UNIVERSITY OF CALIFORNIA SAN DIEGO

Low-density lipoprotein receptor-related protein 1 (LRP1) expression is decreased in a model of Schwann cell differentiation *in vitro* and during early developmental myelination *in vivo*.

A Thesis submitted in partial satisfaction of the requirements for the degree of Master of Science

in

Biology

by

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The Thesis of Katherine Michelle Fichter is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

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University of California San Diego

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Dedication

For my parents Arthur Fichter and Patricia Keil, whose unconditional love, admiration, and support made my education possible. Thank you for never quenching my dreams, even when I wanted to be a professional fiction author. I love you both more than words can describe.

I also give warm thanks to David Cypert, who introduced me to coffee when he accidentally bought me a latte instead of tea. My coffee drinking got me through many final exams, tedious writing sessions (both personal and academic), and gave me an expensive, pretentious hobby to discuss at parties.

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List of Abbreviations

α_2M*: Activated alpha 2-Macroglobulin

ERK1/2: Extracellular signal-related kinases ½

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

IGF-1: Insulin-like growth factor-1

LDL: Low-density lipoprotein

LRP1: Low-density lipoprotein receptor-related protein-1

MAG: Myelin-associated glycoprotein

MBP: Myelin basic protein

MEF: Murine embryonic fibroblast

MMP-9: Matrix metalloproteinase-9

MV: Myelin vesicle

NMDA-R: *N*-methyl-*D*-aspartate receptor

P_0: Myelin protein zero

P0, P7, and P29: Postnatal developmental days 0, 7, and 29

$p75^{NTR}$: p75 neurotrophin receptor

PI3K/Akt: Phosphatidylinositol 3-kinase/protein kinase B

RAP: Receptor-associated protein

SC: Schwann cell

***scLrp1*:** Schwann cell *Lrp1*

TNF-α: Tumor necrosis factor-alpha

UPR: Unfolded protein response

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Acknowledgements

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Abstract of the Thesis

Low-density lipoprotein receptor-related protein 1 (LRP1) expression is decreased in a model of Schwann cell differentiation *in vitro* and during early developmental myelination *in vivo*.

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Master of Science in Biology

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Professor Wendy M. Campana, Chair
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During early postnatal development in rats, Schwann cells form myelin and insulate large-diameter axons to promote saltatory conduction and maintain axonal integrity. After injury in adults, Schwann cells promote transcription of regeneration-associated genes and downregulate myelin program to generate repair Schwann cells. The LRP1 receptor is upregulated after injury and is important to the Schwann cell repair program, but the roles of LRP1 during developmental myelination are unknown. To establish the relationship between LRP1 and myelination, changes in LRP1 gene expression were analyzed over the course of development and in myelinating Schwann cells *in vitro*. An *in vitro* model of developmental myelination was established from primary rat Schwann cells, as confirmed by upregulation of the

myelin marker proteins MBP and P_0 and downregulation of $p75^{NTR}$. LRP1 expression was downregulated after differentiation *in vitro*. Differentiation altered activation of Schwann cell signaling pathways associated with development and nerve injury. Phosphoprotein analysis showed differentiation reduced activation of c-Jun, increased activation of Akt, and did not change activation of ERK1/2. Differentiation also reduced activation of ERK1/2 signaling in response to NRG1 and the LRP1 ligand α_2M*, and furthermore impaired the ability of Schwann cells to internalize myelin debris by phagocytosis. Together, these results indicate that LRP1 expression is inversely associated with myelination, is expressed by non-myelinating Schwann cells, and reduction of LRP1 altered Schwann cell signaling. Given these data, we hypothesize that LRP1 may instead be important to non-myelinating Schwann cell during development and possibly facilitate removal of myelin debris after nerve injury.

Introduction

10.1 Development and anatomy of the peripheral nerve

10.1.1 Overview of the peripheral nerve

Peripheral nerves are structures that conduct information to and from the central nervous system (CNS). They contain the axons of both sensory and motor neurons, which originate in the dorsal root ganglion (DRG) and ventral horn, respectively (Purves et al., 2001). Axons, connective tissue, blood vessels, and glial cells are organized into structures called fascicles (Figure 1), and groups of fascicles form nerves (Jessen et al., 2015; Menorca et al., 2013).

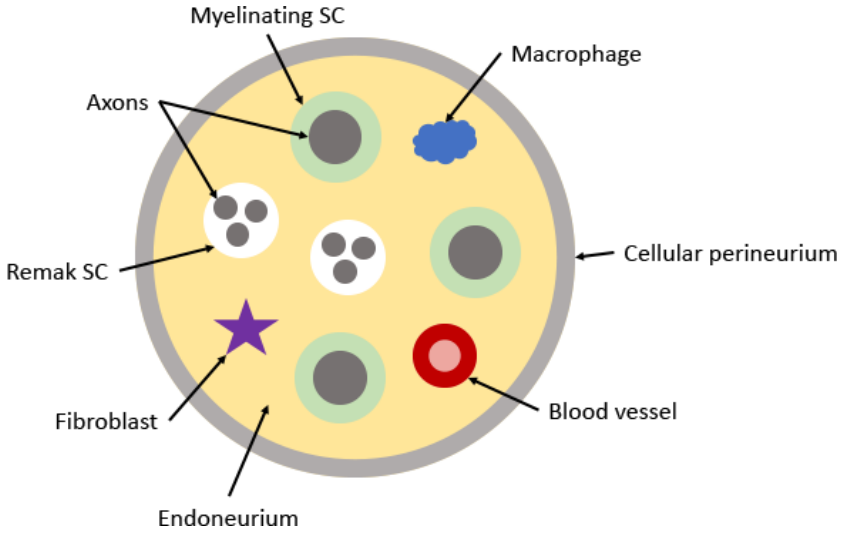

Figure 1. Diagram of an adult peripheral nerve fascicle. Adapted from (Jessen et al., 2015)

Non-neuronal cells in the peripheral nerve include fibroblasts, macrophages, and Schwann cells (SCs). Endoneurial fibroblasts are derived from Schwann cell precursors and secrete the extracellular matrix protein collagen (Jessen & Mirsky, 2005; Joseph et al., 2004), while macrophages are immune cells that originate from blood monocytes and perform phagocytosis (Epelman et al., 2014; van Furth & Cohn, 1968). Myelinating SCs ensheathe large-diameter axons to form the cholesterol-rich membranes of the myelin sheath, which insulates and promotes efficient signal propagation along axons (Jessen et al., 2015; Saher & Simons, 2010).

Non-myelinating SCs form Remak bundles and are also present in the peripheral nerve. The development of SCs into these mature phenotypes is discussed below. We will discuss how cells in the peripheral nerve support development, respond to injury, and promote regeneration.

10.1.2 Origins, development, and functions of Schwann cells

During early embryonic development, **Schwann cell precursors** (SCPs) are derived from **neural crest cells** (Jessen et al., 2015) (Figure 2). Neuregulin-1 (NRG1) signaling is required for gliogenesis and survival of SCPs (Birchmeier & Nave, 2008; Dong et al., 1995). NRG1 signaling required the ErbB3 receptor, and mice genetically deficient for ErbB3 did not develop SCPs. Furthermore, a lack of SCPs contributed to neuronal death in the late stages of embryonic development (Riethmacher et al., 1997), suggesting that neurons also rely on SCPs.

By the late stages of embryonic development, SCPs in normal embryos differentiate into **immature SCs** (Figure 2). Immature SCs were shown to generate autocrine circuits that allowed them to survive in the absence of axons *in vitro* (Meier et al., 1999). Additionally, proliferation of immature SCs was induced by cAMP elevation (Arthur-Farraj et al., 2011; Mogha et al., 2013). Following birth and through postnatal development, immature SCs differentiate into two mature SC phenotypes, myelinating and non-myelinating SCs (Figure 2).

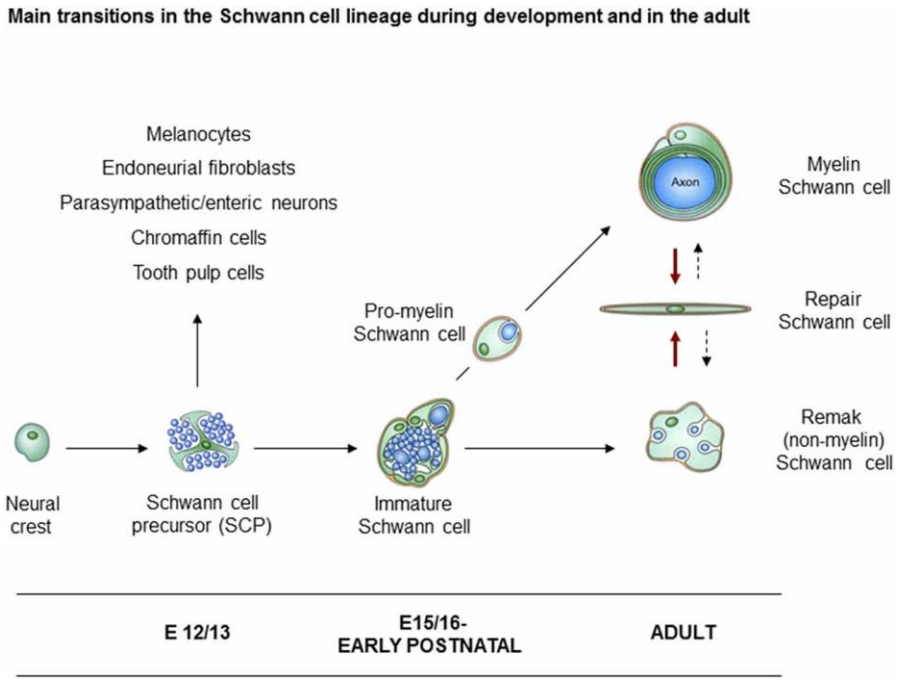

Figure 2. Main transitions in the Schwann cell lineage. From (Jessen & Mirsky, 2019)

While cAMP elevation was shown to induce SC proliferation, high levels of cAMP elevation promoted differentiation into the **myelinating phenotype** instead (Jessen et al., 1991). Neuregulin signaling is also required for developmental myelination. At developmental day 11, ErbB3/4 mutant mice had hypomyelinated sciatic nerves and died shortly thereafter (Brinkmann et al., 2008). Another requirement for developmental myelination is the expression of the transcription factor Krox20; sustained expression of Krox20 is required to maintain the myelin sheath (Decker et al., 2006; Topilko et al., 1994).

Myelinating SCs are highly structured and have both radial and longitudinal polarization (Özçelik et al., 2010). Several layers of myelin membranes are compacted between the outer (abaxonal) and inner (adaxonal) membrane surfaces (Pereira et al., 2012; Salzer, 2015). These membranes are enriched with cholesterol and contain 70% lipid by weight. Additionally, PNS myelin contains the membrane-bound **myelin basic protein** (MBP) and **myelin protein 0** (P_0) (Saher & Simons, 2010).

Myelinating SCs ensheathe large-diameter axons and promote signal propagation by saltatory conduction (Menorca et al., 2013). Compared to healthy control animals, axonal conduction velocity decreased in the sciatic nerves of genetically hypomyelinated mice (de Waegh & Brady, 1990). Furthermore, myelinating SCs are also required to provide trophic support to neurons. This may be because axons are almost completely ensheathed by myelin (Nave, 2010). Dysfunction of SC mitochondria was shown to cause progressive demyelination and subsequent development of peripheral neuropathy in mice (Viader et al., 2013).

Non-myelinating SCs, also known as Remak cells, surround groups of small-diameter axons and form Remak bundles. The level of NRG1 type III released by an axon is a developmental signal that determines whether axons become myelinated or ensheathed (Taveggia et al., 2005). The number of axons grouped into a Remak bundle is dependent on their distance from the spinal cord and the identity of the nerve to which they belong. For example, in Remak bundles in the sciatic nerve can contain over a dozen axons individually ensheathed by the cytoplasm of a single SC (Murinson & Griffin, 2004).

A single Remak bundle can also contain axons derived from multiple types of neurons (Murinson, Hoffman, Banihashemi, Meyer, & Griffin, 2005). However, electrophysiological studies performed on nonhuman primates demonstrated that individual nerve fibers contained both non-myelinated and myelinated regions (Griffin & Thompson, 2008; Peng, Ringkamp, Campbell, & Meyer, 1999), indicating that the developmental processes that determine nerve structure are complex. Furthermore, studies performed on mice showed that normal development of Remak bundle structure and myelination required the gene *Lrp1* (Orita et al., 2013) (see Overview of LRP1). While less is known about Remak cells than myelinating SCs, they have

also been shown to provide metabolic support to axons (Beirowski et al., 2014; Viader et al., 2011).

10.2 Wallerian degeneration after peripheral nerve injury and mechanisms of regeneration

10.2.1 Overview of peripheral nerve injury

Peripheral nerve injury is common, particularly in young men. Most cases are caused by vehicle accidents or trauma (Kouyoumdjian, 2006). While the peripheral nerve is capable of regeneration after injury (see Regeneration of the peripheral nerve after injury), a prolonged period of denervation is associated with poor functional recovery (Fu & Gordon, 1995b, 1995a). Surgical reconstruction aids regeneration by rejoining segments of the injured nerve, therefore decreasing the distance axons are required to regenerate. When reconstruction was delayed, SCs in sectioned peripheral nerves of rats deactivated and experienced increased death. Immediate reconstruction of the nerve promoted axonal outgrowth and protected SCs from deactivation (Tsuda, Kanje, & Dahlin, 2011). Unfortunately, one longitudinal study done on humans found that surgery only facilitated functional recovery in ½ of cases (Kallio & Vastamäki, 1993). Furthermore, nerve injury may lead to **neuropathic pain** (Colloca et al., 2017), a condition that affects an estimated 10-25% of the adult population worldwide (Goldberg & McGee, 2011). Factors that have been reviewed to impact a patient's capacity for regeneration are complicated and are based on the patient's age, type of injury received, and how long reconstruction is delayed after injury (Maripuu et al., 2012). Given these data, the scope and consequences of peripheral nerve injury are great. Both timely reconstruction of peripheral nerves after injury and

interventions designed to improve the nerve's capacity for regeneration may be needed to address the problem of peripheral nerve injury.

10.2.2 Wallerian degeneration and the Schwann cell response to injury

Wallerian degeneration is the coordinated physiological response to nerve injury after which regeneration and functional recovery may occur (Rotshenker, 2011). A latent phase follows injury during which no gross physical changes to the nerve are observed (Waller, 1850). After the 2-day latent period, damaged axons distal to the injury site were shown to undergo degeneration in mice (Beirowski et al., 2005).

Both non-myelinating and myelinating SCs in the distal stump transdifferentiate into the **repair phenotype**, which is specialized to promote regeneration after injury (Gomez-Sanchez et al., 2017; Jopling, Boue, & Belmonte, 2011). The transcription factor c-Jun is robustly activated in SCs after peripheral nerve injury and is a key regulator of transdifferentiation. However, other negative regulators of myelination such as Notch and Sox-2 are also hypothesized to have an important role in nerve repair (Jessen & Mirsky, 2008; Jessen et al., 2015). In SCs, sustained c-Jun activation prevents remyelination by inhibiting expression of the transcription factor Krox20 (Parkinson et al., 2008). Repair SCs provide guidance to regenerating axons, initiate the clearance of myelin debris, and recruit macrophages to the injury site.

Regenerating axons in the proximal stump form structures called **growth cones** and are guided toward the distal stump by SCs and fibroblasts. Repair SCs stimulate axonal growth by producing nerve growth factor (NGF) and by forming bands of Bungner (regeneration tracks) to guide axons toward distal targets (Arthur-Farraj et al., 2012; Heumann et al., 1987). Fibroblasts were shown to generate signals to which SCs respond and subsequently reorganize into

regeneration tracks (Parrinello et al., 2010). Another transcription factor, STAT3, has been shown to protect repair SCs from apoptosis and is required for SC autocrine survival signaling, which is important because SCs may experience prolonged denervation after injury (Benito et al., 2017). Chronic denervation of rat SCs reduced the expression of glial cell line-derived neurotrophic factor (GDNF) and resulted in persistent deficits in SCs' ability to express GDNF (Höke, Gordon, Zochodne, & Sulaiman, 2002), thereby impairing SCs from promoting regeneration after injury. Given these data, the timely and appropriate response of the peripheral nerve environment to injury is crucial for recovery.

Macrophages also play a role in Wallerian degeneration. Resident macrophages in the peripheral nerve became activated in response to injury (Mueller et al., 2003). Additionally, SCs were shown to recruit macrophages to the nerve by secreting leukemia inhibitory factor (LIF) and monocyte chemoattractant protein-1 (MCP-1) (Lutz et al., 2017; Tofaris et al., 2002). The primary function of macrophages is to remove both axonal and myelin debris from the nerve after injury (Hirata & Kawabuchi, 2002; Lutz et al., 2017; Rosenberg, Wolman, Franzini-Armstrong, & Granato, 2012). Clearance of myelin debris is crucial for regeneration because myelin-associated glycoprotein (MAG) has been shown to inhibit neurite outgrowth in adult DRG neurons (Mukhopadhyay, Doherty, Walsh, Crocker, & Filbin, 1994) (see Regeneration of the peripheral nerve after injury).

10.2.3 Mechanisms of myelin clearance by Schwann cells

After injury, SCs initiate the clearance of myelin debris from the peripheral nerve (Perry et al., 1995). Later, the second wave of myelin degradation requires SC recruitment of macrophages (Vargas et al., 2010). SCs use two mechanisms, autophagy and phagocytosis, to

internalize myelin debris. **Phagocytosis** is a process where large particles or debris are recognized and internalized by the cell, then degraded within acidic cellular compartments known as lysosomes (Richards & Endres, 2014; Rosales & Uribe-Querol, 2017). Approximately 4-6 days after injury, SCs use phagocytosis to internalize fragments of myelin membranes called ovoids (Lutz et al., 2017). **Autophagy** is a related process that involves self-degradation. The gene *Atg7* is required for SCs to perform autophagy of myelin debris (Gomez-Sanchez et al., 2015). Additionally, myelin degradation can be enhanced pharmacologically; the antibiotic rapamycin (RAPA) was shown to promote SC autophagy in rats after sciatic nerve transection (Ko et al., 2018).

10.2.4 Regeneration of the peripheral nerve after injury

Axons derived from the CNS demonstrate greater regenerative potential when transplanted into the PNS (David & Aguayo, 1981). This occurs because the CNS environment inhibits regeneration, but also because the PNS environment is better adapted to support it (reviewed by Huebner & Strittmatter, 2009). In the CNS, myelin-associated proteins such as MAG, Nogo-A, and oMgp were shown to inhibit neurite outgrowth (Chen et al., 2000; Kottis et al., 2002; McKerracher et al., 1994). While MAG also inhibited neurite outgrowth in the PNS (Mukhopadhyay et al., 1994), a higher capacity for regeneration in the PNS is possible because SCs and macrophages are more efficient at myelin clearance than oligodendrocytes and microglia (reviewed by Alizadeh, Dyck, & Karimi-Abdolrezaee, 2015).

Oligodendrocytes in the CNS required axons for survival cues and subsequently died after optic nerve transection (Barres et al., 1993). While remyelinating oligodendrocytes are derived from oligodendrocyte progenitor cells (OPCs) (Blakemore & Keirstead, 1999), OPC

differentiation is inhibited by the presence of myelin debris (Kotter, Li, Zhao, & Franklin, 2006; Plemel, Manesh, Sparling, & Tetzlaff, 2013). Inefficient clearance of myelin debris in the CNS therefore represents a challenge to both regeneration and remyelination. This is in contrast to the PNS, where although proliferation decreased after extended denervation, SCs were shown to survive and retain the capacity for remyelination after injury (Gordon, Wood, & Sulaiman, 2018).

10.3 Overview of LDL receptor-related protein-1 (LRP1) and functions in Schwann cells

10.3.1 Overview of LRP1

The low-density lipoprotein receptor-related protein-1 (LRP1) belongs to the LDL receptor family, a group of endocytic scavenger receptors (Canton et al., 2013; Fernandez-Castaneda et al., 2013; Herz & Strickland, 2001). The general structure of LRP1, including the receptor's modular domains and location of the α/β chains, is shown in Figure 3.

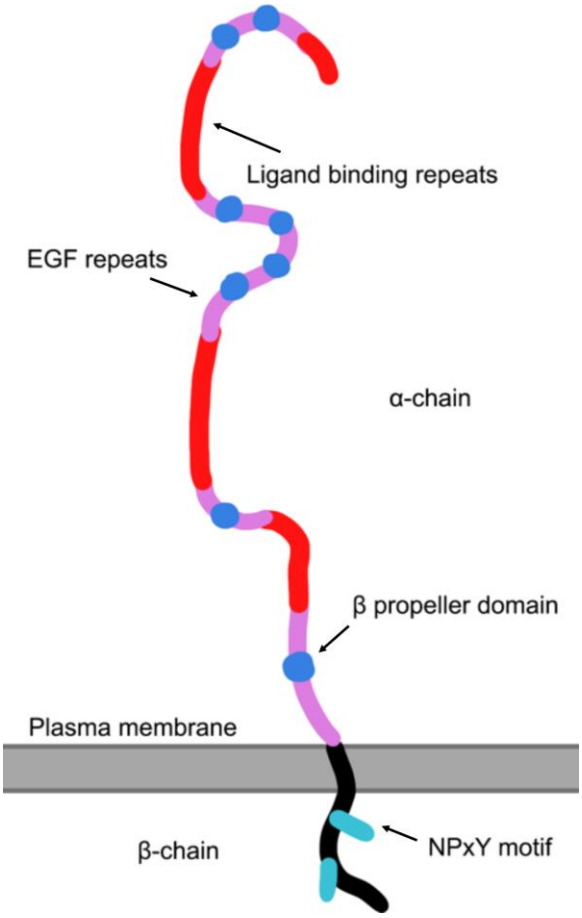

Figure 3. Structure and binding domains of LRP1. Adapted from (Lillis, Van Duyn, Murphy-Ullrich, & Strickland, 2008)

LRP1 is a transmembrane protein that contains two subunits, the α- and β-chains. The 85 kDa β-chain spans the plasma membrane and is bound to the 515 kDa α-chain, which lies outside of the cell (Herz et al., 1990). The LRP1 α-chain contains complement-like cysteine-rich repeats that act as ligand binding sites. Each LRP1 receptor contains multiple binding sites, and each binding site is capable of recognizing multiple ligands such as α_2-Macroglobulin (α_2M), tissue-type plasminogen activator (tPA), matrix metalloproteinase-9 (MMP-9), and the binding inhibitor receptor-associated protein (RAP) (Strickland et al., 2002; Willnow et al., 1994). NPxY motifs on the LRP1 β-chain bind to adaptor and scaffold proteins (Herz & Strickland, 2001) that allow LRP1 to participate in cell signaling (Flütsch et al., 2016; Mantuano, Lam, Shibayama,

Campana, & Gonias, 2015). Additionally, LRP1 is required for embryonic implantation (Herz et al., 1992).

10.3.2 Expression and general functions of LRP1

LRP1 expression in humans is ubiquitous and has been documented in the major organs of the reproductive, secretory, musculoskeletal, immune, and nervous systems (Figure 4). The wide expression of LRP1 explains its broad range of functions, including cholesterol metabolism, reproduction, and immuno-modulation (Brifault, Gilder, Laudati, Banki, & Gonias, 2017; Herz et al., 1992; Rohlmann, Gotthardt, Hammer, & Herz, 1998).

LRP1 is present in both the central and peripheral nervous systems. In the CNS, LRP1 is found in all cell types (Wolf, Lopes, VandenBerg, & Gonias, 1992), including neurons, astrocytes, oligodendrocytes, and microglia (Marzolo, von Bernhardi, Bu, & Inestrosa, 2000). In the PNS, LRP1 expression in SCs is upregulated after injury and LRP1 may play an important role in the SC response to injury (see Functions of LRP1 in Schwann cells).

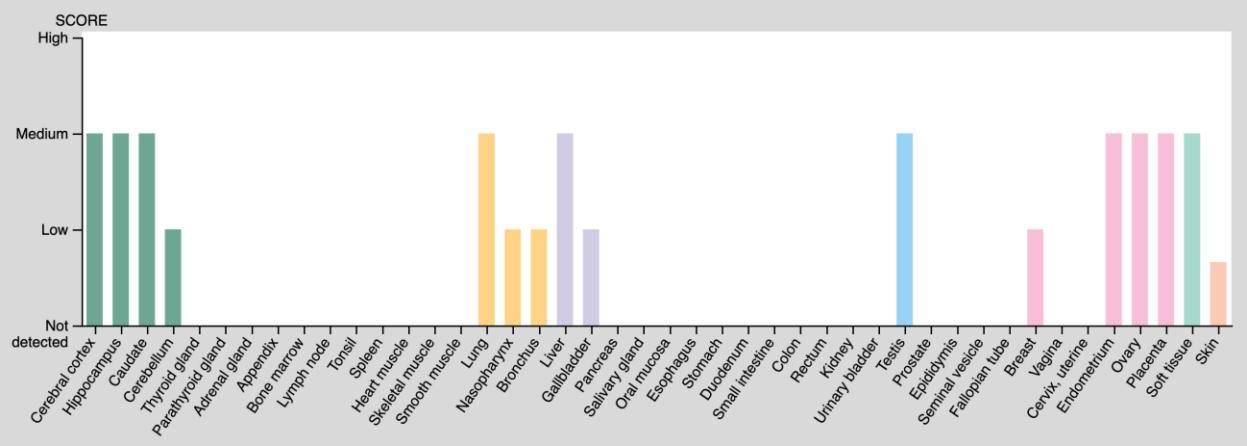

Figure 4. LRP1 protein expression overview by organ. From tissue expression of LRP1 – summary at the Human Protein Atlas; see (Uhlen et al., 2015)

10.3.3 Functions of LRP1 in Schwann cells

Signaling with co-receptors. Scaffold proteins connect the LRP1 β-chain to the *N*-methyl-*D*-aspartate receptor (NMDA-R) to form a co-receptor complex (Mantuano et al., 2015). Together, LRP1 and the NMDA-R detect and activate SC signaling in response to injury (Flütsch et al., 2016) and mediate the functions of LRP1 in SCs described below.

Survival. Denervated primary SCs were shown to promote survival through autocrine signaling (Meier et al., 1999). Because autocrine signaling requires communication between SCs, mechanisms that prevent apoptosis after injury may be important for long-term survival and regeneration. LRP1 expression increased in response to treatment with TNF-α and was shown to protect primary cultured rat SCs from apoptosis through activation of the PI3K/Akt signaling pathway (Campana et al., 2006). The unfolded protein response (UPR) promotes apoptosis by inhibiting activation of the PI3K pathway. LRP1 was shown to block the UPR in cultured mouse SCs (Mantuano et al., 2011). Additionally, trophic factors such as insulin-like growth factor-1 (IGF-1) and prosaposin bind LRP1 and activate the PI3K pathway to regulate SC survival *in vitro* (Campana, Darin, & O'Brien, 1999).

Migration. Furthermore, after injury SCs must guide regenerating axons toward the distal stump. LRP1 has been shown to play a role in primary SC migration *in vitro.* Matrix metalloproteinase-9 (MMP-9) binds to LRP1 with the hemopexin domain and activates signaling, which promoted migration of cultured SCs by regulating activity of the GTPases Rac1 and RhoA (Mantuano, Inoue, et al., 2008; Mantuano, Jo, Gonias, & Campana, 2010). The amino acid glutamate has also been shown to promote migration of SCs *in vitro* by a mechanism that uses LRP1 and the NMDA-R (Campana et al., 2017; Mantuano et al., 2015).

Injury. Previous work demonstrated that while axonal regeneration was accelerated in *Lrp1-/-* mice after injury, deletion of *Lrp1* caused abnormalities in the formation of new Remak bundles and the mice developed neuropathic pain (Poplawski et al., 2018). Even though LRP1 was not required for regeneration, LRP1 appears to regulate functions associated with the SC response to injury and the regeneration of normal peripheral nerve structure.

10.3.4 LRP1 is a potential phagocytosis receptor for myelin in Schwann cells

SCs gain the ability to perform phagocytosis after injury (Gomez-Sanchez et al., 2015). Increasing evidence points to LRP1 as a possible receptor for myelin phagocytosis. Gaultier and others (Gaultier et al., 2009) showed that MBP is an LRP1 ligand and that MBP binding was blocked by the addition of RAP *in vitro*. When the authors treated cultured oligodendrocytes with myelin vesicles (MVs), they observed internalization of MVs by phagocytosis. To show that LRP1 specifically mediated myelin phagocytosis, they used two approaches. One, they used RAP to prevent MVs from binding to LRP1 and showed that phagocytosis did not occur. Two, to confirm these results they also performed LRP1 gene silencing.

SCs have been shown to use receptors other than LRP1 to perform myelin phagocytosis. A second group used a measure of gene enrichment known as fragments per kilobase million (FPKM) to identify phagocytic receptors enriched in purified rat SCs at 0, 3, 5, and 7 days after sciatic nerve crush injury. At 5 days post-injury, Axl and Mertk were the most highly enriched phagocytosis receptors in SCs (Lutz et al., 2017). These data contradict previous studies that showed LRP1 expression in the sciatic nerve, specifically in SCs, increases after injury (Campana et al., 2006). These contradictory data may be due to discrepancies in the age of animals studied, the type of injury performed, and the timing of LRP1 expression. Because

clearance of myelin debris after injury is important for regeneration (Mukhopadhyay et al., 1994), it is important to identify the role of LRP1 in Schwann cell phagocytosis and whether induction of LRP1 expression is capable of enhancing myelin clearance.

10.4 Objectives

LRP1 expression increases in the peripheral nerve after injury and mediates functions of the SC repair program such as signaling, survival, and migration. However, the roles of LRP1 in SC development and myelination are not known. To understand the relationship between LRP1 expression and myelination, our objectives are to (1) produce a model of developmental myelination *in vitro*; (2) analyze LRP1 gene expression over the course of development *in vivo* and determine the relationship between myelination and LRP1 expression; and (3) apply our model to understand how changes in LRP1 expression alter SC behavior following differentiation.

LRP1 is known to be a phagocytic receptor for oligodendrocytes in the CNS, but some data suggest that LRP1 is not important for myelin phagocytosis by SCs. However, our laboratory previously showed that LRP1 is upregulated in SCs after injury. Due to this contradictory data, a role for LRP1 in myelin phagocytosis has yet to be tested experimentally. Because myelin clearance is important for regeneration, we aim to determine whether SCs use LRP1 for phagocytosis of myelin debris, and whether inducing LRP1 expression can enhance SCs' ability to internalize MVs.

Materials & Methods

11.1 Proteins and reagents

α_2-Macroglobulin (α_2M) was purified from human plasma by the method of Imber and Pizzo (Imber & Pizzo, 1981) and activated for binding to LRP1 (to form α_2M*) by reacting with 200 mM methylamine HCl as previously described (Gonias, Reynolds, & Pizzo, 1982). Modification of α_2M by methylamine was confirmed by demonstrating the characteristic increase in α_2M electrophoretic mobility by non-denaturing PAGE (Barrett, Brown, & Sayers, 1979). Bovine pituitary extract (BPE) and laminin were purchased from Sigma-Aldrich. Recombinant human neuregulin-1 (NRG1) was from R&D Systems. Forskolin was purchased from Cell Signaling Technology.

11.2 Schwann cell culture

Rat SCs were isolated from the sciatic nerves of 1-day old Sprague-Dawley rats. Primary SC cultures were purified of fibroblasts with Thy1.1 antibody (M7898; Sigma-Aldrich) and rabbit complement cytolysis (Sigma-Aldrich) as previously described (Campana et al. 1998). Primary cultures consisted of 98% pure SCs as confirmed by I.F. microscopy for S100β. SCs were cultured on 1 μg/μL Poly-*D*-Lysine (PDL)-coated plates and grown in complete medium consisting of low-glucose DMEM, 10% heat inactivated fetal bovine serum (HI-FBS), 1% Penicillin/Streptomycin, 4 μM forskolin, and 21 μg/mL BPE.

For differentiation, SCs were plated on PDL and 0.5 μg/mL laminin-coated flasks in the presence of complete medium. Cells were allowed to adhere and proliferate until they reached the appropriate confluency (70-90%) for each assay. Then, SCs were supplied differentiation medium (advanced DMEM/F-12, 2 mM L-glutamine, 18 μM forskolin, 4 μM NRG1, 1%

Penicillin/Streptomycin) or control medium (low-glucose DMEM, 10% HI-FBS, 1% Penicillin/Streptomycin) for 48 h.

11.3 Immunoblot analysis and phosphoprotein array

Schwann cells were extracted in RIPA buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with complete protease inhibitor cocktail and phosphatase inhibitor (Roche Diagnostics). Protein content was determined by bicinchoninic acid (BCA) assay (ThermoFisher Scientific).

For immunoblot analysis, 5 μg of total protein from each sample was loaded into a 10% Mini-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad) and subjected to gel electrophoresis at 100V for 1 h. Proteins were transferred to a 0.45 μm PVDF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). The membranes were blocked in TBST (Tris-buffered saline, 0.1% Tween-20) containing 5% non-fat milk for 1 h at RT and incubated overnight at 4 ºC with the following primary antibodies: anti-P_0 (ab31851; Abcam), anti-β-actin (3700, Cell Signaling Technology), anti-phospho-Akt (9271; Cell Signaling Technology), anti-phospho-ERK1/2 (4695, Cell Signaling Technology), anti-GAPDH (ab9485, Abcam), and anti-LRP1 β-chain (L2170, Sigma-Aldrich). After three washes in TBST, primary antibodies were detected with species-specific Horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology).

Phosphoprotein array was performed using the Proteome Profiler Human Phospho-Kinase Array kit (ARY003B, R&D Biosystems). Briefly, an equivalent amount of cellular protein (200 μg) was applied to nitrocellulose membranes coated with primary antibodies for the

detection of 43 phosphorylated proteins. Species-specific secondary antibodies coupled to HRP were used according to the manufacturer's instructions.

All membranes were revealed with ECL reagent ProSignal™ (Prometheus) and the Azure C300 Chemiluminescent Western Blot Imaging System (Azure Biosystems). Densitometry analysis was performed with ImageJ software.

11.4 Immunoblot analysis

After treatment, protein samples were lysed in RIPA buffer. Protein concentration was determined by BCA assay and 5 µg total protein from each sample was loaded into a 10% Mini-PROTEAN® TGX™ Precast Protein Gel (456-1034, Bio-Rad) and subjected to gel electrophoresis at 100V for 1 h. Proteins were transferred to a 0.45 µm PVDF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). The membranes were blocked in 5% non-fat milk (NFM) for 1 h and incubated overnight at 4 °C with the following primary antibodies: *anti-P_0* (ab31851; Abcam), *anti-β-actin* (3700, Cell Signaling Technology), *anti-phospho-Akt* (9271; Cell Signaling Technology), *anti-phospho-ERK1/2* (4695, Cell Signaling Technology), *anti-GAPDH* (ab9485, Abcam), and *anti-LRP1 β-chain* (L2170, Sigma-Aldrich). Primary antibodies were detected with a Horseradish peroxidase-conjugated species-specific antibody (Cell Signaling Technology). Conjugated antibodies were detected with ProSignal™ ECL reagent (Prometheus) and the Azure C300 Chemiluminescent Western Blot Imaging System (Azure Biosystems). Densitometry analysis was performed with ImageJ software.

11.5 Gene expression analysis

For gene expression analysis in cultured cells, Schwann cells were washed with PBS and total RNA was isolated and purified using the NucleoSpin® RNA kit (Macherey-Nagel). For analyzing gene expression during development, Sprague-Dawley rats at postnatal days 0, 7, and 29 were sacrificed by decapitation, sciatic nerves were collected, and the epineurium was removed. Nerve pieces were homogenized in Trizol reagent (ThermoFisher Scientific) and total RNA was extracted using the phenol-chloroform method (Chomczynski & Sacchi, 1987) and purified using the NucleoSpin® RNA kit (Macherey-Nagel).

RNA was then reverse transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad). RT-qPCR was performed with TaqMan™ gene expression products and the StepOnePlus Real-Time PCR System (Applied Biosystems) as previously described (Brifault et al., 2017). Relative changes in gene expression were calculated with the $2\text{-}\Delta\Delta C_T$ method using GAPDH mRNA as a normalizer. The following TaqMan™ primers were used: *GAPDH* (Rn99999916), *MBP* (Rn01399616), $p75^{NTR}$ (Rn00561634) and *LRP1* (Rn01503901).

11.6 Fluorescence microscopy

SCs were differentiated on Nunc™ Lab-Tek™ Chamber Slides (154526PK, ThermoFisher Scientific). After differentiation, cells were washed with PBS, fixed in 4% paraformaldehyde (PFA) and permeabilized in 0.1% Triton X-100 (Sigma-Aldrich). The cells were blocked in phosphate buffered saline (PBS) containing 5% normal donkey serum (NDS; Sigma-Aldrich) for 1 h at RT and incubated at 4 °C for 16 h in labeling buffer (PBS, 1% serum, 0.001% Triton X-100) with the following primary antibodies: *anti-*$p75^{NTR}$ (1:500; 07-476; EMD Millipore) and *anti-*P_0 (1:250; ab31851; Abcam). As a control, cells were incubated in labeling

buffer without primary antibodies. After washing with PBS, cells were incubated with a species-specific secondary antibody: Alexa Fluor® 594-conjugated donkey anti-rabbit IgG (1:500; R37119; Invitrogen) or Alexa Fluor® 488-conjugated donkey anti-mouse IgG (1:500; R37114, Invitrogen) for 1 h at RT. Slides were mounted using Fluoroshield Mounting Medium with DAPI (104139; Abcam) and visualized with a Leica DMi8 Inverted Fluorescent Microscope (Leica Biosystems).

11.7 Myelin vesicle purification and labeling

Myelin vesicles (MVs) were purified from the brain (CNS) and sciatic nerve (PNS) according to a modified protocol (Larocca & Norton, 2007). Adult male Sprague-Dawley rats were sacrificed by CO_2 inhalation and decapitation. Harvested sciatic nerves were homogenized in 0.32M sucrose solution with a battery-operated pestle motor mixer (Argos Technologies) or Dounce homogenizer (DWK Life Sciences) and passed through a syringe to ensure homogeneity. Myelin was recovered by sucrose gradient centrifugation and washed extensively in water. The myelin pellets were resuspended in 0.32M sucrose and layered over 0.85M sucrose in a 1:2 ratio before centrifugation at 32,000 rpm for 45 min. The purified myelin layers were collected from the interface and centrifuged again at 32,000 rpm for 20 min. The MVs were resuspended from the pellet in 50 μL of PBS (pH 8) for labeling. MVs were labeled with pHrodo™ Red succinimidyl ester (P36600, ThermoFisher Scientific) as described elsewhere (Hendrickx et al., 2014) to visualize internalization. pHrodo™ dye was dissolved in 1 mL of dimethyl sulfoxide (Sigma-Aldrich) for a final concentration of 1 mg/mL. The reconstituted dye was incubated with MVs for 45 min at RT on a rocking platform. The labeled MVs were centrifuged at 15,000 rpm for 30 min to remove excess dye and resuspended in PBS (pH 7.4) for

biological assays or PBS (pH 4) to determine the degree of fluorescence. Extracellular protein concentration was quantified by BCA assay. The purity of the preparation was analyzed by Coomassie Blue staining and immunoblot analysis for MBP (CNS) or P_0 (PNS) protein. Labeled MVs were used fresh or stored at -80 °C.

11.8 Phagocytosis assays

SCs were treated with 25 μg/mL of PNS myelin and incubated at 37 °C for 1 h to permit internalization and endosome acidification to occur. SCs were briefly washed with PBS and fixed in 4% PFA for 15 min at RT. To explore the phagocytic activity of SCs by immunofluorescence, cells were cultured in PDL- and laminin-coated chamber slides in the presence of control or differentiation medium. After 48 h, SCs were washed with PBS and fixed in 4% PFA. Slides were then mounted with Fluoroshield Mounting Medium with DAPI (104139; Abcam) and allowed to dry overnight. The slides were visualized using a Leica DMi8 Inverted Fluorescent Microscope (Leica Biosystems).

For flow cytometry experiments, SCs were cultured on 6-well plates in control and differentiation medium. After 48 h, cells were treated with PNS myelin as described above. Adherent SCs were detached from the culture plate(s) using 0.25% trypsin and trypsinization was stopped by adding a 10-fold volume of DMEM medium. After centrifugation, SCs were resuspended in PBS and subjected to flow cytometry analysis.

11.9 Statistical analysis

All results are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software Inc.). Comparisons between two groups were made

using two-tailed unpaired *t*-test or Mann-Whitney test when variances within groups were significant. Comparisons between more than two groups were performed using one-way ANOVA followed by Dunnett's *post-hoc* test to compare means to a control. For all tests, significance was set as $p<0.05$.

Results

12.1 Establishing myelinated primary rat Schwann cells *in vitro*

To establish a model for developmental myelination *in vitro*, we prepared primary rat SCs from neonatal rat pups and cultured them as previously described (Brockes, Fields, & Raff, 1979). Primary SCs were cultured in control or differentiation medium for 48 h. Following differentiation, SCs were observed by phase contrast microscopy. Non-differentiated SCs had a rounded morphology and were loosely organized, whereas differentiated SCs had an elongated, spindle-like morphology and formed a wave shape (Figure 5A) consistent with SCs grown on a laminin substrate (Weiner, Fukushima, Contos, Scherer, & Chun, 2001).

To determine whether differentiation induced myelination, we analyzed the expression of myelin protein zero (P_0) and p75 neurotrophin receptor ($p75^{NTR}$) proteins by immunofluorescence. We chose P_0 as a marker for myelination because it is abundantly expressed in PNS myelin (Saher & Simons, 2010), and $p75^{NTR}$ as a negative marker for myelination because it is known to be downregulated during myelination. In contrast to non-differentiated SCs, differentiated SCs upregulated P_0 and downregulated $p75^{NTR}$ as seen by immunofluorescence (Figure 5B).

To quantify the change in expression of $p75^{NTR}$ and myelin genes, we also analyzed the relative abundance of MBP and $p75^{NTR}$ mRNA by RT-qPCR. We used MBP as a secondary marker for myelination because it is one of the major proteins expressed in PNS myelin. Differentiated SCs expressed 79.4 ± 33.2-fold more MBP relative to non-differentiated SCs. In differentiated SCs, relative expression of $p75^{NTR}$ decreased 2.4 ± 0.1-fold (**p<0.05; **p<0.05; Figure 5C, D).

As an additional confirmation of myelin protein expression, we decided to quantify changes in protein expression and performed immunoblot studies to detect $p75^{NTR}$ and P_0 in non-differentiated versus differentiated SCs. Non-differentiated SCs expressed only a faint band at the expected molecular weight of P_0 (25 kDa), while expression of P_0 protein increased in differentiated SCs (Figure 5E). Densitometry analysis revealed a 7.8 ± 1.1-fold increase in P_0 in differentiated SCs compared to non-differentiated SCs (***$p<0.001$; Figure 5F). Together, these data suggest that differentiation downregulated proteins associated with development and repair, increased expression of myelin proteins, and therefore guided primary cultured SCs to a myelinating phenotype *in vitro*.

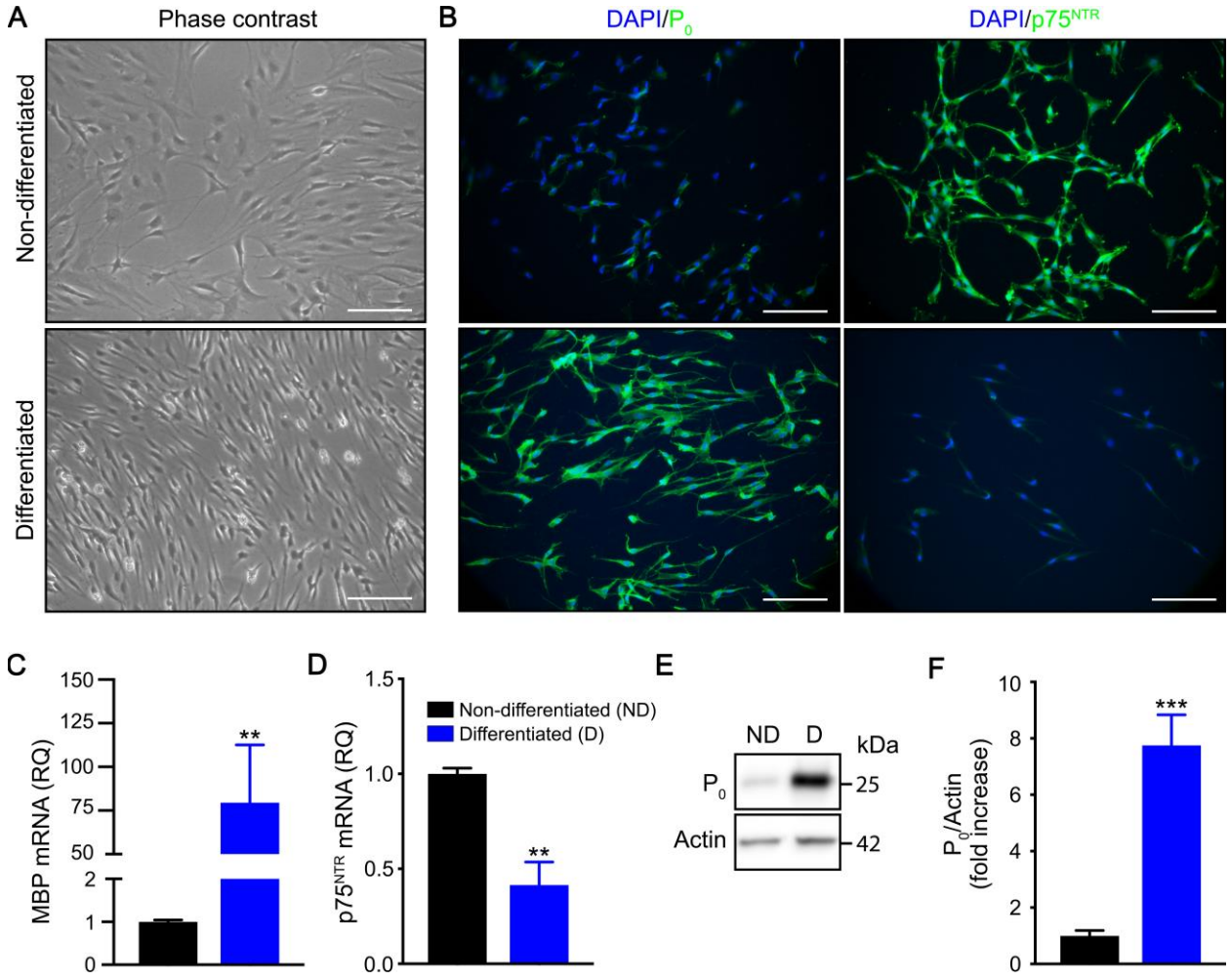

Figure 5. Primary rat Schwann cells are induced to a myelinating phenotype *in vitro*. Primary rat SCs were cultured for 48 h in control or differentiation medium to induce myelination. (**A**) Phase contrast microphotograph of non-differentiated (top panel) or differentiated (bottom panel) SCs. (**B**) Fluorescent imaging of non-differentiated and differentiated SCs. The middle panels show myelin protein zero (P_0, green) and the right panels show p75 neurotrophin receptor ($p75^{NTR}$, green). DAPI (blue) identifies SC nuclei. Scale bars: 100 μm. (**C, D**) RT-qPCR experiments were performed to quantify the relative abundance of (**C**) myelin basic protein (MBP) and (**D**) $p75^{NTR}$ mRNA (mean ± SEM; n=6; $^{**}p<0.05$, $^{**}p<0.05$, Mann-Whitney U-test). (**E**) Cell extracts were immunoblotted to detect P_0 protein. β-Actin was used as a loading control. (**F**) Densitometry analysis was performed to determine the relative level of P_0 protein standardized against the loading control (mean ± SEM; n=4; $^{***}p<0.001$, unpaired *t*-test).

12.2 Differentiation alters Schwann cell signaling pathways *in vitro*

Because signaling plays an important role in the SC response to injury (Campana et al., 2017; Flütsch et al., 2016; Mantuano et al., 2010), we wanted to understand whether or not the signaling pathways that are important for the SC repair program are activated in uninjured myelinating SCs. Primary rat SCs were placed in control or differentiation medium for 48 h. Following differentiation, non-differentiated and differentiated SCs were analyzed using the Phospho-Kinase Array Proteome Profiler kit. The 43 phosphorylated and 2 unphosphorylated total proteins analyzed by the array allowed us to quickly identify proteins that were most highly regulated in our model system.

The array revealed that 8 proteins appeared to be strongly regulated between non-differentiated and differentiated SCs, including phospho-Akt1/2/3, phospho-c-Jun, phospho-CREB, phospho-ERK1/2, phospho-GSK-3α/β, phospho-JNK1/2/3, phospho-STAT3, and phospho-WNK1 (Figure 6A). Densitometry analysis was performed to quantify the change in protein phosphorylation of the 8 regulated targets we identified. Protein phosphorylation increased following differentiation in all of the targets except for c-Jun, where phosphorylation decreased following differentiation (Figure 6B).

To confirm the results of our phospho-array, we analyzed phosphorylation of known SC signaling proteins by immunoblot: Akt, c-Jun, and ERK1/2 (Figure 6C).

Immunoblotting and densitometry analysis confirmed a 2.5 ± 0.3-fold increase of phospho-Akt in differentiated compared to non-differentiated SCs (*p<0.05; Figure 6C, D). The activation of Akt in differentiated SCs is consistent with the observation that the PI3K/Akt signaling pathway regulates myelin sheath thickness in the PNS during development (Domènech-Estévez et al., 2016).

We then decided to analyze c-Jun because it is robustly activated in SCs after injury, suppresses myelination, and activates expression of genes associated with the SC repair phenotype (Arthur-Farraj et al., 2012; Parkinson et al., 2008). As expected, densitometry analysis of the immunoblots for phospho-c-Jun revealed that levels of phospho-c-Jun were 5.6 ± 0.1-fold lower in differentiated cells as compared to non-differentiated cells (**$p<0.05$; Figure 6C, D).

Because extensive roles for the MAPK/ERK signaling pathway during the SC response to injury have been described (Harrisingh et al., 2004; Mantuano et al., 2010; Mantuano, Lam, & Gonias, 2013), we also analyzed ERK1/2 phosphorylation by immunoblot. ERK1/2 phosphorylation appeared to increase in differentiated SCs versus non-differentiated SCs. However, when densitometry analysis was performed, we did not observe a significant difference in ERK1/2 phosphorylation in non-differentiated versus differentiated SCs ($p=0.8872$; Figure 6C, D). These data led to the conclusion that differentiation alters activation of signaling pathways associated with SC survival, repair, and myelination.

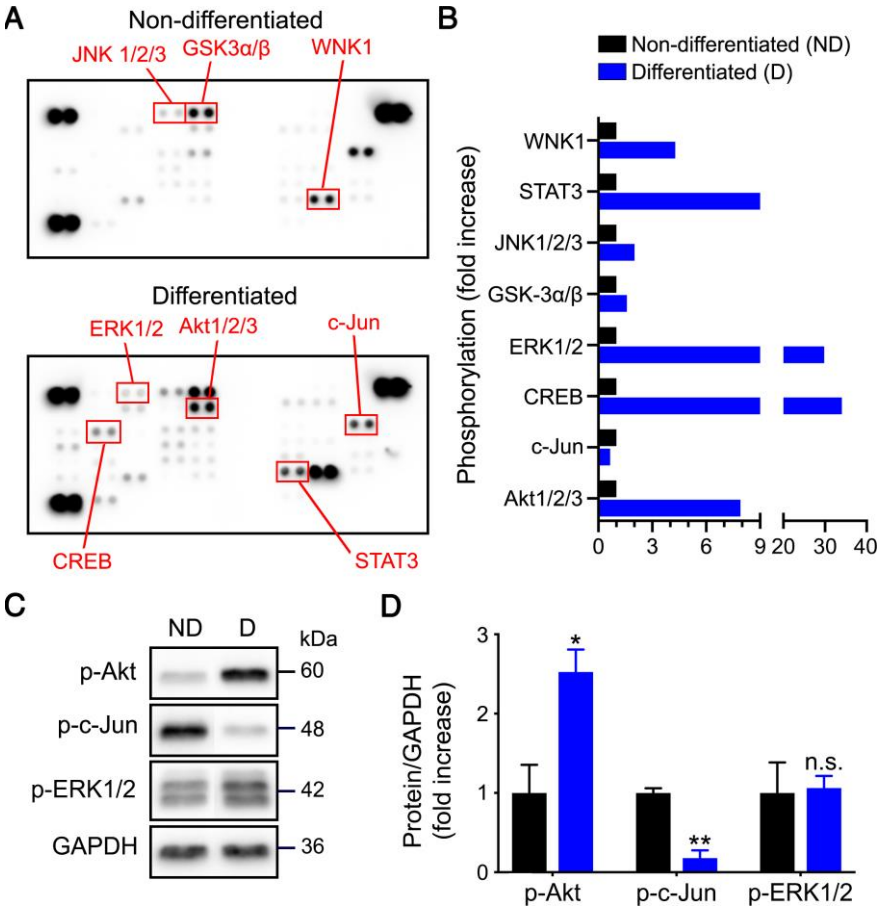

Figure 6. Differentiation *in vitro* alters signaling pathways associated with Schwann cell survival, repair, and myelination after injury. Primary rat SCs were cultured in either complete (non-differentiated SCs) or differentiation (differentiated SCs) medium for 48 h. (**A**) Protein phosphorylation was determined using the Phospho-Kinase Array Proteome Profiler. The membranes have internal controls to ensure equal loading of cell extracts and exposure. The blots are annotated with proteins that demonstrated altered phosphorylation. (**B**) Arrays were subjected to densitometry analysis. Changes in phosphorylation are shown (n=1). (**C**) To confirm the results of the array, immunoblot analysis was performed to detect the indicated proteins, including p-Akt, p-c-Jun, and p-ERK. GAPDH was used as a loading control. (**D**) Densitometry analysis was performed to quantify the relative levels of p-Akt, p-c-Jun, and p-ERK1/2 standardized against the loading control (mean ± SEM; n=3; **p*<0.05, ***p*<0.01, n.s. non-significant, unpaired *t*-test).

12.3 LRP1 expression decreases during the course of development *in vivo* and after differentiation *in vitro*

Previously, our laboratory showed that LRP1 expression in Schwann cells increases *in vivo* following nerve crush injury and *in vitro* after treatment with TNF-α (Campana et al., 2006), suggesting that LRP1 expression may play a role in transitioning Schwann cell phenotype. However, the pattern of LRP1 expression during SC development remains unknown. Therefore, we wanted to see whether or not LRP1 is expressed by developing SCs and after differentiation.

In rats, myelination begins at birth and continues through the first 1-2 weeks of development. We harvested sciatic nerves from rats at postnatal developmental days 0, 7, and 29 (P0, P7, and P29) for gene expression analysis. To demonstrate the progression of myelination, we analyzed changes in the expression of MBP mRNA. By P7, expression of MBP increased 5.6 ± 1.8-fold (**p<0.01). At P29, MBP expression remained 4.8 ± 0.7-fold higher relative to what we observed at P0 (*p<0.05; Figure 7A).

To assess the relationship between development and LRP1 expression, we also analyzed changes in LRP1 mRNA over time. By P7, expression of LRP1 significantly decreased 3.2 ± 0.1-fold (*p<0.05). However, at P29 expression of LRP1 was similar to what we observed at P0 (Figure 7B). These data suggest a negative relationship between myelination and that LRP1 is expressed in the early stages of postnatal development.

Next, we sought to determine whether LRP1 expression decreases following SC differentiation. Primary rat SCs were cultured in control or differentiation medium for 48 h. Differentiated SCs were analyzed for changes in LRP1 gene expression by RT-qPCR and protein expression by immunoblot.

We found that compared to non-differentiated SCs, differentiated SCs expressed 3.9 ± 0.1-fold less LRP1 mRNA (***p<0.001; Figure 7C). To confirm changes in the expression of LRP1 at the protein level, we performed western blot experiments to detect the 85 kDa β-chain of LRP1. In cell extracts isolated from non-differentiated SCs, we clearly detected a band at the expected 85 kDa size for the LRP1 β-chain. By contrast, the intensity of the signal was reduced in differentiated cells (Figure 7D) Densitometry analysis confirmed that differentiated SCs expressed 2.4 ± 0.04 less LRP1 protein than non-differentiated SCs (*p<0.05; Figure 7E). Collectively, these data show that LRP1 expression varied with the differentiation state of SCs. LRP1 expression appears to be highest during early postnatal development *in vivo* and in non-differentiated SCs that are similar to an immature phenotype *in vitro*, and then decreases following both myelination *in vivo* and SC differentiation toward a myelinating phenotype *in vitro*.

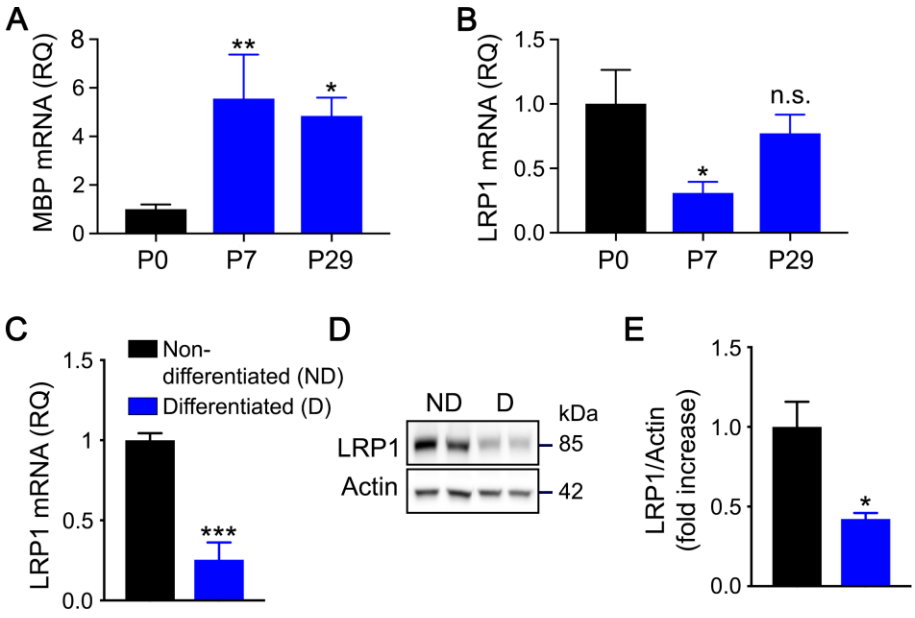

Figure 7. LRP1 expression decreases during development *in vivo* and following differentiation *in vitro*. (**A, B**) Sciatic nerves were harvested from rats at developmental days 0, 7, and 29 (P0, P7, and P29). Total RNA was extracted, and RT-qPCR experiments were conducted to quantify relative levels of (**A**) MBP and (**B**) LRP1 mRNA (mean ± SEM; n=4; *p<0.05, **p<0.01, n.s. non-significant, one-way ANOVA followed by Dunnett's *post-hoc* test). (**C-E**) Schwann cells were cultured in control or differentiation medium. After 48 h mRNA encoding for LRP1 was quantified by RT-qPCR (**C**) (mean ± SEM; n=4; ***p<0.001, unpaired *t*-test). LRP1 protein was detected by immunoblot analysis (**D**), and β-actin was used as a loading control. (**E**) Densitometry analysis was performed to quantify the relative level of LRP1 standardized against the loading control (mean ± SEM; n=4; *p<0.05, unpaired *t*-test).

12.4 ERK1/2 activation by α_2M* and NRG1 is reduced in differentiated Schwann cells *in vitro*

Next, we wanted to show that the decrease in LRP1 protein after differentiation led to a decrease in LRP1-dependent signaling. Primary rat SCs were cultured in control or differentiation medium for 48 h. Following differentiation, SCs were treated with activated 10 nM α_2-Macroglobulin (α_2M*) or 12 nM NRG1 for 15 min. α_2M is an LRP1 ligand (Willnow et al., 1994) previously shown to trigger ERK1/2 phosphorylation in primary SCs (Mantuano et al., 2015). NRG1 was used as a positive control for ERK1/2 phosphorylation.

Figure 8A shows that NRG1 and α2M* induced ERK1/2 phosphorylation in non-differentiated SCs as determined by immunoblot analysis. However, phosphorylation of ERK1/2 was reduced in differentiated SCs. Densitometry analysis confirmed this observation and revealed that in differentiated cells, phospho-ERK1/2 expression was reduced 4.5 ± 0.03 and 6.1 ± 0.01-fold following α_2M* and NRG1 treatment, respectively (Figure 8B). While these data are not yet significant, they suggest that differentiation reduces both LRP1-mediated and NRG1-induced ERK1/2 activation.

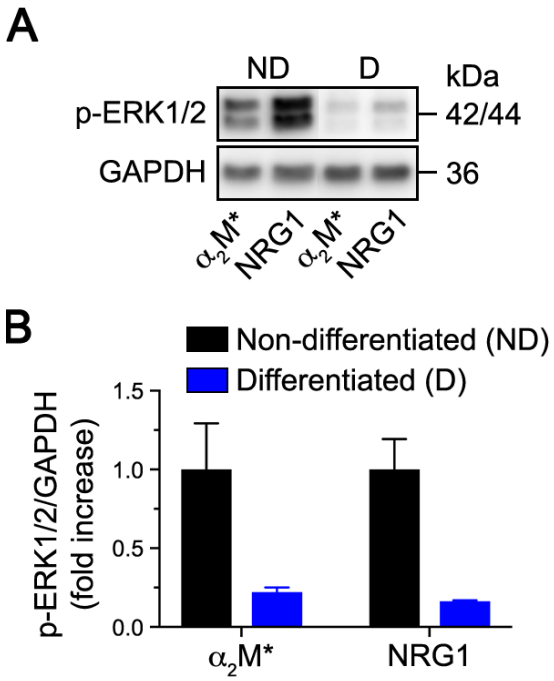

Figure 8. Cell signaling activated by LRP1 ligand is reduced in differentiated Schwann cells. Primary rat SCs were cultured in control or differentiation medium for 48 h. Following differentiation, SCs were treated with 10 nM α_2M* or 12 nM NRG1 for 15 min. (**A**) Protein extracts were subjected to immunoblot analysis to detect p-ERK. GAPDH was used as a loading control. Representative immunoblots of 2 independent experiments are shown. (**B**) Densitometry analysis was performed to quantify p-ERK standardized to the loading control (mean ± SEM, n=2).

12.5 Phagocytosis of myelin vesicles is impaired in differentiated Schwann cells *in vitro*

SCs were shown to use autophagy and phagocytosis to clear myelin debris after injury (Lutz et al., 2017). Previous work also showed that oligodendrocytes, astrocytes, and fibroblasts in the CNS use LRP1 to internalize myelin debris by phagocytosis (Gaultier et al., 2009). In this context, we decided to determine whether our model could be applied to study the role of LRP1 in phagocytosis of myelin vesicles (MVs) by SCs. MVs were prepared from rat sciatic nerves and labeled with pHrodo™, which is a dye that fluoresces under low-pH conditions such as within lysosomes and therefore allowed us to visualize only the MVs internalized by SCs.

SCs were treated with MVs for 1 h and immunofluorescence studies were performed to visualize the internalized MVs. Representative immunofluorescence images are shown (Figure 9A, B). We observed a sub-population of non-differentiated SCs that internalized MVs, as indicated by pHrodo™ labelling (red) around the nucleus (DAPI, blue) (Figure 9A). Because no fluorescence was observed in differentiated SCs, this strongly suggested that internalization of MVs by phagocytosis is impaired in SCs after differentiation (Figure 9B). To quantify these data, we performed flow cytometry experiments. The height of peaks on the graph represent cell counts. We consistently observed a lower cell count in the differentiated samples. Along the X axis, we observed a shift in fluorescence intensity of non-differentiated cells treated with MVs. This showed that internalization of MVs occurred. To quantify the number of non-differentiated cells that internalized MVs, we calculated the area under the peak and found that 11.4% of non-differentiated cells internalized MVs (Figure 9C). Together, these preliminary data suggest that PNS myelin is internalized by non-differentiated SCs, but not differentiated SCs.

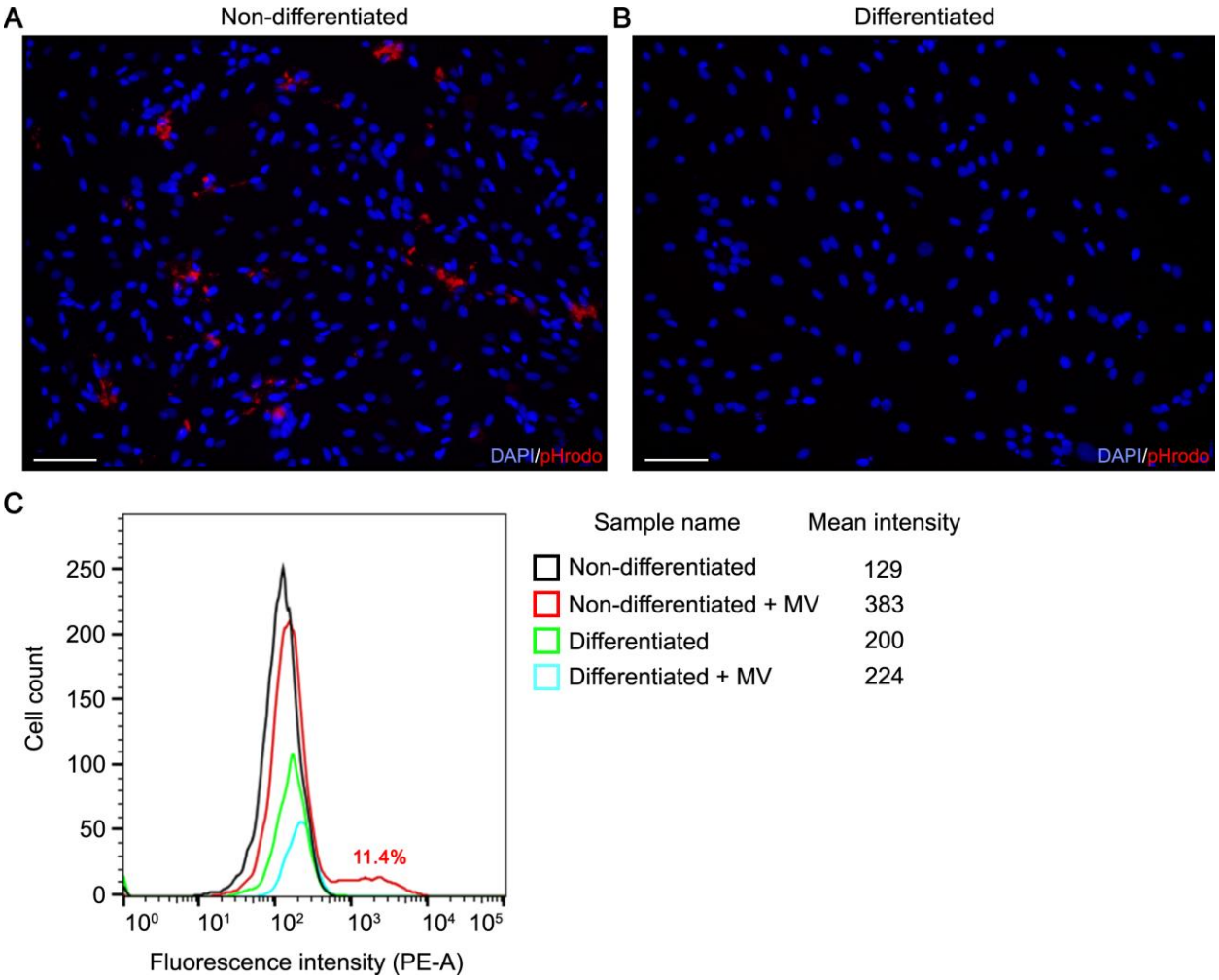

Figure 9. Phagocytosis of myelin vesicles is impaired in differentiated primary rat Schwann cells *in vitro*. Primary rat SCs were cultured in control or differentiation medium for 48 h and treated for 1 h at 37 ºC with 25 µg/mL PNS myelin vesicles (MVs) labeled with pHrodo™ Red Succinimidyl ester. (**A, B**) Representative images of immunofluorescence studies showing the internalized MVs (pHrodo, red). DAPI (blue) identifies SC nuclei. Images are representative of 2 independent experiments. Scale bars: 75 µm. (**C**) After treatment, non-differentiated and differentiated SCs were prepared for flow cytometry analysis and lifted from the culture plate using 0.25% trypsin. Flow cytometry was performed to show MV internalization. A shift in the mean fluorescence intensity indicates that internalization occurred in 11.4% of non-differentiated cells treated with MVs (red line).

Discussion

13.1 Development of control and differentiation media

Mouse SCs required NRG1 and cAMP to myelinate (Arthur-Farraj et al., 2011). Therefore, we supplemented differentiation medium with forskolin, which is known to increase cAMP (Dolci, Pesce, & De Felici, 1993), and with NRG1. The role of NRG1 in developmental myelination has been well established (Brinkmann et al., 2008; Riethmacher et al., 1997).

SCs' response to cAMP is dose-dependent. At lower concentrations, cAMP induces proliferation of SCs. Instead, high levels of cAMP were shown to induce differentiation (Jessen et al., 1991). SC complete medium contains 4 μM forskolin and is designed to support proliferation *in vitro.* To suppress proliferation and promote differentiation, we supplemented differentiation medium with 18 μM forskolin instead.

Because SCs cultured in complete medium received a lower dose of forskolin, they proliferated much faster than SCs in differentiation medium. To suppress proliferation but maintain non-differentiated SCs *in vitro,* we also prepared control medium that lacked forskolin and bovine pituitary extract (BPE).

13.2 Differentiation is a model for developmental myelination in primary rat Schwann cells

This model is significant because it allows us to study myelinating Schwann cells *in vitro* without the influence of neurons. Because myelination was established in homogenous cultures, the principal advantage of this model is that our results specifically reflect the behavior of myelinating Schwann cells. However, the absence of neurons is a potential limitation for this model because communication between SCs and neurons is known to be important for

development *in vivo* (Corfas, 2004). Furthermore, the absence of neurons in our model prevents us from studying the regulation and maintenance of SC structure *in vitro*. To address this potential limitation, we supplemented differentiation medium with NRG1, a factor derived from axons that is required for developmental myelination (Brinkmann et al., 2008). Previously established models of developmental myelination used co-cultures of neurons and either primary SCs or immortalized SC lines (Hyung et al., 2015; Takaku et al., 2018). These models have a broad range of applications, from strategies that enhance myelination or permit the study of demyelinating neuropathies *in vitro*.

Given our goal to establish a model for developmental myelination *in vitro,* we isolated primary SCs from the sciatic nerves of neonatal rats. During peripheral nerve development, immature SCs differentiate into both myelinating and non-myelinating SCs (Jessen et al., 2015). After injury in adults, both myelinating and non-myelinating SCs differentiate into repair SCs (Gomez-Sanchez et al., 2017). Because multiple phenotypes exist for SCs both during development and after injury, we analyzed the expression of phenotype markers in non-differentiated and differentiated SCs to confirm that we directed immature SCs toward a myelinating phenotype.

We showed that differentiated SCs upregulated MBP and P_0 (Figure 5), both of which are proteins found in PNS myelin (Saher & Simons, 2010). Furthermore, differentiated SCs downregulated expression of both $p75^{NTR}$ mRNA and protein (Figure 5B, D), which is consistent with the observation that $p75^{NTR}$ is downregulated during myelination (Jessen & Mirsky, 2008). Because differentiation medium induced myelin gene expression in our model system, we concluded that we successfully differentiated SCs toward a myelinating phenotype *in vitro.*

However, in addition to expressing $p75^{NTR}$, non-differentiated SCs also expressed markers of repair SCs. For example, we observed activation of c-Jun in non-differentiated SCs (Figure 6). Because c-Jun is a transcription factor known to reprogram SCs into repair SCs after injury (Arthur-Farraj et al., 2012; Hantke et al., 2014), this finding was consistent with a repair phenotype and distinct from the myelination model we established *in vitro*. Furthermore, although myelin phagocytosis is a function associated with injury and not development (Gomez-Sanchez et al., 2015; Lutz et al., 2017), we also observed phagocytosis in non-differentiated SCs (Figure 9). Because non-differentiated SCs expressed markers of more than one phenotype, we chose the neutral terms "non-differentiated" and "differentiated" to describe our cells.

13.3 ERK1/2 activation decreased after differentiation and may be downregulated to protect Schwann cells from demyelination

Differentiated SCs downregulated both LRP1 mRNA and proteins (Figure 7), so we wanted to understand how this affected signaling. Because previous work has shown that α_2M* activated ERK1/2 through LRP1 (Mantuano, Mukandala, Li, Campana, & Gonias, 2008), we treated differentiated SCs with 10 nM α_2M*. We also treated differentiated SCs with 12 nM NRG1 as a positive control for ERK1/2 activation, as NRG1 is known to activate the ERK/MAPK pathway through the ErbB3 receptor (Newbern et al., 2011).

After differentiation, we observed decreased activation of ERK1/2 in response to treatment with both activated α_2M* and NRG1 (Figure 8). We were surprised to observe that ERK1/2 activation in response to NRG1 also decreased after differentiation. Previously, Raf overexpression in mice was shown to increase levels of ERK1/2 activation and triggered demyelination (Napoli et al., 2012). It is possible LRP1 and other receptors that activate ERK

signaling, such as ErbB3, are downregulated to control the level of ERK1/2 activation during development and protect SCs from demyelination.

13.4 Other signaling pathways are regulated by SC differentiation

Using our model of developmental myelination, we also identified increased phosphorylation of WNK1 in differentiated SCs by phospho-array (Figure 6A, B). WNK1 is a serine/threonine protein kinase associated with a human hypertensive disorder known as Gordon's syndrome. WNK1 mutations contribute to hypertension by disrupting blood pressure control mechanisms in the kidneys (Wilson et al., 2001). However, previous work also demonstrated that *Wnk1* is widely expressed outside the kidneys and generates tissue-specific isoforms (O'Reilly, Marshall, Speirs, & Brown, 2003). Of relevance to our research, the *Wnk1/Hsn2* isoform is expressed in the nervous system of mice, including SCs, and mutations in the *Hsn2* exon are associated with human hereditary sensory neuropathy type II (Shekarabi et al., 2008). Other work in mammals identified WNK1 in the rat brain where it was shown to phosphorylate MBP (Xu et al., 2000), but not much is known about WNK1 in SCs. Excitingly, the role of WNK1 in SC development and the signaling pathways associated with WNK1 represent an unexplored area for future research.

13.5 LRP1 expression varies with Schwann cell phenotype

Extensive roles for LRP1 in the SC repair program have been described *in vivo* after injury and in primary rat SCs (Campana et al., 2006; Flütsch et al., 2016; Mantuano, Inoue, et al., 2008), but the roles for LRP1 in SC development and myelination are not known. We showed that LRP1 expression in the sciatic nerve was highest at birth (P0) and significantly decreased

after 7 days of development (Figure 7B). However, the relative level of LRP1 mRNA at P29 was not significantly different from the level of LRP1 at birth (Figure 7B), suggesting that LRP1 expression was lowest during early development. Because 90% of nucleated cells in the peripheral nerve are SCs (reviewed by Campana, 2007), we believe the change of LRP1 expression in the sciatic nerve reflects a change of expression in SCs. Because LRP1 expression decreased after differentiation *in vitro* (Figure 7), we hypothesize that the increased expression of LRP1 at P29 is specifically due to the proliferation of non-myelinating SCs.

Because LRP1 was downregulated during early development (Figure 7B) and upregulated in non-myelinating SCs during late development, LRP1 may not be required for developmental myelination. Previous work has shown that mice with conditional deletion of *scLrp1* had normal numbers of myelinating axons, but thinner myelin sheaths (Orita et al., 2013). In the PNS, myelin structure is regulated by Akt signaling and accordingly, myelination was blocked by the addition of the inhibitor LY294002 (Domènech-Estévez et al., 2016). Consequently, our laboratory demonstrated that activation of Akt by LRP1 was also blocked by LY294002 (Campana et al., 2017). If these signaling pathways are connected, then it is possible for LRP1 to regulate myelin structure through this mechanism. However, this is unlikely given that protein expression of LRP1 is significantly downregulated during differentiation (Figure 7D, E).

Conditional deletion of *scLrp1* also showed that LRP1 is required for correct Remak bundle formation in mice (Orita et al., 2013). Because LRP1 was predominantly expressed by non-myelinating SCs, the most likely role for LRP1 during development is the regulation of Remak bundle structure. Consistent with this hypothesis, LRP1 is known to regulate the formation of new Remak bundles after injury (Poplawski et al., 2018).

13.6 A potential role for LRP1 in the phagocytosis of myelin debris by Schwann cells after injury

SCs initiate the clearance of myelin debris from the peripheral nerve after injury (Perry et al., 1995). Two mechanisms for these observation have been described: autophagy (Gomez-Sanchez et al., 2015) and phagocytosis (Lutz et al., 2017). LRP1 is a phagocytosis receptor (Strickland et al., 2002) known to be upregulated in the sciatic nerve after injury (Campana et al., 2006). Previously, MBP derived from CNS myelin was shown to be an LRP1 ligand. Further experiments then showed that LRP1 was a phagocytosis receptor for myelin in oligodendrocytes, the myelinating glia of the CNS (Gaultier et al., 2009). Given this context, we hypothesized that SCs also use LRP1 to remove myelin debris by phagocytosis.

Our experiments with non-differentiated and differentiated SCs revealed that differentiation impaired the ability of SCs to internalize myelin debris (Figure 9). However, we only demonstrated that a sub-population of non-differentiated cells were capable of myelin phagocytosis (Figure 9A). One explanation for this observation is that while non-differentiated SCs expressed higher levels of LRP1 protein than differentiated SCs (Figure 7D, E), we did not measure whether these receptors are present on the cell surface and available to bind myelin.

Furthermore, previous work claimed that LRP1 was not enriched in SCs after injury, and investigated other phagocytosis receptors that remove myelin debris in SCs (Lutz et al., 2017). These data contradict the observation that LRP1 expression in the sciatic nerve and in SCs increased after crush injury (Campana et al., 2006). However, discrepancies in the methods used to collect these data may explain the contradictory results. For example, our laboratory analyzed LRP1 expression after axotomy in neonates and adults. We showed that LRP1 gene expression

after axotomy is enhanced in adults and does not change in neonates. However, the Lutz group performed sciatic nerve crush injury to rats at P18, which may explain why no enrichment of LRP1 was observed. These data suggest that the type of injury and maturity of the animal impact the expression of LRP1 and consequently, whether it may function as a phagocytic receptor. It is also possible that after injury, SCs use LRP1 primarily as a signaling receptor, and instead use other phagocytosis receptors to remove myelin debris. Therefore, future experiments are necessary to clarify the scientific role of LRP1 in myelin phagocytosis.

13.7 Future directions

Our model of developmental myelination *in vitro* can help us determine the mechanisms myelinating SCs use to respond to injury. Unpublished work in our laboratory showed that a high dose of the amino acid glutamate triggered activation of c-Jun in differentiated SCs but did not downregulate myelin gene expression. Glutamate is known to activate the LRP1 and NMDA-R signaling complex (Campana et al., 2017), implicating a potential role for these receptors in the SC response to injury. Previously, TNF-α treatment was shown to induce LRP1 expression in primary SCs (Campana et al., 2006). By treating differentiated SCs with TNF-α, we may determine if activation of c-Jun is enhanced by LRP1 expression, and whether enhanced c-Jun activation is sufficient to downregulate myelin gene expression.

Previous research showed that sustained activation of ERK1/2 in response to Raf overexpression triggered demyelination (Napoli et al., 2012). Therefore, we hypothesized that SCs downregulate LRP1 and ErbB3 during differentiation to prevent demyelination caused by over-activation of ERK1/2. To test this hypothesis, we will begin by analyzing changes in the

expression of ErbB3 during differentiation. Then, we can induce ERK1/2 activation to measure demyelination and determine whether LRP1 and ErbB3 are involved.

To decipher the involvement of LRP1 in phagocytosis of myelin, we will determine whether SCs use LRP1 to internalize myelin and whether upregulation of LRP1 after injury promotes phagocytosis. Because P_0 is more abundant in peripheral myelin than MBP, we can show that P_0 is an LRP1 ligand and that RAP blocks them from binding to each other. Then, flow cytometry experiments with RAP will show whether peripheral MVs specifically bind to LRP1 and become internalized by SCs. Furthermore, treatment with TNF-α has been shown to increase LRP1 mRNA expression in Schwann cells (Campana et al., 2006). To understand whether upregulation of LRP1 in SCs after injury contributes to phagocytosis, we would apply TNF-α to differentiated SCs prior to treatment with MVs and see if injury enhances the ability of SCs to internalize MVs.

References

Alizadeh, A., Dyck, S. M., & Karimi-Abdolrezaee, S. (2015). Myelin damage and repair in pathologic CNS: challenges and prospects. *Frontiers in Molecular Neuroscience*, *8*, 35. https://doi.org/10.3389/fnmol.2015.00035

Arthur-Farraj, P. J., Latouche, M., Wilton, D. K., Quintes, S., Chabrol, E., Banerjee, A., Woodhoo, A., Jenkins, B., Rahman, M., Turmaine, M., Wicher, G. K., Mitter, R., Greensmith, L., Behrens, A., Raivich, G., Mirsky, R., & Jessen, K. R. (2012). c-Jun reprograms Schwann cells of injured nerves to generate a repair cell essential for regeneration. *Neuron*, *75*(4), 633–647. https://doi.org/10.1016/j.neuron.2012.06.021

Arthur-Farraj, P. J., Wanek, K., Hantke, J., Davis, C. M., Jayakar, A., Parkinson, D. B., Mirsky, R., & Jessen, K. R. (2011). Mouse schwann cells need both NRG1 and cyclic AMP to myelinate. *Glia*, *59*(5), 720–733. https://doi.org/10.1002/glia.21144

Barres, B. A., Jacobson, M. D., Schmid, R., Sendtner, M., & Raff, M. C. (1993). Does oligodendrocyte survival depend on axons? *Current Biology*, *3*(8), 489–497. https://doi.org/10.1016/0960-9822(93)90039-Q

Barrett, A. J., Brown, M. A., & Sayers, C. A. (1979). The electrophoretically "slow" and "fast" forms of the alpha 2-macroglobulin molecule. *The Biochemical Journal*, *181*(2), 401–418.

Beirowski, B., Adalbert, R., Wagner, D., Grumme, D. S., Addicks, K., Ribchester, R. R., & Coleman, M. P. (2005). The progressive nature of Wallerian degeneration in wild-type and slow Wallerian degeneration (WldS) nerves. *BMC Neuroscience*, *6*, 6. https://doi.org/10.1186/1471-2202-6-6

Beirowski, B., Babetto, E., Golden, J. P., Chen, Y.-J., Yang, K., Gross, R. W., Patti, G. J., & Milbrandt, J. (2014). Metabolic regulator LKB1 is crucial for Schwann cell–mediated axon maintenance. *Nature Neuroscience*, *17*(10), 1351–1361. https://doi.org/10.1038/nn.3809

Benito, C., Davis, C. M., Gomez-Sanchez, J. A., Turmaine, M., Meijer, D., Poli, V., Mirsky, R., & Jessen, K. R. (2017). STAT3 Controls the Long-Term Survival and Phenotype of Repair Schwann Cells during Nerve Regeneration. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *37*(16), 4255–4269. https://doi.org/10.1523/JNEUROSCI.3481-16.2017

Birchmeier, C., & Nave, K.-A. (2008). Neuregulin-1, a key axonal signal that drives Schwann cell growth and differentiation. *Glia*, *56*(14), 1491–1497. https://doi.org/10.1002/glia.20753

Blakemore, W. F., & Keirstead, H. S. (1999). The origin of remyelinating cells in the central nervous system. *Journal of Neuroimmunology*, *98*(1), 69–76. https://doi.org/10.1016/S0165-5728(99)00083-1

Brifault, C., Gilder, A. S., Laudati, E., Banki, M., & Gonias, S. L. (2017). Shedding of membrane-associated LDL receptor-related protein-1 from microglia amplifies and sustains

neuroinflammation. *The Journal of Biological Chemistry*, *292*(45), 18699–18712. https://doi.org/10.1074/jbc.M117.798413

Brinkmann, B. G., Agarwal, A., Sereda, M. W., Garratt, A. N., Müller, T., Wende, H., Stassart, R. M., Nawaz, S., Humml, C., Velanac, V., Radyushkin, K., Goebbels, S., Fischer, T. M., Franklin, R. J., Lai, C., Ehrenreich, H., Birchmeier, C., Schwab, M. H., & Nave, K. A. (2008). Neuregulin-1/ErbB Signaling Serves Distinct Functions in Myelination of the Peripheral and Central Nervous System. *Neuron*, *59*(4), 581–595. https://doi.org/10.1016/J.NEURON.2008.06.028

Brockes, J. P., Fields, K. L., & Raff, M. C. (1979). Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. *Brain Research*, *165*(1), 105–118. https://doi.org/10.1016/0006-8993(79)90048-9

Campana, W. M. (2007). Schwann cells: Activated peripheral glia and their role in neuropathic pain. *Brain, Behavior, and Immunity*, *21*(5), 522–527. https://doi.org/10.1016/j.bbi.2006.12.008

Campana, W. M., Darin, S. J., & O'Brien, J. S. (1999). Phosphatidylinositol 3-kinase and Akt protein kinase mediate IGF-I- and prosaptide-induced survival in schwann cells. *Journal of Neuroscience Research*, *57*(3), 332–341. https://doi.org/10.1002/(SICI)1097-4547(19990801)57:3<332::AID-JNR5>3.0.CO;2-0

Campana, W. M., Li, X., Dragojlovic, N., Janes, J., Gaultier, A., & Gonias, S. L. (2006). The Low-Density Lipoprotein Receptor-Related Protein Is a Pro-Survival Receptor in Schwann Cells: Possible Implications in Peripheral Nerve Injury. *Journal of Neuroscience*, *26*(43), 11197–11207. https://doi.org/10.1523/JNEUROSCI.2709-06.2006

Campana, W. M., Mantuano, E., Azmoon, P., Henry, K., Banki, M. A., Kim, J. H., Pizzo, D. P., & Gonias, S. L. (2017). Ionotropic glutamate receptors activate cell signaling in response to glutamate in Schwann cells. *The FASEB Journal*, *31*(4), 1744–1755. https://doi.org/10.1096/fj.201601121R

Canton, J., Neculai, D., & Grinstein, S. (2013). Scavenger receptors in homeostasis and immunity. *Nature Reviews Immunology*, *13*(9), 621–634. https://doi.org/10.1038/nri3515

Chen, M. S., Huber, A. B., van der Haar, M. E., Frank, M., Schnell, L., Spillmann, A. A., Christ, F., & Schwab, M. E. (2000). Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. *Nature*, *403*(6768), 434–439. https://doi.org/10.1038/35000219

Chomczynski, P., & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*, *162*(1), 156–159. https://doi.org/10.1016/0003-2697(87)90021-2

Colloca, L., Ludman, T., Bouhassira, D., Baron, R., Dickenson, A. H., Yarnitsky, D., Freeman, R., Truini, A., Attal, N., Finnerup, N. B., Eccleston, C., Kalso, E., Bennett, D. L., Dworkin, R. H., & Raja, S. N. (2017). Neuropathic pain. *Nature Reviews. Disease Primers*, *3*, 17002.

https://doi.org/10.1038/nrdp.2017.2

Corfas, G. (2004). Mechanisms and Roles of Axon-Schwann Cell Interactions. *Journal of Neuroscience*, *24*(42), 9250–9260. https://doi.org/10.1523/JNEUROSCI.3649-04.2004

David, S., & Aguayo, A. J. (1981). Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. *Science (New York, N.Y.)*, *214*(4523), 931–933. https://doi.org/10.1126/SCIENCE.6171034

de Waegh, S., & Brady, S. T. (1990). Altered slow axonal transport and regeneration in a myelin-deficient mutant mouse: the trembler as an in vivo model for Schwann cell-axon interactions. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *10*(6), 1855–1865. https://doi.org/10.1523/JNEUROSCI.10-06-01855.1990

Decker, L., Desmarquet-Trin-Dinh, C., Taillebourg, E., Ghislain, J., Vallat, J.-M., & Charnay, P. (2006). Peripheral Myelin Maintenance Is a Dynamic Process Requiring Constant Krox20 Expression. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *26*(38), 9771–9779. https://doi.org/10.1523/JNEUROSCI.0716-06.2006

Dolci, S., Pesce, M., & De Felici, M. (1993). Combined action of stem cell factor, leukemia inhibitory factor, and cAMP on in vitro proliferation of mouse primordial germ cells. *Molecular Reproduction and Development*, *35*(2), 134–139. https://doi.org/10.1002/mrd.1080350206

Domènech-Estévez, E., Baloui, H., Meng, X., Zhang, Y., Deinhardt, K., Dupree, J. L., Einheber, S., Chrast, R., & Salzer, J. L. (2016). Akt Regulates Axon Wrapping and Myelin Sheath Thickness in the PNS. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *36*(16), 4506–4521. https://doi.org/10.1523/JNEUROSCI.3521-15.2016

Dong, Z., Brennan, A., Liu, N., Yarden, Y., Lefkowitz, G., Mirsky, R., & Jessen, K. R. (1995). Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation, and maturation of rat schwann cell precursors. *Neuron*, *15*(3), 585–596. https://doi.org/10.1016/0896-6273(95)90147-7

Epelman, S., Lavine, K. J., & Randolph, G. J. (2014). Origin and functions of tissue macrophages. *Immunity*, *41*(1), 21–35. https://doi.org/10.1016/j.immuni.2014.06.013

Fernandez-Castaneda, A., Arandjelovic, S., Stiles, T. L., Schlobach, R. K., Mowen, K. A., Gonias, S. L., & Gaultier, A. (2013). Identification of the low density lipoprotein (LDL) receptor-related protein-1 interactome in central nervous system myelin suggests a role in the clearance of necrotic cell debris. *The Journal of Biological Chemistry*, *288*(7), 4538–4548. https://doi.org/10.1074/jbc.M112.384693

Flütsch, A., Henry, K., Mantuano, E., Lam, M. S., Shibayama, M., Takahashi, K., Gonias, S. L., & Campana, W. M. (2016). Evidence that LDL receptor-related protein 1 acts as an early injury detection receptor and activates c-Jun in Schwann cells. *NeuroReport*, *27*(18), 1305–1311. https://doi.org/10.1097/WNR.0000000000000691

Fu, S. Y., & Gordon, T. (1995a). Contributing factors to poor functional recovery after delayed nerve repair: prolonged axotomy. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *15*(5 Pt 2), 3876–3885. https://doi.org/10.1523/JNEUROSCI.15-05-03876.1995

Fu, S. Y., & Gordon, T. (1995b). Contributing factors to poor functional recovery after delayed nerve repair: prolonged denervation. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *15*(5), 3886–3895. https://doi.org/10.1523/JNEUROSCI.15-05-03886.1995

Gaultier, A., Wu, X., Le Moan, N., Takimoto, S., Mukandala, G., Akassoglou, K., Campana, W. M., & Gonias, S. L. (2009). Low-density lipoprotein receptor-related protein 1 is an essential receptor for myelin phagocytosis. *Journal of Cell Science*, *122*(Pt 8), 1155–1162. https://doi.org/10.1242/jcs.040717

Goldberg, D. S., & McGee, S. J. (2011). Pain as a global public health priority. *BMC Public Health*, *11*, 770. https://doi.org/10.1186/1471-2458-11-770

Gomez-Sanchez, J. A., Carty, L., Iruarrizaga-Lejarreta, M., Palomo-Irigoyen, M., Varela-Rey, M., Griffith, M., Hantke, J., Macias-Camara, N., Azkargorta, M., Aurrekoetxea, I., De Juan, V. G., Jefferies, H. B. J., Aspichueta, P., Elortza, F., Aransay, A. M., Martínez-Chantar, M. L., Baas, F., Mato, J. M., Mirsky, R., Woodhoo, A., & Jessen, K. R. (2015). Schwann cell autophagy, myelinophagy, initiates myelin clearance from injured nerves. *The Journal of Cell Biology*, *210*(1), 153–168. https://doi.org/10.1083/jcb.201503019

Gomez-Sanchez, J. A., Pilch, K. S., van der Lans, M., Fazal, S. V., Benito, C., Wagstaff, L. J., Mirsky, R., & Jessen, K. R. (2017). After Nerve Injury, Lineage Tracing Shows That Myelin and Remak Schwann Cells Elongate Extensively and Branch to Form Repair Schwann Cells, Which Shorten Radically on Remyelination. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *37*(37), 9086–9099. https://doi.org/10.1523/JNEUROSCI.1453-17.2017

Gonias, S. L., Reynolds, J. A., & Pizzo, S. V. (1982). Physical properties of human alpha 2-macroglobulin following reaction with methylamine and trypsin. *Biochimica et Biophysica Acta*, *705*(3), 306–314.

Gordon, T., Wood, P., & Sulaiman, O. A. R. (2018). Long-Term Denervated Rat Schwann Cells Retain Their Capacity to Proliferate and to Myelinate Axons in vitro. *Frontiers in Cellular Neuroscience*, *12*, 511. https://doi.org/10.3389/fncel.2018.00511

Griffin, J. W., & Thompson, W. J. (2008). Biology and pathology of nonmyelinating Schwann cells. *Glia*, *56*(14), 1518–1531. https://doi.org/10.1002/glia.20778

Hantke, J., Carty, L., Wagstaff, L. J., Turmaine, M., Wilton, D. K., Quintes, S., Koltzenburg, M., Baas, F., Mirsky, R., & Jessen, K. R. (2014). c-Jun activation in Schwann cells protects against loss of sensory axons in inherited neuropathy. *Brain*, *137*(11). https://doi.org/10.1093/brain/awu257

Harrisingh, M. C., Perez-Nadales, E., Parkinson, D. B., Malcolm, D. S., Mudge, A. W., & Lloyd, A. C. (2004). The Ras/Raf/ERK signalling pathway drives Schwann cell dedifferentiation. *The EMBO Journal*, *23*(15), 3061–3071. https://doi.org/10.1038/sj.emboj.7600309

Hendrickx, D. A. E., Schuurman, K. G., van Draanen, M., Hamann, J., & Huitinga, I. (2014). Enhanced uptake of multiple sclerosis-derived myelin by THP-1 macrophages and primary human microglia. *Journal of Neuroinflammation*, *11*, 64. https://doi.org/10.1186/1742-2094-11-64

Herz, J., Clouthier, D. E., & Hammer, R. E. (1992). LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation. *Cell*, *71*(3), 411–421. https://doi.org/10.1016/0092-8674(92)90511-A

Herz, J., Kowal, R. C., Goldstein, J. L., & Brown, M. S. (1990). Proteolytic processing of the 600 kd low density lipoprotein receptor-related protein (LRP) occurs in a trans-Golgi compartment. *The EMBO Journal*, *9*(6), 1769–1776. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2112085

Herz, J., & Strickland, D. K. (2001). LRP: a multifunctional scavenger and signaling receptor. *The Journal of Clinical Investigation*, *108*(6), 779–784. https://doi.org/10.1172/JCI13992

Heumann, R., Korsching, S., Bandtlow, C., & Thoenen, H. (1987). Changes of nerve growth factor synthesis in nonneuronal cells in response to sciatic nerve transection. *The Journal of Cell Biology*, *104*(6), 1623–1631. https://doi.org/10.1083/JCB.104.6.1623

Hirata, K., & Kawabuchi, M. (2002). Myelin phagocytosis by macrophages and nonmacrophages during Wallerian degeneration. *Microscopy Research and Technique*, *57*(6), 541–547. https://doi.org/10.1002/jemt.10108

Höke, A., Gordon, T., Zochodne, D. W., & Sulaiman, O. A. R. (2002). A Decline in Glial Cell-Line-Derived Neurotrophic Factor Expression Is Associated with Impaired Regeneration after Long-Term Schwann Cell Denervation. *Experimental Neurology*, *173*(1), 77–85. https://doi.org/10.1006/EXNR.2001.7826

Huebner, E. A., & Strittmatter, S. M. (2009). Axon regeneration in the peripheral and central nervous systems. *Results and Problems in Cell Differentiation*, *48*, 339–351. https://doi.org/10.1007/400_2009_19

Hyung, S., Yoon Lee, B., Park, J.-C., Kim, J., Hur, E.-M., & Francis Suh, J.-K. (2015). Coculture of Primary Motor Neurons and Schwann Cells as a Model for In Vitro Myelination. *Scientific Reports*, *5*(1), 15122. https://doi.org/10.1038/srep15122

Imber, M. J., & Pizzo, S. V. (1981). Clearance and binding of two electrophoretic "fast" forms of human alpha 2-macroglobulin. *The Journal of Biological Chemistry*, *256*(15), 8134–8139.

Jessen, K. R., & Mirsky, R. (2005). The origin and development of glial cells in peripheral nerves. *Nature Reviews Neuroscience*, *6*(9), 671–682. https://doi.org/10.1038/nrn1746

Jessen, K. R., & Mirsky, R. (2008). Negative regulation of myelination: Relevance for development, injury, and demyelinating disease. *Glia*, *56*(14), 1552–1565. https://doi.org/10.1002/glia.20761

Jessen, K. R., & Mirsky, R. (2019). Schwann Cell Precursors; Multipotent Glial Cells in Embryonic Nerves. *Frontiers in Molecular Neuroscience*, *12*, 69. https://doi.org/10.3389/FNMOL.2019.00069

Jessen, K. R., Mirsky, R., & Lloyd, A. C. (2015). Schwann Cells: Development and Role in Nerve Repair. *Cold Spring Harbor Perspectives in Biology*, *7*(7), a020487. https://doi.org/10.1101/cshperspect.a020487

Jessen, K. R., Mirsky, R., & Morgan, L. (1991). Role of Cyclic AMP and Proliferation Controls in Schwann Cell Differentiation. *Annals of the New York Academy of Sciences*, *633*(1 Glial-Neurona), 78–89. https://doi.org/10.1111/j.1749-6632.1991.tb15597.x

Jopling, C., Boue, S., & Belmonte, J. C. I. (2011). Dedifferentiation, transdifferentiation and reprogramming: three routes to regeneration. *Nature Reviews Molecular Cell Biology*, *12*(2), 79–89. https://doi.org/10.1038/nrm3043

Joseph, N. M., Mukouyama, Y.-S., Mosher, J. T., Jaegle, M., Crone, S. A., Dormand, E.-L., Lee, K.-F., Meijer, D., Anderson, D. J., & Morrison, S. J. (2004). Neural crest stem cells undergo multilineage differentiation in developing peripheral nerves to generate endoneurial fibroblasts in addition to Schwann cells. *Development (Cambridge, England)*, *131*(22), 5599–5612. https://doi.org/10.1242/dev.01429

Kallio, P. K., & Vastamäki, M. (1993). An Analysis of the Results of Late Reconstruction of 132 Median Nerves. *Journal of Hand Surgery*, *18*(1), 97–105. https://doi.org/10.1016/0266-7681(93)90205-T

Ko, P.-Y., Yang, C.-C., Kuo, Y.-L., Su, F.-C., Hsu, T.-I., Tu, Y.-K., & Jou, I.-M. (2018). Schwann-Cell Autophagy, Functional Recovery, and Scar Reduction After Peripheral Nerve Repair. *Journal of Molecular Neuroscience*, *64*(4), 601–610. https://doi.org/10.1007/s12031-018-1056-8

Kotter, M. R., Li, W.-W., Zhao, C., & Franklin, R. J. M. (2006). Myelin impairs CNS remyelination by inhibiting oligodendrocyte precursor cell differentiation. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *26*(1), 328–332. https://doi.org/10.1523/JNEUROSCI.2615-05.2006

Kottis, V., Thibault, P., Mikol, D., Xiao, Z.-C., Zhang, R., Dergham, P., & Braun, P. E. (2002). Oligodendrocyte-myelin glycoprotein (OMgp) is an inhibitor of neurite outgrowth. *Journal of Neurochemistry*, *82*(6), 1566–1569. https://doi.org/10.1046/j.1471-4159.2002.01146.x

Kouyoumdjian, J. A. (2006). Peripheral nerve injuries: A retrospective survey of 456 cases. *Muscle & Nerve*, *34*(6), 785–788. https://doi.org/10.1002/mus.20624

Larocca, J. N., & Norton, W. T. (2007). Isolation of Myelin. *Current Protocols in Cell Biology*,

33(1), 3.25.1-3.25.19. https://doi.org/10.1002/0471143030.cb0325s33

Lillis, A. P., Van Duyn, L. B., Murphy-Ullrich, J. E., & Strickland, D. K. (2008). LDL receptor-related protein 1: unique tissue-specific functions revealed by selective gene knockout studies. *Physiological Reviews*, *88*(3), 887–918. https://doi.org/10.1152/physrev.00033.2007

Lutz, A. B., Chung, W.-S., Sloan, S. A., Carson, G. A., Zhou, L., Lovelett, E., Posada, S., Zuchero, J. B., & Barres, B. A. (2017). Schwann cells use TAM receptor-mediated phagocytosis in addition to autophagy to clear myelin in a mouse model of nerve injury. *Proceedings of the National Academy of Sciences*, *114*(38), E8072–E8080. https://doi.org/10.1073/PNAS.1710566114

Mantuano, E., Henry, K., Yamauchi, T., Hiramatsu, N., Yamauchi, K., Orita, S., Takahashi, K., Lin, J. H., Gonias, S. L., & Campana, W. M. (2011). The unfolded protein response is a major mechanism by which LRP1 regulates Schwann cell survival after injury. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *31*(38), 13376–13385. https://doi.org/10.1523/JNEUROSCI.2850-11.2011

Mantuano, E., Inoue, G., Li, X., Takahashi, K., Gaultier, A., Gonias, S. L., & Campana, W. M. (2008). The hemopexin domain of matrix metalloproteinase-9 activates cell signaling and promotes migration of schwann cells by binding to low-density lipoprotein receptor-related protein. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *28*(45), 11571–11582. https://doi.org/10.1523/JNEUROSCI.3053-08.2008

Mantuano, E., Jo, M., Gonias, S. L., & Campana, W. M. (2010). Low density lipoprotein receptor-related protein (LRP1) regulates Rac1 and RhoA reciprocally to control Schwann cell adhesion and migration. *The Journal of Biological Chemistry*, *285*(19), 14259–14266. https://doi.org/10.1074/jbc.M109.085126

Mantuano, E., Lam, M. S., & Gonias, S. L. (2013). LRP1 assembles unique co-receptor systems to initiate cell signaling in response to tissue-type plasminogen activator and myelin-associated glycoprotein. *The Journal of Biological Chemistry*, *288*(47), 34009–34018. https://doi.org/10.1074/jbc.M113.509133

Mantuano, E., Lam, M. S., Shibayama, M., Campana, W. M., & Gonias, S. L. (2015). The NMDA receptor functions independently and as an LRP1 co-receptor to promote Schwann cell survival and migration. *Journal of Cell Science*, *128*(18), 3478–3488. https://doi.org/10.1242/jcs.173765

Mantuano, E., Mukandala, G., Li, X., Campana, W. M., & Gonias, S. L. (2008). Molecular dissection of the human α2-macroglobulin subunit reveals domains with antagonistic activities in cell signaling. *Journal of Biological Chemistry*, *283*(29), 19904–19911. https://doi.org/10.1074/jbc.M801762200

Maripuu, A., Björkman, A., Björkman-Burtscher, I. M., Mannfolk, P., Andersson, G., & Dahlin, L. B. (2012). Reconstruction of sciatic nerve after traumatic injury in humans - factors influencing outcome as related to neurobiological knowledge from animal research. *Journal*

of Brachial Plexus and Peripheral Nerve Injury, *7*(1), 7. https://doi.org/10.1186/1749-7221-7-7

Marzolo, M. P., von Bernhardi, R., Bu, G., & Inestrosa, N. C. (2000). Expression of a2-macroglobulin receptor/low density lipoprotein receptor-related protein (LRP) in rat microglial cells. *Journal of Neuroscience Research*, *60*(3), 401–411. https://doi.org/10.1002/(SICI)1097-4547(20000501)60:3<401::AID-JNR15>3.0.CO;2-L

McKerracher, L., David, S., Jackson, D. L., Kottis, V., Dunn, R. J., & Braun, P. E. (1994). Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. *Neuron*, *13*(4), 805–811. https://doi.org/10.1016/0896-6273(94)90247-X

Meier, C., Parmantier, E., Brennan, A., Mirsky, R., & Jessen, K. R. (1999). Developing Schwann Cells Acquire the Ability to Survive without Axons by Establishing an Autocrine Circuit Involving Insulin-Like Growth Factor, Neurotrophin-3, and Platelet-Derived Growth Factor-BB. *Journal of Neuroscience*, *19*(10), 3847–3859. https://doi.org/10.1523/JNEUROSCI.19-10-03847.1999

Menorca, R. M. G., Fussell, T. S., & Elfar, J. C. (2013). Nerve physiology: mechanisms of injury and recovery. *Hand Clinics*, *29*(3), 317–330. https://doi.org/10.1016/j.hcl.2013.04.002

Mogha, A., Benesh, A. E., Patra, C., Engel, F. B., Schöneberg, T., Liebscher, I., & Monk, K. R. (2013). Gpr126 functions in Schwann cells to control differentiation and myelination via G-protein activation. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *33*(46), 17976–17985. https://doi.org/10.1523/JNEUROSCI.1809-13.2013

Mueller, M., Leonhard, C., Wacker, K., Ringelstein, E. B., Okabe, M., Hickey, W. F., & Kiefer, R. (2003). Macrophage Response to Peripheral Nerve Injury: The Quantitative Contribution of Resident and Hematogenous Macrophages. *Laboratory Investigation*, *83*(2), 175–185. https://doi.org/10.1097/01.LAB.0000056993.28149.BF

Mukhopadhyay, G., Doherty, P., Walsh, F. S., Crocker, P. R., & Filbin, M. T. (1994). A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron*, *13*(3), 757–767. https://doi.org/10.1016/0896-6273(94)90042-6

Murinson, B. B., & Griffin, J. W. (2004). C-Fiber Structure Varies with Location in Peripheral Nerve. *Journal of Neuropathology & Experimental Neurology*, *63*(3), 246–254. https://doi.org/10.1093/jnen/63.3.246

Murinson, B. B., Hoffman, P. N., Banihashemi, M. R., Meyer, R. A., & Griffin, J. W. (2005). C-fiber (Remak) bundles contain both isolectin B4-binding and calcitonin gene-related peptide-positive axons. *The Journal of Comparative Neurology*, *484*(4), 392–402. https://doi.org/10.1002/cne.20506

Napoli, I., Noon, L. A., Ribeiro, S., Kerai, A. P., Parrinello, S., Rosenberg, L. H., Collins, M. J., Harrisingh, M. C., White, I. J., Woodhoo, A., & Lloyd, A. C. (2012). A Central Role for the ERK-Signaling Pathway in Controlling Schwann Cell Plasticity and Peripheral Nerve Regeneration In Vivo. *Neuron*, *73*(4), 729–742.

https://doi.org/10.1016/J.NEURON.2011.11.031

Nave, K.-A. (2010). Myelination and the trophic support of long axons. *Nature Reviews Neuroscience*, *11*(4), 275–283. https://doi.org/10.1038/nrn2797

Newbern, J. M., Li, X., Shoemaker, S. E., Zhou, J., Zhong, J., Wu, Y., Bonder, D., Hollenback, S., Coppola, G., Geschwind, D. H., Landreth, G. E., & Snider, W. D. (2011). Specific Functions for ERK/MAPK Signaling during PNS Development. *Neuron*, *69*(1), 91–105. https://doi.org/10.1016/J.NEURON.2010.12.003

O'Reilly, M., Marshall, E., Speirs, H. J. L., & Brown, R. W. (2003). WNK1, a Gene within a Novel Blood Pressure Control Pathway, Tissue-Specifically Generates Radically Different Isoforms with and without a Kinase Domain. *Journal of the American Society of Nephrology*, *14*(10), 2447–2456. https://doi.org/10.1097/01.ASN.0000089830.97681.3B

Orita, S., Henry, K., Mantuano, E., Yamauchi, K., De Corato, A., Ishikawa, T., Feltri, M. L., Wrabetz, L., Gaultier, A., Pollack, M., Ellisman, M., Takahashi, K., Gonias, S. L., & Campana, W. M. (2013). Schwann cell LRP1 regulates remak bundle ultrastructure and axonal interactions to prevent neuropathic pain. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *33*(13), 5590–5602. https://doi.org/10.1523/JNEUROSCI.3342-12.2013

Özçelik, M., Cotter, L., Jacob, C., Pereira, J. A., Relvas, J. B., Suter, U., & Tricaud, N. (2010). Pals1 is a major regulator of the epithelial-like polarization and the extension of the myelin sheath in peripheral nerves. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *30*(11), 4120–4131. https://doi.org/10.1523/JNEUROSCI.5185-09.2010

Parkinson, D. B., Bhaskaran, A., Arthur-Farraj, P. J., Noon, L. A., Woodhoo, A., Lloyd, A. C., Feltri, M. L., Wrabetz, L., Behrens, A., Mirsky, R., & Jessen, K. R. (2008). c-Jun is a negative regulator of myelination. *The Journal of Cell Biology*, *181*(4), 625–637. https://doi.org/10.1083/JCB.200803013

Parrinello, S., Napoli, I., Ribeiro, S., Digby, P. W., Fedorova, M., Parkinson, D. B., Doddrell, R. D. S., Nakayama, M., Adams, R. H., & Lloyd, A. C. (2010). EphB Signaling Directs Peripheral Nerve Regeneration through Sox2-Dependent Schwann Cell Sorting. *Cell*, *143*(1), 145–155. https://doi.org/10.1016/J.CELL.2010.08.039

Peng, Y. B., Ringkamp, M., Campbell, J. N., & Meyer, R. A. (1999). Electrophysiological Assessment of the Cutaneous Arborization of Aδ-Fiber Nociceptors. *Journal of Neurophysiology*, *82*(3), 1164–1177. https://doi.org/10.1152/jn.1999.82.3.1164

Pereira, J. A., Lebrun-Julien, F., & Suter, U. (2012). Molecular mechanisms regulating myelination in the peripheral nervous system. *Trends in Neurosciences*, *35*(2), 123–134. https://doi.org/10.1016/J.TINS.2011.11.006

Perry, V. H., Tsao, J. W., Fearn, S., & Brown, M. C. (1995). Radiation-induced reductions in macrophage recruitment have only slight effects on myelin degeneration in sectioned

peripheral nerves of mice. *The European Journal of Neuroscience*, *7*(2), 271–280. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7538855

Plemel, J. R., Manesh, S. B., Sparling, J. S., & Tetzlaff, W. (2013). Myelin inhibits oligodendroglial maturation and regulates oligodendrocytic transcription factor expression. *Glia*, *61*(9), 1471–1487. https://doi.org/10.1002/glia.22535

Poplawski, G., Ishikawa, T., Brifault, C., Lee-Kubli, C., Regestam, R., Henry, K. W., Shiga, Y., Kwon, H., Ohtori, S., Gonias, S. L., & Campana, W. M. (2018). Schwann cells regulate sensory neuron gene expression before and after peripheral nerve injury. *Glia*, *66*(8), 1577–1590. https://doi.org/10.1002/glia.23325

Purves, D., Augustine, G. J., Fitzpatrick, D., Katz, L. C., LaMantia, A.-S., McNamara, J. O., & Williams, S. M. (2001). *Neuroscience* (2nd ed.). Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK11008/

Richards, D. M., & Endres, R. G. (2014). The Mechanism of Phagocytosis: Two Stages of Engulfment. *Biophysical Journal*, *107*(7), 1542. https://doi.org/10.1016/J.BPJ.2014.07.070

Riethmacher, D., Sonnenberg-Riethmacher, E., Brinkmann, V., Yamaai, T., Lewin, G. R., & Birchmeier, C. (1997). Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature*, *389*(6652), 725–730. https://doi.org/10.1038/39593

Rohlmann, A., Gotthardt, M., Hammer, R. E., & Herz, J. (1998). Inducible inactivation of hepatic LRP gene by cre-mediated recombination confirms role of LRP in clearance of chylomicron remnants. *The Journal of Clinical Investigation*, *101*(3), 689–695. https://doi.org/10.1172/JCI1240

Rosales, C., & Uribe-Querol, E. (2017). Phagocytosis: A Fundamental Process in Immunity. *BioMed Research International*, *2017*, 1–18. https://doi.org/10.1155/2017/9042851

Rosenberg, A. F., Wolman, M. A., Franzini-Armstrong, C., & Granato, M. (2012). In vivo nerve-macrophage interactions following peripheral nerve injury. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *32*(11), 3898–3909. https://doi.org/10.1523/JNEUROSCI.5225-11.2012

Rotshenker, S. (2011). Wallerian degeneration: the innate-immune response to traumatic nerve injury. *Journal of Neuroinflammation*, *8*(1), 109. https://doi.org/10.1186/1742-2094-8-109

Saher, G., & Simons, M. (2010). *Cholesterol and Myelin Biogenesis*. https://doi.org/10.1007/978-90-481-8622-8_18

Salzer, J. L. (2015). Schwann Cell Myelination. *Cold Spring Harbor Perspectives in Biology*, *7*(8), a020529. https://doi.org/10.1101/cshperspect.a020529

Shekarabi, M., Girard, N., Rivière, J.-B., Dion, P., Houle, M., Toulouse, A., Lafrenière, R. G., Vercauteren, F., Hince, P., Laganiere, J., Rochefort, D., Faivre, L., Samuels, M., & Rouleau, G. A. (2008). Mutations in the nervous system--specific HSN2 exon of WNK1

cause hereditary sensory neuropathy type II. *The Journal of Clinical Investigation*, *118*(7), 2496–2505. https://doi.org/10.1172/JCI34088

Strickland, D. K., Gonias, S. L., & Argraves, W. S. (2002). Diverse roles for the LDL receptor family. *Trends in Endocrinology and Metabolism: TEM*, *13*(2), 66–74. https://doi.org/10.1016/S1043-2760(01)00526-4

Takaku, S., Yako, H., Niimi, N., Akamine, T., Kawanami, D., Utsunomiya, K., & Sango, K. (2018). Establishment of a myelinating co-culture system with a motor neuron-like cell line NSC-34 and an adult rat Schwann cell line IFRS1. *Histochemistry and Cell Biology*, *149*(5), 537–543. https://doi.org/10.1007/s00418-018-1649-x

Taveggia, C., Zanazzi, G., Petrylak, A., Yano, H., Rosenbluth, J., Einheber, S., Xu, X., Esper, R. M., Loeb, J. A., Shrager, P., Chao, M. V., Falls, D. L., Role, L., & Salzer, J. L. (2005). Neuregulin-1 type III determines the ensheathment fate of axons. *Neuron*, *47*(5), 681–694. https://doi.org/10.1016/j.neuron.2005.08.017

Tofaris, G. K., Patterson, P. H., Jessen, K. R., & Mirsky, R. (2002). Denervated Schwann cells attract macrophages by secretion of leukemia inhibitory factor (LIF) and monocyte chemoattractant protein-1 in a process regulated by interleukin-6 and LIF. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *22*(15), 6696–6703. https://doi.org/20026699

Topilko, P., Schneider-Maunoury, S., Levi, G., Baron-Van Evercooren, A., Chennoufi, A. B. Y., Seitanidou, T., Babinet, C., & Charnay, P. (1994). Krox-20 controls myelination in the peripheral nervous system. *Nature*, *371*(6500), 796–799. https://doi.org/10.1038/371796a0

Tsuda, Y., Kanje, M., & Dahlin, L. B. (2011). Axonal outgrowth is associated with increased ERK 1/2 activation but decreased caspase 3 linked cell death in Schwann cells after immediate nerve repair in rats. *BMC Neuroscience*, *12*, 12. https://doi.org/10.1186/1471-2202-12-12

Uhlen, M., Fagerberg, L., Hallström, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szigyarto, C. A.-K., Odeberg, J., Djureinovic, D., Takanen, J. O., Hober, S., Alm, T., Edqvist, P.-H., Berling, H., Tegel, H., Mulder, J., Rockberg, J., Nilsson, P., Schwenk, J. M., Hamsten, M., von Feilitzen, K., Forsberg, M., Persson, L., Johansson, F., Zwalen, M., von Heijne, G., Nielsen, J., & Pontén, F. (2015). Tissue-based map of the human proteome. *Science*, *347*(6220), 1260419–1260419. https://doi.org/10.1126/science.1260419

van Furth, R., & Cohn, Z. A. (1968). The origin and kinetics of mononuclear phagocytes. *The Journal of Experimental Medicine*, *128*(3), 415–435. https://doi.org/10.1084/JEM.128.3.415

Vargas, M. E., Watanabe, J., Singh, S. J., Robinson, W. H., & Barres, B. A. (2010). Endogenous antibodies promote rapid myelin clearance and effective axon regeneration after nerve injury. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(26), 11993–11998. https://doi.org/10.1073/pnas.1001948107

Viader, A., Golden, J. P., Baloh, R. H., Schmidt, R. E., Hunter, D. A., & Milbrandt, J. (2011). Schwann cell mitochondrial metabolism supports long-term axonal survival and peripheral nerve function. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *31*(28), 10128–10140. https://doi.org/10.1523/JNEUROSCI.0884-11.2011

Viader, A., Sasaki, Y., Kim, S., Strickland, A., Workman, C. S., Yang, K., Gross, R. W., & Milbrandt, J. (2013). Aberrant Schwann Cell Lipid Metabolism Linked to Mitochondrial Deficits Leads to Axon Degeneration and Neuropathy. *Neuron*, *77*(5), 886–898. https://doi.org/10.1016/J.NEURON.2013.01.012

Waller, A. V. (1850). Experiments on the section of the glossopharyngeal and hypoglossal nerves of the frog, and observations of the alterations produced thereby in the structure of their primitive fibres. *Philosophical Transactions of the Royal Society of London*, *140*, 423–429. https://doi.org/10.1098/rstl.1850.0021

Weiner, J. A., Fukushima, N., Contos, J. J., Scherer, S. S., & Chun, J. (2001). Regulation of Schwann cell morphology and adhesion by receptor-mediated lysophosphatidic acid signaling. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *21*(18), 7069–7078. https://doi.org/10.1523/JNEUROSCI.21-18-07069.2001

Willnow, T. E., Orth, K., & Herz, J. (1994). Molecular dissection of ligand binding sites on the low density lipoprotein receptor-related protein. *The Journal of Biological Chemistry*, *269*(22), 15827–15832. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7515061

Wilson, F. H., Disse-Nicodème, S., Choate, K. A., Ishikawa, K., Nelson-Williams, C., Desitter, I., Gunel, M., Milford, D. V., Lipkin, G. W., Achard, J. M., Feely, M. P., Dussol, B., Berland, Y., Unwin, R. J., Mayan, H., Simon, D. B., Farfel, Z., Jeunemaitre, X., & Lifton, R. P. (2001). Human hypertension caused by mutations in WNK kinases. *Science (New York, N.Y.)*, *293*(5532), 1107–1112. https://doi.org/10.1126/science.1062844

Wolf, B. B., Lopes, M. B., VandenBerg, S. R., & Gonias, S. L. (1992). Characterization and immunohistochemical localization of alpha 2-macroglobulin receptor (low-density lipoprotein receptor-related protein) in human brain. *The American Journal of Pathology*, *141*(1), 37–42. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1632469

Xu, B., English, J. M., Wilsbacher, J. L., Stippec, S., Goldsmith, E. J., & Cobb, M. H. (2000). WNK1, a novel mammalian serine/threonine protein kinase lacking the catalytic lysine in subdomain II. *The Journal of Biological Chemistry*, *275*(22), 16795–16801. https://doi.org/10.1074/jbc.275.22.16795