based on the evidence of the role of Pax3 in other adult tissue stem and progenitor cells, it was hypothesised that the cells in adult peripheral nerve that express Pax3 may be peripheral glioblasts. Here, methods have been developed for identification and visualisation of Pax3 expressant cells in normal 60 day old mouse peripheral nerve that allowed morphological and phenotypic distinctions to be made between Pax3 expressing cells and other nonmyelinating Schwann cells. The distinctions described provide compelling support for a resident glioblast population in adult mouse peripheral nerve.

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## Introduction

The *paired-box homeotic gene 3* (*PAX3*) encodes the PAX3 transcription factor that is known to regulate transcriptional activation or repression of a broad spectrum of downstream genes in a variety of developmental pathways. *PAX3* functions after embryogenesis relate to regulatory roles in the ontogeny of stem cells throughout the postnatal lifespan of the organism. The roles of *PAX3/Pax3* are well defined across a variety of adult tissue lineages [1–12]. From these studies, it can be concluded that the overarching purpose for continued expression of *PAX3/Pax3* in adult tissues is primarily for maintenance of a progenitor cell population. In adult progenitor cells it is said that *PAX3/Pax3* protects the 'stemness' of the cell through regulation of downstream target genes involved in the maintenance of an undifferentiated phenotype and in its absence, cells acquire the characteristics of a mature cell [13].

*Pax3* is known to be expressed in a characteristic, temporal pattern in Schwann cells of the developing peripheral nervous system [14]. Kioussi and colleagues [14] report that *Pax3* RNA is associated with nonmyelinating Schwann cells (NMSCs) of 30 day old mouse sciatic nerve; this report of continued *Pax3* expression in adult cells of neural crest origin was thought intriguing. Therefore, investigations focused on determination of the *Pax3* mRNA transcripts and double-labeling of Pax3 with other early immature Schwann cell markers in normal 60 day old mouse sciatic nerve and results demonstrate that cells that express Pax3 are characterised by a peripheral glioblast phenotype.

## Results

### *Pax3* mRNA Transcripts are Expressed in 60 Day Old Mouse Sciatic Nerve

There are conflicting reports about the expression of *PAX3/Pax3* in Schwann cells of adult peripheral nerve [14–16]; so, the initial aim of the investigations was to report the *Pax3* mRNA transcripts in normal mouse sciatic nerve. To identify all possible *Mus musculus* mRNA transcripts, the mouse genome sequence available on the NCBI was interrogated for all possible splice sites. Three mouse transcripts have been sequenced to date; *Pax3c* and *Pax3d* are expressed in embryonic cells of the myogenic and melanogenic lineages [17] and *Pax3β*, which encodes a transcriptionally inactive isoform, is expressed in embryonic myogenic precursors [18]. Barber et al. [17] have reported a *Pax3f* transcript, expressed in the embryonic day 9.5 mice and although exact sequence data is unavailable, it is thought that the transcript is generated by splicing exon 5 directly to exon 9 using the known splice donor and acceptor sequences.

To delineate whether the production of additional mouse transcripts of *Pax3* is possible, a comparison of human and mouse nucleotide sequences was undertaken using the NCBI BLAST database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to search for mouse consensus donor and acceptor splice sequences contained within the *Pax3* locus. The amino acid sequences from 197–215 of human PAX3a or 197–206 of PAX3b are not homologous to those of mouse Pax3 [19,20] and there is no record of a *Mus musculus* transcript *Pax3a* or *Pax3b*. The mouse *Pax3* gene shows a lack of consensus splice site elements required for production of homologous *Pax3e*, *Pax3g* and *Pax3h* transcripts as those produced

in humans; moreover, the mouse *Pax3* genomic sequence diverges from the human gene in the 3' region from which these transcripts are produced and shows less than 70% homology to the human sequence (Murine clone RP24-529B23 Chromosome 1).

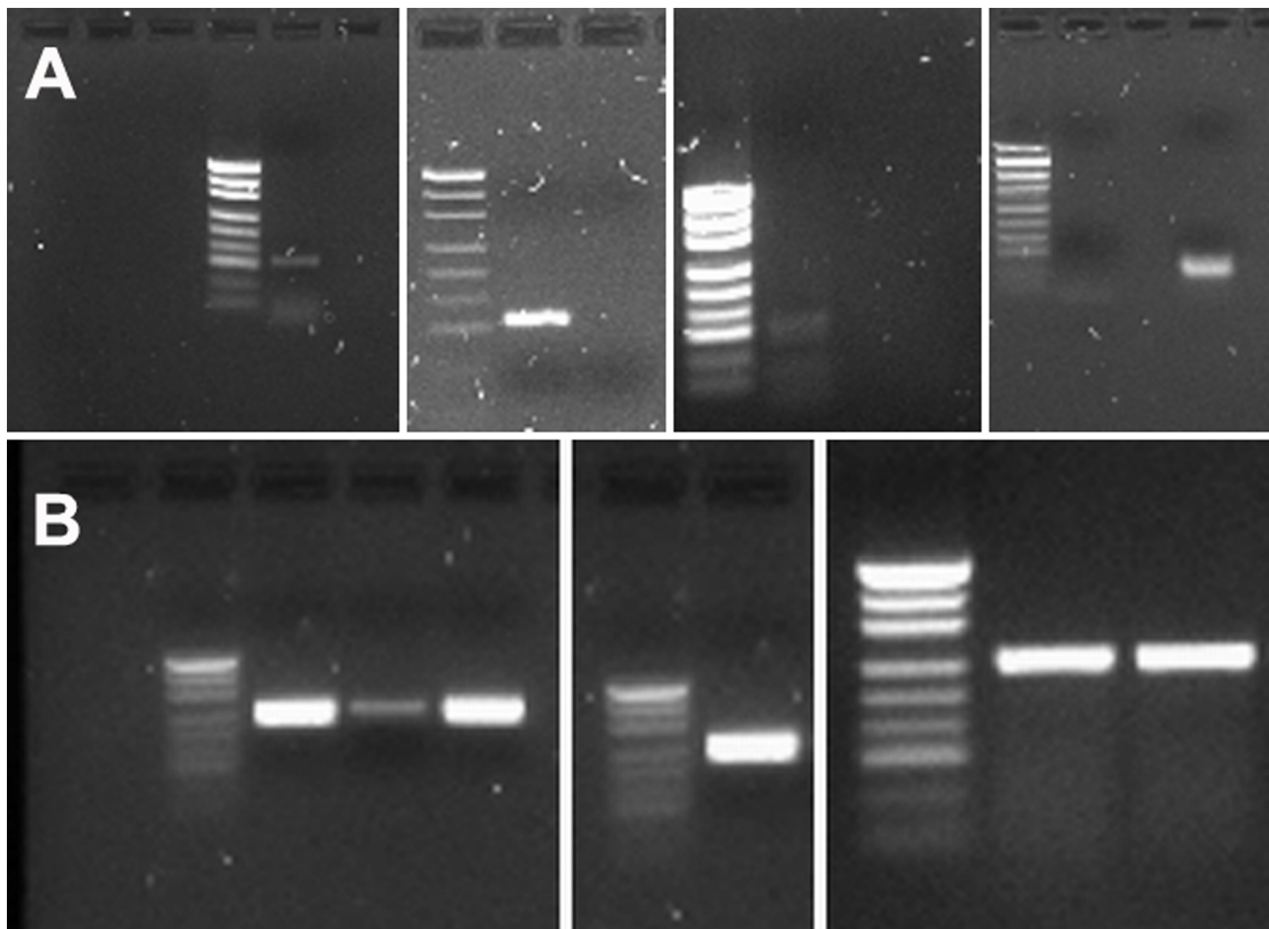
As such, specific primers were designed to amplify the mRNA of mouse *Pax3c*, *Pax3d*, *Pax3f* and *Pax3g* transcripts. RT-PCR results confirmed that 2 alternate *Pax3* mRNA transcripts were expressed in 60 day old mouse sciatic nerve (n = 6). *Pax3c* or *Pax3d* transcripts were detectable in 4/6 individual nerves, although co-expression of both transcripts was not observed. In 2/6 nerves analysed, *Pax3* mRNA was undetectable. In all nerves tested, PCR amplification of *Pax3g* and *Pax3f* mRNA products were undetectable (Fig. 1).

### The Morphology of 60 Day Old Mouse NMSCs of Sciatic Nerve

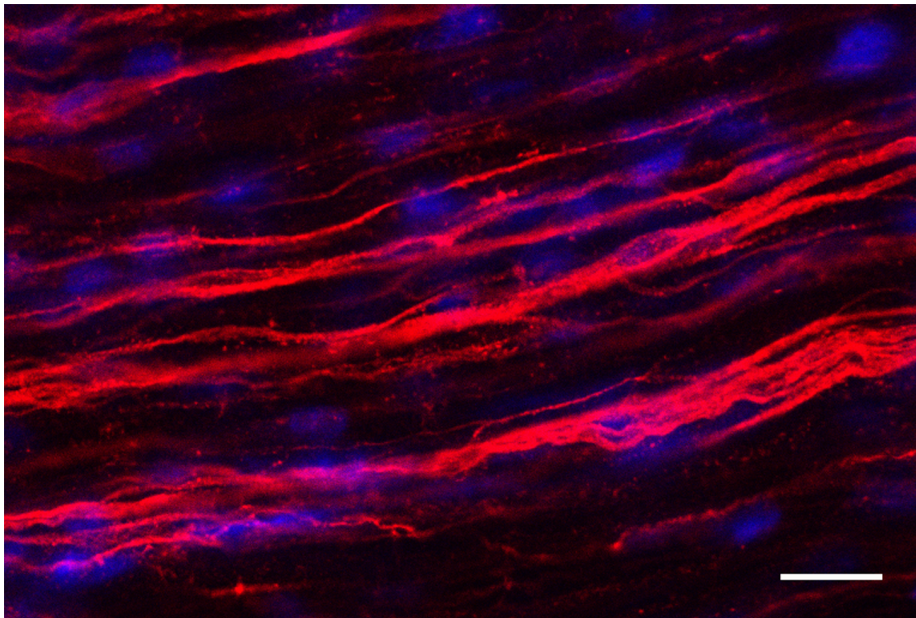
In the adult peripheral nervous system, C-fibre neurons are not myelinated and are organised into a bundle in which many nerve fibres are ensheathed by one NMSC for conduction of peripheral afferent signals. NMSCs have a characteristic morphology that consists of long branching networks of cytoplasmic processes which coalesce in a plexiform manner with adjacent nonmyelinated bundles [21,22]. To date, the morphology of mouse NMSCs that make up nonmyelinated bundles have been loosely characterised.

In this study, mouse NMSCs were clearly visualised by fluorescence microscopy of teased sciatic nerve fibre specimens (data not shown); the morphology of the cell is stated as 2–4  $\mu\text{m}$  in diameter across the cytoplasmic extensions and 4–5  $\mu\text{m}$  in diameter across the nuclear region. The length of the cell is between 80–200  $\mu\text{m}$  and the nucleus is between 12–20  $\mu\text{m}$  in length. The nucleus of the NMSC is centrally located (as opposed to the peripheral location of the nucleus of a myelinating Schwann cell) and the nonmyelinated C-fibres that traverse longitudinally across the NMSC nucleus form it into a characteristic 'cigar' or spindle shape such as has been described for rat NMSCs [23].

It is known that nonmyelinated bundles contain two classes of C-fibres, those dependent on nerve growth factor (NGF) that express low-affinity nerve growth factor receptor (p75<sup>Ngfr</sup>) and those dependent on glial-derived neurotrophic factor that express glial-derived neurotrophic factor family receptor- $\alpha 1$  [22]. It is also known that p75<sup>Ngfr</sup> is expressed on the NMSC plasmalemma adjacent to a NGF-dependent C-fibre it ensheathes [23]; therefore, p75<sup>Ngfr</sup> was chosen to label NMSCs associated with NGF-dependent C-fibres. Here, the plexiform nature of nonmyelinated NGF-dependent C-fibres, described by Carlsen, Behse [21] in human and Murinson et al. [22] in rat, was seen in 60 day old mouse by the immunofluorescence labeling (Fig. 2).



**Figure 1. *Pax3* transcripts are expressed in normal adult mouse peripheral nerve.** Gel electrophoresis of PCR amplification products of *Pax3* transcripts from normal mouse sciatic nerves. All PCR products are compared to pUC DNA ladder. **A)** *Pax3c* (117 bp) was expressed in 3/6 nerves tested while *Pax3d* (97 bp) was expressed in 1/6 nerves tested. *Pax3* products were not amplified in 2/6 nerves tested. **B)** Positive controls for relative amounts of *Gapdh* product amplified from the total RNA of the six nerve lysates are shown. doi:10.1371/journal.pone.0059184.g001



**Figure 2. The complex structure of nonmyelinated nerve bundles in normal adult mouse peripheral nerve.** A micrograph of a whole mount sciatic nerve fascicle preparation labeled with p75Ngfr (red) reveals the plexiform comingling and exchange of NGF-dependent C-fibers between adjacent nonmyelinated bundles. Cell nuclei are visualised using Hoechst DNA dye (blue). Images were acquired using scanning laser confocal microscopy. Optical plane = 1.5  $\mu\text{m}$ . Pinhole aperture = 3.0. Scale bar represents 20  $\mu\text{m}$ . doi:10.1371/journal.pone.0059184.g002

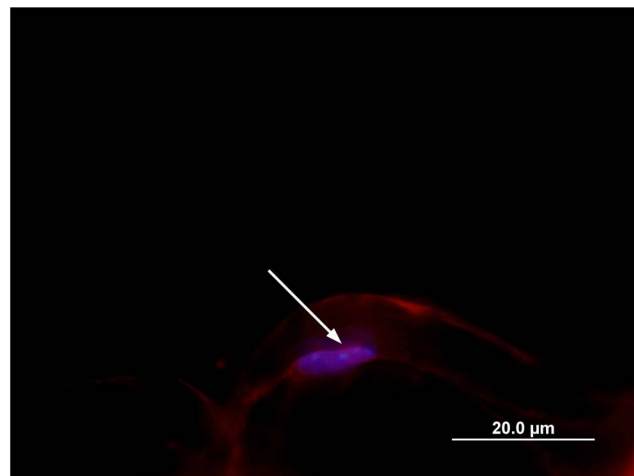
### Characterisation of Cells that Express Pax3 in Normal Adult Mouse Sciatic Nerve

RT-PCR results verified that *Pax3c* and *Pax3d* transcripts were present in 60 day old mouse sciatic nerve (Fig. 1), thus, it remained to confirm the presence of the proteins encoded by these transcripts in the mouse tissue. A mouse monoclonal IgG2a isotype-specific antibody directed at amino acids that form the transactivation domain of the Pax3 protein [24] was employed and when used with the isotype-specific anti-mouse IgG2a secondary antibody, non-specific background staining of endogenous mouse tissue IgGs and other components was minimised.

Development of the Pax3 immunofluorescent labeling method commenced using frozen sections of mouse sciatic nerve pre- and/or postfixed with 4% w/v paraformaldehyde (PFA) and a secondary indirect immunofluorescence procedure. In both tangential and longitudinal sections, a nuclear Pax3 label was undetectable. As indicated by the RT-PCR results, Pax3 expression levels were expected to be relatively low, thus, a tertiary (avidin/biotin) indirect immunofluorescence procedure was also performed on the frozen sections. When this method was analysed, levels of non-specific background staining were high and a nuclear Pax3 label remained undetectable (data not shown). Next, individual 2 mm lengths of fascicles from adult mouse sciatic nerve were pre- and/or postfixed in PFA and teased into individual Schwann cell/axons and indirect immunofluorescence methods were tested for Pax3 labeling. Various tissue permeabilisation protocols were also assessed as to their effects on cellular and extracellular integrity, nonspecific staining and intensity of immunofluorescent Pax3 label. Results showed that all methods trialed had a Pax3 label of low intensity and, in many specimens, Schwann cell structure was not optimal (data not shown). Positive control samples were processed during each of the teased fibre immunolabeling experiments using Krox24, a transcription factor reported expressed in Schwann cells of adult peripheral nerve [25,26]. In the positive control samples, nuclei that expressed Krox24 were

clearly distinguishable and strongly immunofluorescent (Fig. 3). It was thus concluded that the PFA fixation method was linked to difficulties associated with the lack of optimal Pax3 immunofluorescent labeling in both the frozen and teased fibre samples prepared.

An alternate method, consisting of a short post-fixation of dried whole mount sciatic nerve fascicles with 4°C acetone was subsequently found to preserve both tissue morphology and Pax3 antigenicity, therefore, a Pax3 labeling procedure was developed using this method of fixation and analysed using



**Figure 3. Krox24 immunofluorescence in adult mouse peripheral nerve.** A sciatic nerve fascicle post-fixed for 2 hours in PFA was labeled with an antibody targeted at the Krox24 transcription factor. In this micrograph, a myelinating Schwann cell nucleus (indicated by the arrow) shows Krox24 positivity. doi:10.1371/journal.pone.0059184.g003

scanning laser confocal microscopy. Results showed strong Pax3 immunoreactivity in cell nuclei randomly distributed along the length of the 60 day old sciatic nerve trunk. In the whole mount mouse sciatic nerve fascicle specimens analysed, relatively 2% of cell nuclei were positive for Pax3 when compared to the total number of Hoechst stained nuclei visible along the length of the nerve (Fig. 4D, 5B); in the nerve fascicle preparations examined (approximately 2 mm length) there was an average of 9 cells per specimen that showed Pax3 positivity. Notably, the Pax3 expressing cells did not have a characteristic NMSC morphology. The shape of the nucleus was distinctly oval or round and colocalisation with p75Ngfr revealed that Pax3+ cells lacked p75Ngfr labeled bipolar cytoplasmic extensions (Fig. 5). In the three nerve preparations examined for colocalisation of Pax3 and p75Ngfr, 100% of the Pax3+ cells (n = 28) co-expressed p75Ngfr in the nucleus (Fig. 4B). It was evident that relatively 98% of Schwann cells associated with NGF-dependent C-fibres did not express Pax3.

It was hypothesised that cells of adult nerve that express Pax3 would co-express peripheral glioblast markers, therefore colocalisation studies were performed using antibodies against Pax3 and a marker of neural crest cells, transcription factor SRY-related high-mobility group box-2 (Sox2). To date, Sox2 expression has been thought limited to embryonic glioblasts [27]; however, it is detected here for the first time in mouse sciatic nerve of 60 day old animals. Across the three Pax3 and Sox2 double-labeled whole mount sciatic nerve fascicles examined, 100% of cells that had nuclear Pax3 expression also had nuclear Sox2 expression (n = 22) (Fig. 6) and again, cells that had Pax3/Sox2 positivity were approximately 2% of the total cells imaged.

## Discussion

This study was primarily concerned with the expression of a developmental transcription factor, Pax3, in adult mouse peripheral nerve. No studies have discussed Pax3 protein expression in normal adult mouse NMSCs and thus it was compelling to investigate and contemplate the implications of Pax3 expression, as previously reported, knowing the roles of Pax3/Pax3 across other adult lineages such as melanocyte and skeletal muscle. It is important to note that initial development of the methods were based on an assumption that all of the P60 NMSC of peripheral nerve would express Pax3/Pax3, as it had been reported previously that the expression in P30 nerves was linked to inhibition of key myelination genes [14]. Our findings, which refute this, were tested using a variety of rigorously optimised methods. Taken together, the immunofluorescent labeling and RT-PCR results support one of the key findings of the study, namely, that Pax3 expression is demonstrated in only 2% of adult NMSCs. That Pax3 does not have a continuing role in the inhibition of myelination genes within adult NMSCs after P30 is supported by reports that cell fate specification to the myelinating phenotype occurs between E16 and birth and is principally linked to associated nerve fibre caliber and dose-dependent secretion of axonal factors [28–30].

The Pax3 expression pattern seen in adult peripheral nerve is similar to that seen in adult skeletal muscle where Pax3/7 expressing stem (satellite) cells account for up to 4% of the total myonuclei [31]. That Pax3 transcripts were below the level of detection in some of the nerves tested here by RT-PCR, was confirmed by the fact that approximately 2% of cells within the nerve trunk express Pax3 at age 60 days. These findings are in agreement with other studies which failed to label Pax3/Pax3 in adult peripheral nerve [15,16].

## Pax3 Expressing Cells in Adult Mouse Peripheral Nerve Co-express Stem Cell Markers

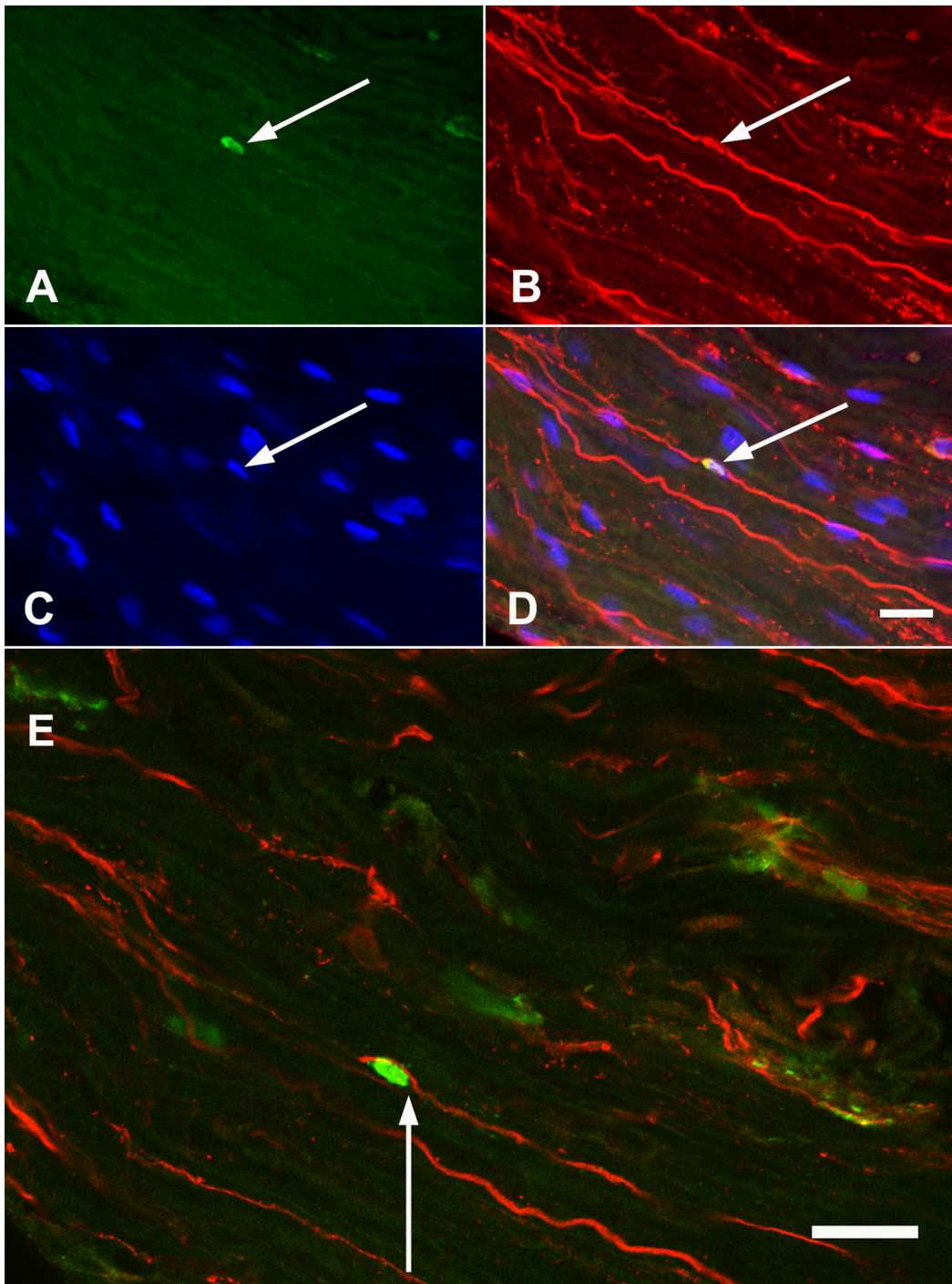
Based on the evidence of the role of Pax3 in other adult tissue stem and progenitor cells, and taken together with evidence that a population of cells exist in adult peripheral nerve that express Pax3 [14], it was hypothesised that these Pax3+ cells may be peripheral glioblasts. Therefore, aims of the investigations continued to focused on identification, visualisation and initial characterisation of the cells of adult mouse peripheral nerve that express Pax3. The most significant finding is a population of cells that co-express Pax3, Sox2 and p75Ngfr have been identified. These proteins are commonly expressed in multipotent cells in a variety of tissues [32–35] and while the role of Pax3 in Schwann cells remains largely undetermined, its overarching role in other tissues is maintenance of progenitor cells across the life span [9]. In Schwann cells, Sox2 has been shown to increase responsiveness to proliferative stimuli, prevent myelin gene expression and inhibit differentiation [27,36]. SOX2 is one of the four Yamanaka factors, or genes whose expression is artificially forced to induce non-pluripotent adult somatic cells into pluripotent stem cells (iPSCs) *in vitro*. In the progress toward clinical application of iPSCs, both SOX2 and PAX3 have key roles in the generation, identification and maintenance of patient-specific iPSCs *in vitro* [37–39]. The demonstration of the novel subset of cells described in these investigations, although preliminary, is thought initial, sound evidence of the existence of peripheral glioblasts in adult mouse peripheral nerve.

## Pax3 Expressing Cells are Distinct from Other NMSCs of Adult Peripheral Nerve

Labeling procedures allowed several distinctions to be made between Pax3 expressing cells and other NMSCs. Firstly, it was seen that the Pax3+ cells co-expressed p75Ngfr in the nucleus but lacked p75Ngfr+ bipolar cytoplasmic extensions. While it is possible that Pax3 expressant cells may associate with glial-derived neurotrophic factor dependent C-fibres (future studies should address this question), the p75Ngfr nuclear expression pattern would be curious; on the other hand, the p75Ngfr/Pax3+ nuclear expression is consistent with that of a peripheral glioblast [40] and is similar to denervated Schwann cells *in vitro* which when released from axonal contact upregulate p75Ngfr and secrete nerve growth factor for autocrine survival [41].

The morphologic and phenotypic differences of the Pax3 expressing cells described here lend credence to the theory that peripheral glioblasts may be retained in peripheral nerve after birth [42,43]. Transition from the embryonic Schwann precursor to the ‘committed’, or immature Schwann phenotype progresses at embryonic day 12 in the mouse, at which time changes are associated with the establishment of an autocrine survival circuit. Where precursor Schwann undergo apoptosis in the absence of axonal trophic support, immature Schwann cells survive via autocrine secretion of growth factors such as neurotrophin-3, a ligand of p75Ngfr; at this stage, the fated peripheral glioblasts express Pax3, p75Ngfr and Sox2 and are capable of self-survival [29,44–50]. Thus, it may be theorised that a population of these cells are retained into adulthood and may be the origin of the Pax3/Sox2/p75Ngfr expressant cells described here.

Finally, in the mouse after birth, a subset of Schwann cells that associate with C-fibres differentiate toward a nonmyelinating phenotype, re-establish dependency on paracrine signaling for survival [51] and form the peripheral nonmyelinated nerve bundles. Of note is the finding that 60 day old NMSCs that form the nonmyelinated bundles associated with NGF-dependent C-

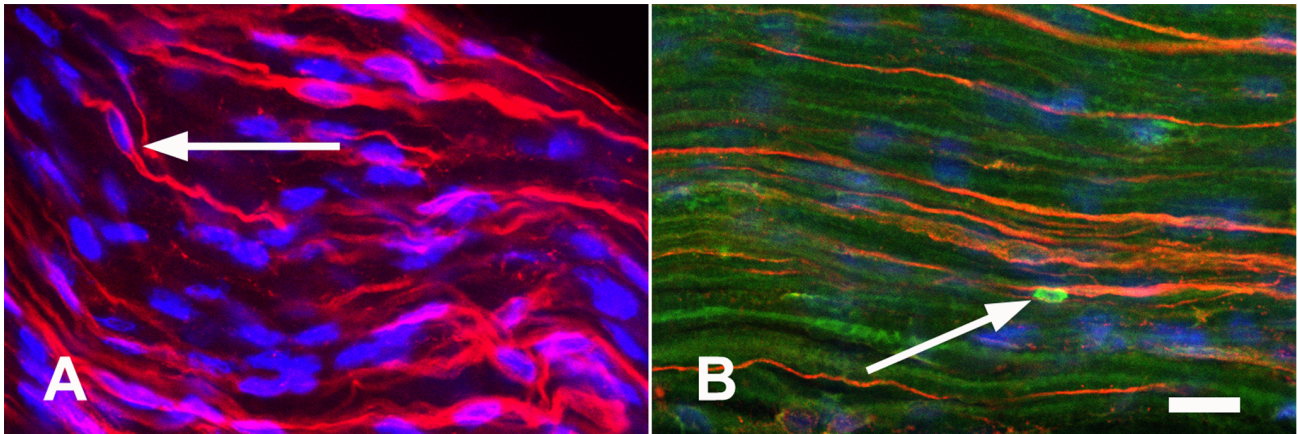


**Figure 4. p75Ngfr and Pax3 co-localisation in normal adult mouse peripheral nerve.** **A)** The Pax3 expressing cell (indicated by the arrow) is oval and has nuclear expression of p75Ngfr (**B**). **D)** The merged images of Pax3 (**A**), p75Ngfr (**B**) and Hoechst DNA dye (**C**) immunofluorescence. **E)** The Pax3+ nucleus indicated (green) was imaged on a different focal plane than **A-D** such that it could be more clearly evident that it does not have p75Ngfr cytoplasmic extensions (arrow). Images were acquired using scanning laser confocal microscopy. *Optical plane = 1.5  $\mu$ m. Pinhole aperture = 3.5. Scale bar represents 20  $\mu$ m.*  
doi:10.1371/journal.pone.0059184.g004

fibres did not express Pax3, which indicates that expression is down-regulated from postnatal day 30 when Kiousi et al. [14] last report its expression. Thus, Pax3 appears to have a temporal postnatal role in the suppression of myelination genes in a large subset of NMSCs much the same as it does in myelinating Schwann cells [14].

#### Prospective Roles for Resident Glioblasts in Adult Peripheral Nerve

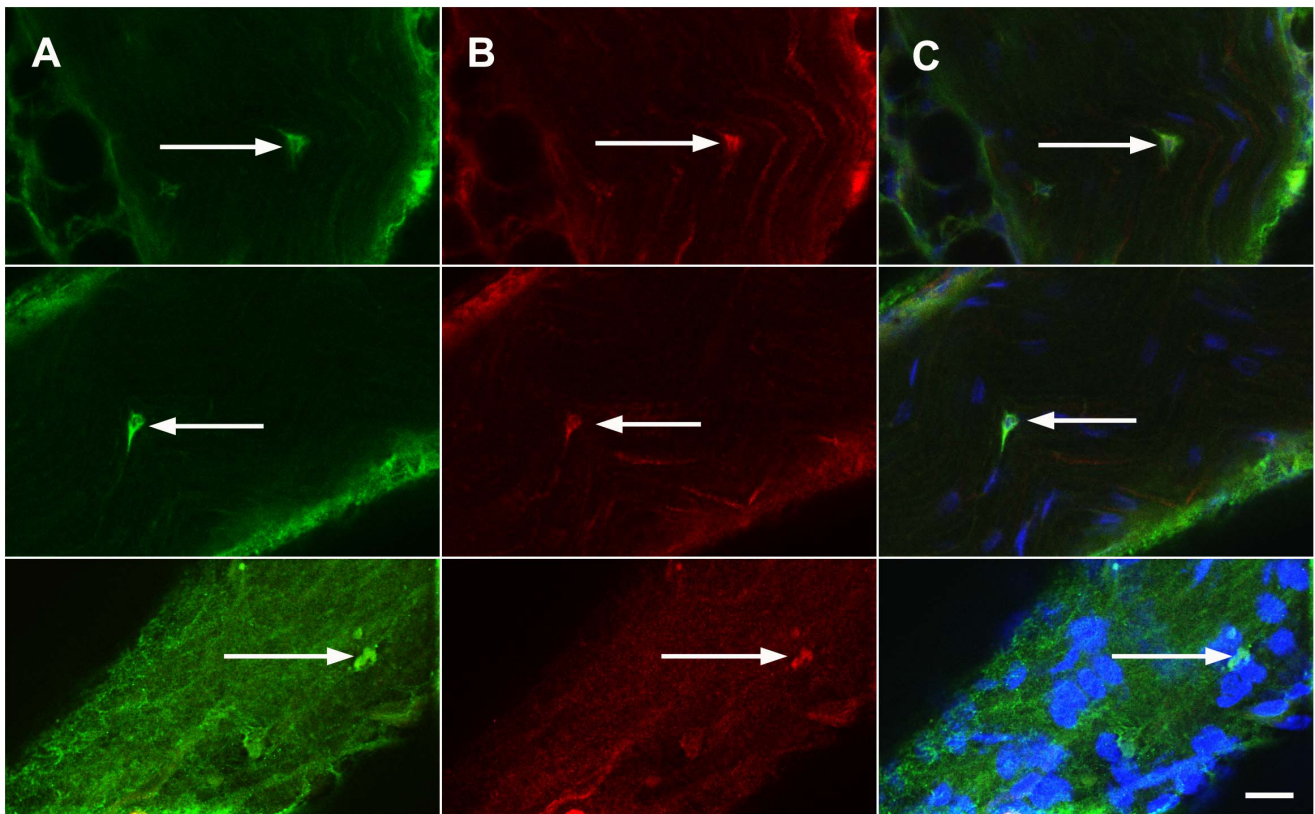
Peripheral nerve injuries, in which the nerve trunk is severed, result in separation of the axon from the nucleus and a subsequent inflammatory response called Wallerian degeneration. A funda-



**Figure 5. Distinctions between Pax3 expressant cells and the NMSC associated with NGF-dependent C-fibres.** **A)** The cell indicated by the arrow has the characteristic morphology of the NMSCs associated with NGF-dependent C-fibres. Note the spindle shaped nucleus (blue) and p75+ cytoplasmic extensions (red). **B)** The cell indicated by the arrow typifies the morphology of the Pax3+ cells imaged in the study. The nucleus is oval and there are no associated p75+ cytoplasmic extensions apparent. doi:10.1371/journal.pone.0059184.g005

mental characteristic of Wallerian degeneration is the reported plasticity of adult myelinating Schwann cells to revert from the myelinogenic transcriptional program (or differentiated state) into the cell cycle and back [52]. Briefly, myelinating Schwann cell nuclei enter the DNA synthesis phase while the myelin sheath is

relatively intact [53], although re-entry into the cell cycle represents a commitment to demyelination [54]. In the distal stump of the transected nerve, cells discard degraded myelin into cytoplasmic ovoids and initialise autophagocytosis of myelin proteins and lipids [55–58] while concomitant inhibition of genes



**Figure 6. Transcription factors Pax3 and Sox2 co-localise in the nuclei of cells of adult mouse peripheral nerve.** Whole mount sciatic nerve fascicles co-immunolabeled with Pax3 (green), Sox2 (red) and Hoechst DNA dye (blue) revealed that Pax3 expressant nuclei co-express stem cell marker Sox2 (indicated by the arrows). Images were acquired using scanning laser confocal microscopy. *Optical Plane* = 1.5  $\mu$ m. *Pinhole aperture* = 3.5. Scale bar represents 20  $\mu$ m. doi:10.1371/journal.pone.0059184.g006

encoding for myelin proteins occurs [59–62]. *Pax3* is upregulated at this stage followed by induction of the characteristic immature Schwann cell markers [14]. These immature Schwann cells proliferate within the persisting basal lamina [63,64] which provides a pathway that proximally regenerating axons use to reach the original target tissue [55,65–68]. As Schwann cells begin to produce and store myelin for remyelination of regenerating axons, *Pax3* levels temporally peak; by contrast, as myelination nears completion, *Pax3* is re-silenced [14]. While theories for the function of *Pax3* in myelinated nerve regeneration include prevention of premature myelogenesis and/or orchestration of Schwann progeny migration, development of efficacious methods for identification and visualisation of *Pax3* expressing cells in adult peripheral nerve, a primary result of this work, will facilitate future studies of the role of *Pax3* in myelinated peripheral nerve regeneration.

Here, the question arises as to why the peripheral nerve trunk would harbour resident glioblasts when myelinating Schwann cells have the capability to de-differentiate. Several suppositions come to mind. Resident glioblasts would proliferate (initially) at a greater rate than a lost myelinating cell, which must extrude and degrade myelin debris before it undergoes proliferation and thus resident glioblasts could be an early source of regenerative Schwann cells. In support of this notion is a study by Griffin et al [69] in which paranodal de-myelination was induced (the myelinated Schwann cells were not lost) and results demonstrated that supernumerary Schwann cells had migrated through the endoneurium to overlie areas of paranodal de-myelination. The origin of the supernumerary Schwann cells remain unknown, however, the authors suggest that they may have arisen from adjacent nonmyelinated bundles as the supernumerary cells appeared situated outside the basal lamina of the damaged myelinated Schwann cells.

Furthermore, there are very few studies outlining the mechanisms of nonmyelinated nerve regeneration and it is possible that resident glioblasts may have a role along these lines. Support for this comes from two studies. Neurofibromatosis Type 1 (NF1) is characterised by loss of the *NF1* gene that encodes neurofibromin [27,70,71] and persons affected are predisposed to develop benign peripheral nerve sheath tumours (or neurofibromas), myeloid leukemia, hyperpigmentation of the skin and learning disabilities [72–74]; moreover, persons with a loss of heterozygosity of *NF1* alleles develop malignant peripheral nerve sheath tumours [75]. Neurofibromas consist primarily of NMSCs [75–77] where malignant transformation is linked to NMSC hyperproliferation and detrimental effects on adjacent cells [78]. Importantly, in neurofibroma, it has been found that tumours have a significant population of stem cells [79] and although the origin of the stem cells remains unknown, Pongpudpunth et al [79] proposed that “formation of neurofibromas may be linked to alterations in the self-renewal program of peripheral nerve progenitor cells”.

Finally, a recent study has demonstrated a population of *Pax3*+ immature Schwann cells in the cutaneous nerve plexus of the dermis [80]; interestingly, these cells are associated with the unmyelinated nerves of the hair follicle [80,81]. Based on an understanding of the prominent structural plasticity of the innervation of the hair follicle during hair cycle changes, it is possible that the *Pax3*+ Schwann cells are resident glioblasts that have a role in the regeneration of unmyelinated nerves during each hair cycle. The *Pax3*-GFP model holds much potential for the future study of both the *Pax3*+ Schwann cells described by Djian-Zaouche et al [80] and the further characterisation of the *Pax3*+ cells described here.

## Conclusion

In 2008, Griffin and Thompson stated that “the possibility of a population of Schwann cell precursors in adult nerves is largely unexplored” [54]. The current investigations were intended to build on the previous work of others who showed that *Pax3*, a classic stem/progenitor cell marker, is detected in adult peripheral nerve trunk. Importantly, our results provide compelling evidence for the existence of glioblasts in adult mouse peripheral nerve and may support the long held theory that embryonic glioblasts are retained in adult peripheral nerve much the same as occurs in other adult *Pax3*-regulated tissue lineages. Thus, studies are ongoing to further characterise the subset of adult peripheral glial cells described here.

Methods were also developed and described that allowed the visualisation and further characterisation of NMSCs that associate with NGF-dependent C-fibres of normal 60 day old mouse peripheral nerve. To date, neurological studies of this kind have been performed on larger animals such as frog, rat, cat and dog. The intricate and complex morphological characteristics of mouse NMSCs described here, along with the procedures for imaging these cells, provides a foundation for further studies of NMSCs in mouse and may be particularly useful for studies using transgenic animals.

## Materials and Methods

### Animals

Experimental procedures were carried out in accordance with the provisions of the National Health and Medical Research Council Australian Code for Responsible Conduct of Research (2007), the Australian code of practice for the care and use of animals for scientific purposes (2004) and the Animal Welfare Act (2002). Experimentation was approved by the Edith Cowan University Animal Ethics Committee (project approval code 06-A7 ZIMAN). The age of the animals was chosen to reflect the cellular makeup of adult or mature tissue. All the investigations described were undertaken using 60 day old male mice that were provided by the Animal Resources Centre (Canning Vale, Western Australia).

### Isolation of RNA from Sciatic Nerve Specimens

Mice were sacrificed by CO<sub>2</sub> narcosis at 20%/min v/v and the sciatic nerves were rapidly excised in an aseptic field. Nerves were dissected and ligated under a Leica Zoom 2000 dissecting microscope, with care taken to remove connective fascia from the epineurium. Freshly removed nerve tissue was immediately frozen by immersion in liquid nitrogen and stored at –80°C until further use. Total RNA was isolated from single sciatic nerves using TriReagent (Molecular Research Center, Inc.) and homogenisation with a glass-col mortar and pestle. For each extraction, RNA purity and concentration were assessed using a Bioanalyzer (Agilent).

### RT-PCR

First strand cDNA was synthesised from 2 µg of isolated RNA using an OmniScript system (Qiagen) and an oligo(dT)<sub>18</sub> primer (10 µM) (Qiagen). Reverse transcription was carried out at 37°C for 1 hour in a total volume of 20 µl. Negative controls included reactions without Omniscript reverse transcriptase. PCR amplifications were performed using a TaqDNA Polymerase Kit (Qiagen) and a negative control (without template DNA) was included in every experiment. The PCR reaction was conducted with the following oligonucleotides, designed using OligoAnalyser 3.1 (Integrated DNA Technologies) and Primer-BLAST (NCBI):

*Pax3c*: (F) 5'-ACCAGGCATGGATTTTCAAG;  
 (R) 5'-AACGTCCAAGGCTTACTTTG.  
*Pax3d*: (F) 5'-CCTCAGGTAATGGGACTTCT;  
 (R) 5'-AATGAAAGGCACCTTTGTCCA.  
*Pax38*: (F) 5'-CTGTGTCCAGATCCCAGCA;  
 (R) 5'-GAGATAATGAAAGGCACCTGAG.  
*Pax3f*: (F) 5'-CAGATGAAGGCTCCGATATTGAC;  
 (R) 5'-CTGGCTTGAGATAATGAAAGGC.

Internal controls for cDNA were performed using PCR amplification of mouse housekeeping gene *Gapdh* and the primers used were as follows:

*Gapdh*: (F) 5'-GTGAAGGTCGGTGTGAACG;  
 (R) 5'-ATTTGATGTTAGTGGGGTCTCG.

Positive controls for primers were performed using total RNA isolated from embryonic day 11 mice and PCR negative controls eliminated cDNA as primer template from each PCR reaction. PCR products were resolved on 1.5% w/v agarose gels and visualised under UV light using a Geldoc system. PCR products were sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems) and an ABI Prism 3730 48 capillary sequencer. Sequences were aligned with known sequences in GenBank using the multiAlign tool in Angis, available on GenBank.

### Preparation of Frozen Nerve Sections

To prepare fresh frozen sections of sciatic nerve, animals were sacrificed by cervical dislocation. The sciatic nerves were surgically excised, immersed in Tissue Tek O.C.T. (Sakura Finetek Europe) and frozen in liquid nitrogen cooled N-methyl butane (Sigma). Tissue blocks were cryosectioned at 9  $\mu$ m onto SuperFrost slides (Menzel-Gläser), dried and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PFA) for 30 minutes. Sections were washed in phosphate buffered saline (PBS) 3 times for 5 minutes prior to subsequent processing or storage at  $-80^{\circ}\text{C}$ . To prepare pre-fixed frozen sections, animals were anaesthetised with Nembutal (Abbott) and transcardially perfused through the left ventricle; a constant flow (10 mL/min) of PBS (10 mL) followed by ice cold PFA in 0.1 M phosphate buffer at pH 7.4 (50 mL) was established using a peristaltic pump. Sciatic nerves were surgically excised, post-fixed in PFA for 6 hours before immersion in 30% sucrose for 48 hours. Tissues were rinsed in PBS, immersed in Tissue Tek O.C.T. and frozen in liquid nitrogen cooled N-methyl butane prior to cryosectioning at 9  $\mu$ m onto SuperFrost slides (Menzel-Gläser). Slides were dried prior to processing or storage at  $-80^{\circ}\text{C}$ .

### Preparation of Teased Nerve Specimens

To prepare pre-fixed teased nerves, animals were anaesthetised with 75  $\mu\text{g/g}$  Nembutal (Abbott) and transcardially perfused through the left ventricle; a constant flow (10 mL/min) of PBS (10 mL) followed by ice cold paraformaldehyde (PFA) in 0.1 M phosphate buffer at pH 7.4 (50 mL) was established using a peristaltic pump. Sciatic nerves were immediately excised and postfixed in 4% w/v PFA for 18 hours at  $4^{\circ}\text{C}$ . Nerves were rinsed in PBS and prepared onto chilled polylysine slides (Menzel-Gläser) where fascicles were cut into 2 mm lengths and individual fibres were teased apart along the length by 0.2 mm entomology pins. Preparations were dried for 18 hours before immunohistochemical processing or storage at  $-80^{\circ}\text{C}$ .

### Preparation of Whole Mount Nerve Fascicle Specimens

Whole mount preparations were prepared using freshly excised nerves which were obtained from animals sacrificed using  $\text{CO}_2$  narcosis. Sciatic nerves were excised and prepared on chilled

polylysine slides where fascicles were separated and cut into 2 mm segments and mounted by the epineurium. Slides were dried overnight, post-fixed in acetone for 10 minutes at  $-20^{\circ}\text{C}$  and rinsed in PBS at pH 7.4, before immunohistochemical processing or storage at  $-80^{\circ}\text{C}$ .

### Antibodies Used for Immunofluorescence

Primary antibodies used were mouse monoclonal IgG2a anti-quail Pax3 (1:10; Developmental Studies Hybridoma Bank); rabbit polyclonal anti-mouse Krox24 (1:250; Aviva Systems Biology); rabbit polyclonal anti-mouse Sox2 (1:200; Sapphire Bioscience) and rabbit polyclonal anti-mouse p75 nerve growth factor receptor (1:500; Chemicon). Species specific secondary antibodies used were AlexaFluor488-conjugated to goat anti-mouse IgG2a (1:500; Molecular Probes) and AlexaFluor546-conjugated to goat anti-rabbit IgG (1:500; Molecular Probes).

### Procedure for Immunofluorescent Staining of Whole Mount Nerve Fascicles

Slides were rehydrated in Tris buffered saline (TBS) and permeabilised in 0.01% v/v Triton X100 (for 45 minutes at  $25^{\circ}\text{C}$ ). Slides were washed in TBS 3 times for 10 minutes each prior to incubation in 10% v/v normal goat serum for 6 hours at  $25^{\circ}\text{C}$ . Primary antibodies were individually or simultaneously incubated with 0.2% v/v TritonX100 for 18 hours at  $4^{\circ}\text{C}$ . Specimens were washed in 0.05% v/v TBS/Tween20, 6 times for 30 minutes each, using gentle agitation. Secondary antibody incubation was done thereafter at  $25^{\circ}\text{C}$  for 20 minutes. Specimens were washed in TBS/Tween20, 6 times for 30 minutes using gentle agitation where the last wash contained Hoechst DNA dye 33342 (1 ng/ml). Coverslips were mounted with FluorSave medium (Calbiochem). Negative controls were processed at the same time but were not incubated with primary or secondary antibody.

### Microscopy

Fluorescently labeled tissues were viewed with an Olympus BX51 microscope connected to an Olympus DP71 digital camera and digital images were collected in the Olympus analySIS FIVE program and transferred to the IrfanView (4.27) program for montage construction. The contrast and brightness of these images were unaltered. Whole mount specimens were imaged with a BioRad MRC 1000/1024 UV laser scanning confocal microscope on a Nikon Diaphot 300 with either a 40X objective (with zoom) or 60X immersion objective (without zoom) using a 351- and 488-nanometer argon laser and a 543-nanometer helium/neon laser. Gain and black level adjustments were performed to improve analogue to digital signal conversion and background noise was eliminated using a KALMAN filter. Z-stacks were collected using various step-sizes and KALMAN averaging was performed manually for each step. Digital images were collected and compiled in greyscale and subsequently pseudocoloured with hues approximate to the fluorescence emission spectra of the respective fluorophores using the Confocal Assistant<sup>TM</sup> (4.02) program. Images were transferred to Adobe Photoshop (7.0) and IrfanView (4.27) programs for montage construction. The images were unaltered.

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## Author Contributions

Conceived and designed the experiments: MRZ JAB. Performed the experiments: MRZ JAB. Analyzed the data: MRZ JAB. Contributed reagents/materials/analysis tools: MRZ JAB. Wrote the paper: MRZ JAB.

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