A NOVEL NON-VIRAL KNOCKDOWN STRATEGY TARGETING RETINOBLASTOMA PROTEIN FOR REGENERATION OF PERIPHERAL AXONS

by

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Abstract

Pripheral nerve degeneration as a result of traumatic nerve injury is a common neurological condition that is characterized by varying degrees of sensory, autonomic, and motor dysfunction. Due to the high prevalence of peripheral nerve damage, there is a pressing need to develop novel therapies that augment the growth capacity of peripheral nerves in order to achieve optimal functional recovery. Tumor suppressor proteins are a class of anti-proliferative molecules that primarily function to hinder cancer development, but whose experimental suppression has been correlated with increased levels of peripheral axon regeneration. Retinoblastoma protein (Rb1) is one such tumor suppressor, whose role in the regeneration of peripheral nerves has yet to be fully elucidated. Rb1, by binding to the transcription factor E2F1, impedes its ability to promote the production of proteins necessary for cell cycle progression (Harbour et al., 1999). By inhibiting the action of Rb1 via transfection with small-interfering RNAs (siRNAs), our lab has previously highlighted Rb1 as a promising new molecular target for enhancing axon outgrowth (Christie et al., 2014).

Through the use of a novel, non-viral siRNA transfection technique, the current study sought to investigate the impact of a delayed knockdown of Rb1 after injury on the regeneration of distal sensory axons in the skin. Here we show that repeated injections of siRNA into the mouse hind paw followed by electroporation is capable of producing a partial knockdown of Rb1 mRNA in the ipsilateral lumbar dorsal root ganglia of intact, healthy mice, as well as those that had previously undergone a sciatic nerve injury. In order to assess the effects of a partial, delayed knockdown of Rb1 on the regeneration of epidermal sensory axons and functional recovery after nerve injury, we applied the aforementioned transfection technique in the weeks following a sciatic nerve crush and subsequently evaluated regenerative and functional outcomes at multiple time points through behavioural and electrophysiological testing, and histological indices.

Immunohistochemical analysis of the epidermis in footpad biopsies harvested 28 days post-crush showed that mice treated with Rb1-targeted siRNA possessed a significantly greater number of axon profiles crossing the dermal-epidermal junction than did those treated with scrambled control siRNA sequences. However, when innervation levels were examined at day 40, this pro-growth effect in the Rb1 siRNA-treated cohort was lost, indicating that the regenerative benefits observed on day 28 did not endure over an extended timeline. These histological findings were mirrored by the behavioural tests of thermal and mechanical sensitivity as well as by the electrophysiological measures of nerve conduction, which all lacked substantial between-group differences between the Rb1 siRNA and scrambled siRNA-treated cohorts, which would have indicated an improvement in functional recovery following Rb1 knockdown.

Altogether, the findings in this study suggest that hind paw injections of siRNA coupled with electroporation represents a novel and less-invasive means of procuring a partial genetic knockdown in sensory neuron cell bodies. We also conclude that although a delayed knockdown Rb1 improves the ability of regenerating axons to reinnervate the epidermis following injury, these early benefits do not persist at later stages beyond the point of siRNA treatment cessation. Taken in conjunction with previous findings reported by Christie et al. (2014), this suggests that there is an optimal time during which Rb1 suppression is most advantageous for promoting regrowth after injury. Knowledge of how the timing of molecular interventions affects regenerative outcome is clinically relevant and may aid in the development of optimized protocols for pharmacological intervention or gene therapy that could accompany existing surgical techniques for improved patient recovery after peripheral nerve trauma.

Preface

The research presented in this thesis was conducted at the University of Alberta under the supervision of Dr. Douglas Zochodne. The research project received research ethics approval from the University of Alberta Animal Research Ethics Board and Animal Care and Use Committee (ACUC) and was carried out in accordance with the University of Alberta Health Sciences Laboratory Animal Services (HSLAS) standards and guidelines. This thesis is an original work by Kasia Zubkow, with edits suggested by the examination committee.

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Glossary of Terms & Abbreviations

Apaf1	Apoptosis protease activating factor 1
APC	Adenomatous polyposis coli
CDK	Cyclin dependent kinase
cDNA	Complementary DNA
CMAP	Compound motor action potential
CNS	Central nervous system
DNA	Deoxyribonucleic acid
DP-1	Dimerization partner
DRG	Dorsal root ganglia
E2F	E2 promotor binding factor; family of transcription factors which includes E2F 1, 2, 3a, 3b, 4, 5, 6, 7a, 7b, and 8
GW9662	Pharmacological antagonist of PPARγ
HDAC1	Histone deacetylase 1
mRNA	Messenger RNA
p53	Tumor protein 53 (also known as TP53)
p107	Retinoblastoma-like protein 1
p130	Retinoblastoma-like protein 2
PGP9.5	Protein gene product 9.5
PIP(3)	Phosphatidylinositol (3,4,5)-triphosphate
PNS	Peripheral nervous system
PPARγ	Peroxisome proliferator-activated receptor γ

PTEN	Phosphatase and tensin homolog
qRT-PCR	Quantitative real time polymerase chain reaction
RAG	Regeneration associated gene
Rb1	Retinoblastoma protein
RFE	Relative fold expression
RNA	Ribonucleic acid
RNAi	RNA interference
siRNA	Small interfering RNA
SNAP	Sensory nerve action potential
TUNEL	Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling

Introduction

Damage to peripheral nerves, whether it be by direct physical insult, or by chronic degeneration due to an ongoing disease process or drug exposure, is a neurological affliction that commonly presents in clinical settings. Indeed, it is estimated that 2 to 3% of all patients received into level 1 trauma centers have peripheral nerve injuries, and that approximately 2.4% of the general population has peripheral neuropathy of one form or another (Robinson, 2000; Hughes, 2002).

Although the healing and repair process following a nerve injury can be prompted by microsurgical reattachment and repair, decompression, or the introduction of nerve grafts or conduits, reinnervation of target muscles, skin, and organs is often suboptimal, and patients may be left with lingering disability and dysfunction (Grinsell & Keating, 2014). Chronic sensory symptoms associated with nerve damage can include sensitivity (allodynia), paresthesia, which is defined as abnormal sensation such as numbness, tingling, or prickling, and neuropathic pain (Watson & Dyck, 2015). Motor symptoms include muscle weakness, atrophy, and paralysis (Grinsell & Keating, 2014).

Despite the significant advancements in and optimization of surgical nerve repair procedures, there remains a striking lack of therapeutic approaches directed at augmenting molecular regenerative pathways. The aim of this thesis is to address an aspect of the molecular challenges preventing sensory axon regeneration in the peripheral nervous system (PNS) following nerve injury and to explore a novel means by which pro-growth molecular pathways may be disinhibited through the use of non-viral RNA interference (RNAi) directed at a selected inhibitor of regeneration, retinoblastoma protein (Rb1).

1. Background

1.1 Anatomy of peripheral nerves

Peripheral nerve trunks are comprised of a combination of autonomic, sensory, and motor axons running to and from peripheral innervation targets (Kiernan & Rajakumar, 2014; Martini, Timmons, & Tallitsch, 2014). Anatomically, peripheral nerves are made up of their constituent axons organized into bundles, or fascicles, within three distinct layers of connective tissue (Kiernan & Rajakumar, 2014; Martini, Timmons, & Tallitsch, 2014). Surrounding the entire nerve is the epineurium, a tough, fibrous sheath that encompasses and protects all the fascicles contained within the nerve as well as some blood vessels that supply it. Each fascicle is then wrapped in its own perineurium, a layer of connective tissue that is composed of perineurial cells, collagen, and elastic fibers (Kiernan & Rajakumar, 2014; Martini, Timmons, & Tallitsch, 2014). The perineurium helps to form what is known as the "blood-nerve barrier," which protects the peripheral axons from various immunological and chemical components that are in the general circulation (Peltonen, Alanne, & Peltonen, 2013). Lastly, each individual axon within a fascicle is associated with an endoneurial sheath (Kiernan & Rajakumar, 2014; Martini, Timmons, & Tallitsch, 2014), a delicate arrangement of collagenous and elastic tissue fibers that encircles the axon and its accompanying glial cells. Together, the epineurium, perineurium, and endoneurium serve to maintain the organization and integrity of peripheral nerve axons and provide an important means of support following injury.

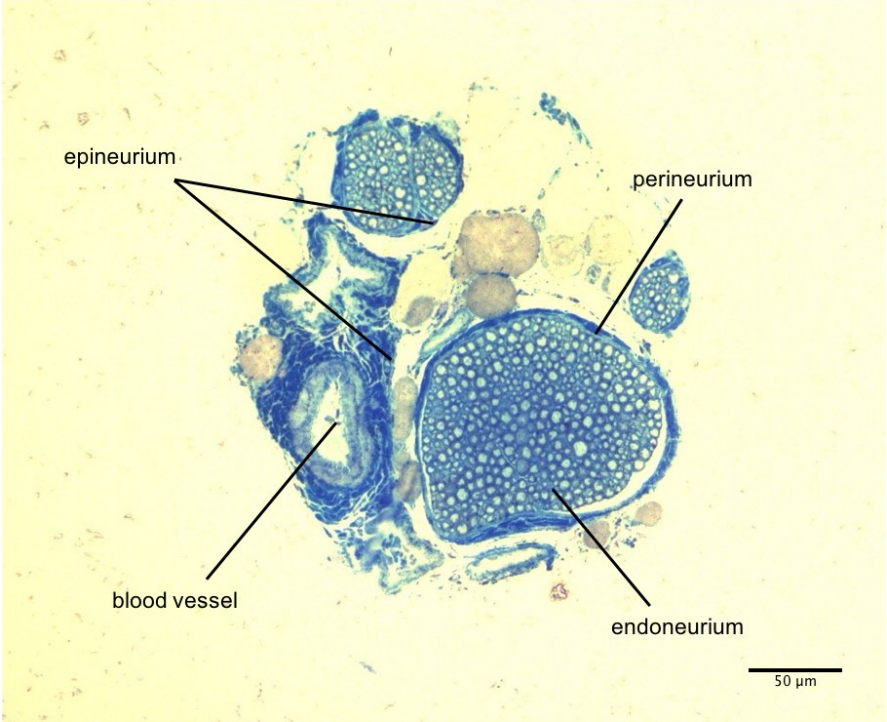

Figure 1. *Cross-sectional anatomy of a peripheral nerve*. This semi-thin epon section (stained with toluidine blue) taken from an intact mouse sural nerve at 20X magnification illustrates the three connective tissue layers of a peripheral nerve trunk – the epineurium, perineurium, and endoneurium – as well as some of the accompanying vasculature.

1.2 Types of nerve injuries

For the purposes of this article and the experiments therein described, peripheral nerve degeneration due to trauma will be the focus, as opposed to peripheral neuropathy, which is pathological degradation that occurs due to a disorder or disease. A traumatic nerve injury may be defined as damage to a peripheral nerve that occurs as a consequence of kinetic energy applied to the nerve or limb (Robinson, 2000).

There are several types of nerve injuries, the severity of which determines the likelihood and success of subsequent regeneration. Nerve injuries are often classified based on the degree of axon loss as well as by the level of disruption of the three connective tissue layers – the epineurium, perineurium, and endoneurium – described above. The Seddon classification of

peripheral nerve injuries proposes three levels of injury (Robinson, 2000; Zochodne, 2008). The mildest, called neurapraxia, involves temporary motor and sensory conduction block, but is not connected with any axonal degeneration (Robinson, 2000). Rather, neurapraxia is due to damage to the myelin sheath, thus disrupting the normal flow of action potentials down the axon (Robinson, 2000; Zochodne, 2008). This type of injury typically occurs in consequence of prolonged compression or distortion of the nerve, and recovery usually takes place within several weeks without any intervention (Robinson, 2000; Zochodne, 2008). Neurapraxia is also known as a 'first-degree' nerve injury under the Sunderland classification scheme (see Table 1).

Axonotmesis refers to injury to a peripheral nerve that can be brought about by mechanical trauma, such as stretch, crush, or percussion injuries, as well as by ischemic or chemical insult (Robinson, 2000; Zochodne, 2008). In contrast to neurapraxia, axonotmesis includes a variable degree of axonal disruption, which results in Wallerian-like degeneration of the nerve segment that is distal to the site of injury (Zochodne, 2008). That being said, the connective tissue layers remain relatively intact, thus providing a conduit for subsequent axon regeneration (Zochodne, 2008; Grinsell & Keating, 2014). Since Seddon's initial classification, axonotmesis has been broken down further into three subclasses or 'degrees' of injury, which ranges from second-degree, in which there is axonal damage with little to no endoneurial interruption, to fourth-degree injury which involves severe disruption of the endoneurium and perineurium, with only the epineurium remaining intact (Robinson, 2000; Zochodne, 2008). The Seddon and Sunderland classification schemes of peripheral nerve injuries are summarized in Table 1.

The final and most serious class of nerve injury is neurotmesis, or fifth-degree injury. In this type of injury, the nerve trunk and all its corresponding connective tissues, including the

epineurium, are severed (Robinson, 2000; Grinsell & Keating, 2014). As a result, the proximal and distal ends or 'stumps' become disconnected, and often times retract from one another (Zochodne, 2008). This loss of connection prompts the distal stump, that is the portion of the nerve that is no longer in contact with its corresponding cell bodies in the dorsal root ganglia, to undergo Wallerian degeneration. Wallerian degeneration is the term used to describe the organized process of axon degradation and its associated events following nerve transection (Zochodne, 2008). Even with surgical intervention, axon regeneration following this type of injury is limited, making the prospect of recovery of function bleak. Indeed, it is estimated that as little as 10% of transected axons will eventually regain contact with their targets following this type of injury (Witzel et al., 2005).

Seddon classification	Sunderland classification	Pathology	Clinical Features & Prognosis
Neurapraxia	First degree	Myelin injury or ischemia; no Wallerian degeneration	Temporary axon conduction block Excellent recovery in weeks to months
Axonotmesis		Axon loss (Wallerian-like degeneration) Variable stromal disruption	Good to poor, depending upon integrity of supporting structures and distance to innervation target
	Second degree	Axon loss Endoneurial tubes intact Perineurium intact Epineurium intact	Good, depending on distance to target
	Third degree	Axon loss Endoneurial tubes disrupted Perineurium intact Epineurium intact	Poor Axonal misdirection Surgery may be required
	Fourth degree	Axon loss Endoneurial tubes disrupted Perineurium disrupted Epineurium intact	Poor Axonal misdirection Surgery usually required
Neurotmesis	Fifth degree	Axon loss (Wallerian degeneration) Endoneurial tubes severed Perineurium severed Epineurium severed	No spontaneous recovery Surgery required Prognosis after surgery guarded

Table 1. *Summary of the Seddon and Sunderland classifications of peripheral nerve injuries*. Adapted from Robinson LR (2000) Traumatic injury to peripheral nerves. Muscle & Nerve 23:868-873.

The ensuing experiments will utilize axonotmesis in the form of a surgically-inflicted sciatic nerve crush to study peripheral axon regeneration and skin reinnervation following injury and the impact of specific molecular manipulation using RNA interference. Sciatic nerve crush will be severe enough to elicit Wallerian-like degeneration, but not so extreme as to make regeneration unlikely; the plan is to render a commonly used and easily reproducible model of peripheral nerve injury (Dubovy, 2011). Since axonal regeneration often occurs following a sciatic nerve crush, the process of reinnervation and functional recovery may be assessed and measured through both behavioural and electrophysiological testing techniques.

1.3 Peripheral nerve regeneration and associated challenges

When a peripheral nerve is axotomized or partially injured, Wallerian or Wallerian-like degeneration occurs as a result (Zochodne, 2008; Dubovy, 2011). Wallerian-like degeneration is a similar process to Wallerian degeneration, except that it occurs in the context of a partial nerve injury without transection. Degeneration of distal axons whose connection with their proximal counterparts is disrupted or severed is an orchestrated process that is critical if subsequent regeneration is to occur (Bisby & Chen, 1990; Levy, Kubes, & Zochodne, 2001; Dubovy, 2011). This process entails fragmentation of the distal nerve stump and is associated with myelin degradation, Schwann cell dedifferentiation and proliferation, and macrophage infiltration (Dahlin & Brandt, 2004; Zochodne 2012). Once the axonal and myelin debris are cleared, a task that takes approximately 1-2 weeks, growth cones will emerge from the proximal stump of the injured nerve, and the axons will slowly begin to regenerate (Navarro, Vivó, & Valero-Cabré, 2007; Krishnan et al., 2015).

Although primarily used in relation to neurons in the brain and spinal cord, the term 'neural plasticity' may be generally defined as changes to neuronal cell morphology, gene expression, and cell-cell interaction that allow neurons to respond and adapt to environmental stimuli (Hirai et al., 2017). Based on this description, it may be argued that peripheral sensory neurons also exhibit neural plasticity, especially after a traumatic nerve injury. Following insult, their cell bodies in the dorsal root ganglia immediately undergo a series of morphological changes altogether known as the "cell body reaction," which is eventually succeeded by axon sprouting (Zochodne, 2012). These morphological changes are accompanied by an upregulation in the expression of regeneration-associated genes, as well as by signalling changes between the axons and their neighboring Schwann cells, thereby influencing their interaction so as to promote regeneration (Zochodne, 2012). Therefore, within the context of this thesis, the term 'plasticity' when applied to primary sensory neurons in the PNS is referring to the combination of the above-mentioned changes, which facilitate axon sprouting and regeneration in the wake of an injurious event.

Following traumatic nerve injury, the damaged peripheral neurons must transition from a stable transmission state to a highly active, regenerative phenotype (Zochodne, 2012). This inevitably involves drastic changes to their structural and molecular machinery thereby necessitating considerable adjustments to the neurons' transcriptional program. Specifically, there is an upregulation in the expression of regeneration-associated genes (RAGs), or genes that contribute to the regenerative response (Zochodne, 2008). Molecules such as tubulin, growth-associated protein 43 (GAP43), heat shock protein 27 (HSP27), and activated transcription factor 3 (ATF3) are just a few of the many RAGs that are upregulated as a result of peripheral axon

damage and that help to facilitate new growth (Tsujino et al., 2000; Seijffers, Allchorne & Woolf, 2006; Navarro, Vivó, & Valero-Cabré, 2007; Ma et al., 2011; Zochodne, 2012).

The success of these regenerative events is often hampered by a number of intrinsic and extrinsic obstacles. Although the PNS is widely referred to as having superior regenerative capabilities, in actuality there are many impediments to complete functional recovery after nerve trauma that can leave patients with permanent motor, sensory, or autonomic deficits. These hurdles include, but are not limited to, the slow rate of axonal elongation, which in mammals typically occurs at a rate of 1-3 mm/day, distance to end targets, inappropriate or misguided reinnervation, inflammation and scarring of the surrounding connective tissue, and parent neuronal cell death (Navarro, Vivó, & Valero-Cabré, 2007; Zochodne, 2012; Wu & Murashov, 2013). It is also generally recognized that the longer it takes for complete reinnervation to take place, the poorer the regenerative outcome will be (Fu & Gordon, 1995a; Fu & Gordon, 1995b; Zochodne, 2012; Krishnan et al., 2015). The reason for this failure is multifactorial. Following the initial insult, the expression of RAGs peaks at approximately 1-2 weeks post-injury but then gradually declines over the ensuing months, making it difficult for neurons to sustain a steady rate of regeneration over a prolonged period of time (Tsujino et al., 2000; Krishnan et al., 2015). Extended denervation, or loss of nerve supply, can also lead to changes in the microenvironment that may make it less hospitable to incoming axons (Fu & Gordon, 1997; Höke et al., 2002). Schwann cells, the principal glial cell type of the PNS, that are chronically denervated enter a state of dormancy that is accompanied by a downregulation of secreted growth factors, thus impairing their ability to support regenerating axons (Höke et al., 2002; Faroni et al., 2015). In addition to the loss of distal glial support, changes to the target tissues themselves, such as atrophy of the myofibers that constitute muscles, may impede the success of eventual

reinnervation and recovery of function (Fu & Gordon, 1995b; Höke & Brushart, 2010; Faroni et al., 2015).

Due to the aforementioned drawbacks associated with delayed end-organ contact and a protracted regenerative timeline, molecular interventions aimed at enhancing neural plasticity and augmenting the pace of regeneration would be therapeutically advantageous. Potential molecular targets for achieving these goals are tumor suppressor proteins. Within mitotically-competent non-neuronal cells, tumor suppressor proteins such as p53, APC, PTEN, and Rb1 function to regulate cell growth and prevent excessive proliferation that may otherwise lead to cancer (Sun & Yang, 2010; Krishnan et al., 2015). Although the expression of these antioncogenes is beneficial under healthy conditions, within injured peripheral neurons these molecules may actively suppress cellular machinery and pathways that could foster plasticity, repair, and growth. An example of this is the tumor suppressor phosphatase and tensin homolog (PTEN). Through its dephosphorylating action on PIP(3), PTEN functions as a negative regulator of PI3K/Akt signaling, a molecular pathway that is important in cell growth, survival, and proliferation (Chalhoub & Baker, 2009; Christie et al., 2010). Under normal conditions, the expression of PTEN is a critical player to preventing tumorigenesis, however within the context of peripheral nerve injury its inhibitory effect on the PI3K/Akt pathway can greatly hinder regenerative efforts (Christie et al., 2010; Krishnan et al., 2015). Indeed, it has been demonstrated both in *vitro* and in *vivo* that inactivating or reducing the activity of PTEN following nerve injury, either through administration of small-interfering RNAs (siRNAs) or the PTEN pharmacological inhibitor bpV(pic), leads to an increase in the number and length of outgrowing axons (Christie et al., 2010; Krishnan et al., 2015).

Unlike PTEN, Rb1 is a tumor suppressor molecule whose role in the adult peripheral nervous system post-injury has yet to be well characterized. A study performed by Christie and colleagues (2014) was the first to suggest that an acute knockdown of Rb1 immediately after nerve injury is advantageous for regenerating axons at the site of injury (Christie et al., 2014). That being said, little is known about the effect of Rb1 suppression on the regeneration of axons further distal to the site of injury, or of the impact that a chronic, prolonged knockdown could have on skin reinnervation as it proceeds well after the initial injury events. Understanding the impact of delayed or postponed intervention after injury when some degree of regeneration is underway is critically important and addresses a common clinical scenario.

1.4 Retinoblastoma protein

Retinoblastoma (Rb1), a 928-amino acid-long protein transcribed from the retinoblastoma gene, was discovered in 1986 and was the first tumor suppressor to be described (Friend et al., 1986; Dyson, 2016). A member of the family of pocket proteins along with p107 and p130, Rb1 is made up of 3 distinct protein domains, each possessing multiple protein-binding surfaces (Dick & Rubin, 2013). The existence of these numerous binding sites is central to Rb1's molecular function as a scaffold for protein interactions and complex formation (Rubin, 2013). Rb1's ability to recruit histone deacetylases and other chromatin-modifying proteins to promoter regions in order to repress E2F-dependent transcription is just one example of this propensity for forming multiple and oftentimes simultaneous protein interactions (Trouche et al., 1997; Brehm et al., 1998). The activity of Rb1 can be modulated through numerous post-translational modifications such as acetylation, methylation, and phosphorylation (Chan et al., 2001; Carr et al., 2011; Dick & Rubin, 2013; Dyson, 2016). Of these modifications,

phosphorylation is especially important as at least thirteen different phosphorylation sites have been described on Rb1, each differentially affecting the conformation of Rb1 and thus its function (Rubin, 2013; Engel et al., 2015). In most cases, phosphorylation at the various sites has an inhibitory role on Rb1 by creating interdomain interactions that prevent certain proteins from binding (Harbour et al., 1999; Rubin, 2013; Engel et al., 2015). Overall, the multiple binding surfaces and the diverse post-translational modifications taken together with the fact that Rb1 has been documented to interact with nearly 200 different proteins, highlights the intricacy of Rb1 function and its potential to fulfill a multi-faceted role in cellular signaling pathways (Rubin, 2013).

In normal adult tissues Rb1 is ubiquitously expressed, however, as alluded to above, its relative activity within specific cell types is regulated by its phosphorylation status (Cordon-Cardo & Richon, 1994; Classon & Harlow, 2002). Depending on the tissue, developmental stage, and cellular context, Rb1 is involved in a myriad of physiological processes including cell proliferation, differentiation, senescence, tissue morphogenesis, genomic stability, and apoptosis (Engel et al., 2015). Given the general lack of literature addressing Rb1 functioning within the adult peripheral nervous system, a portion of what has been established about the role of Rb1 with respect to cell cycle regulation and embryonic development will be briefly summarized.

1.5 Rb1 in cell proliferation & cancer

Retinoblastoma protein is best known for its action as a negative regulator of cell division through its interaction with and inhibition of E2F1 (Harbour et al., 1999; Dick & Rubin, 2013). E2F1 is one of ten proteins in the E2 promoter family of transcription factors, and it in particular has been shown to be crucial in mediating the transcription of genes whose expression is critical

for cell cycle progression through the G1/S phase checkpoint (Ertosun, Hapil, & Nidai, 2015). Examples of genes regulated by E2F1 promoter activity are those corresponding to DNA polymerase α and cyclin A, which function in DNA replication and cell cycle progression, respectively (DeGregori et al., 1995; Yam, Fung, & Poon 2002).

The binding of Rb1 with E2F1 is dependent upon its phosphorylation status, which is mediated by the action of cyclin-dependent kinases (CDKs) (Harbour et al., 1999). Through the stimulation of growth factor receptors, mitogenic signaling pathways eventually lead to the production of cyclin D1 (Whittaker et al., 2004). By complexing with CDK4 and 6, these proteins form an activated kinase complex which then phosphorylates Rb1 (Harbour et al., 1999). As a result of this event, Rb1 releases histone deacetylase 1 (HDAC1), rendering chromatin more transcriptionally active and allowing for the transcription and translation of cyclin E (Harbour et al., 1999). Cyclin E then associates with CDK2 to once again phosphorylate Rb1, thereby causing it to release the transcription factor E2F1 (Harbour et al., 1999; Whittaker et al., 2004). E2F1 then dimerizes with a dimerization partner protein (DP-1) to form an active heterodimer that is able to bind the E2F promoter regions within target genes (Helin et al., 1993; Ertosun, Hapil, & Nidai, 2015). Through this series of interactions and discrete phosphorylation events, the transcriptional regulation imposed by Rb1 is attenuated, and the cell is able to proceed into S phase during which it will duplicate its genetic material and commit to cell division (Harbour et al., 1999).

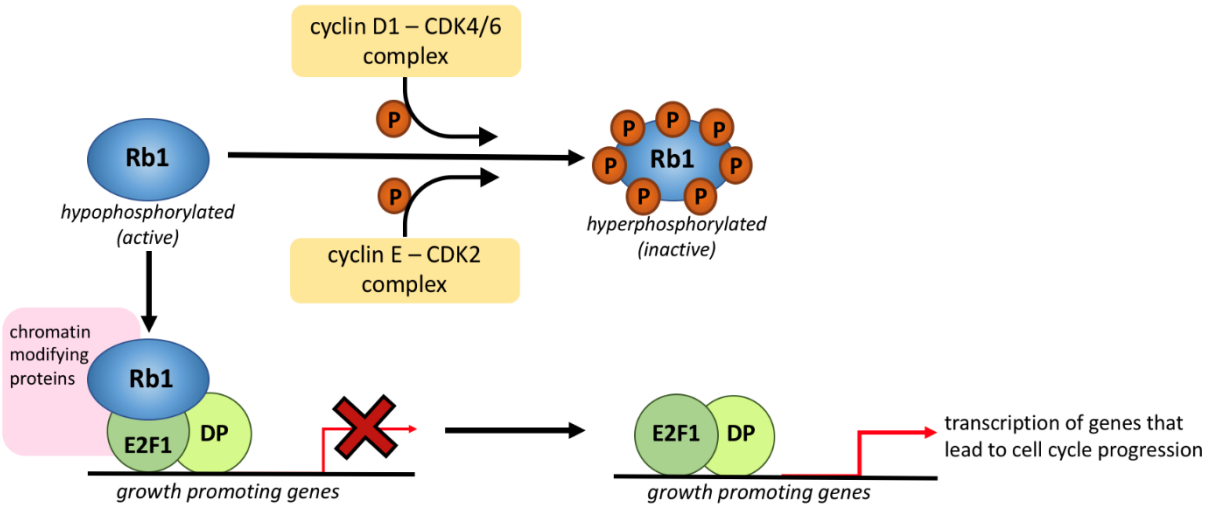

Figure 2. *The canonical Rb1/E2F1 pathway of G1/S phase checkpoint regulation.*

Because of its central role in blocking excessive cell proliferation and promoting cell cycle exit, the expression and proper functioning of Rb1 is especially vital to preventing tumorigenesis within developing tissues. The consequences of a loss or lack of Rb1 is clearly exhibited in retinoblastoma, a type of eye malignancy that typically occurs in childhood due to a loss-of-function mutation on chromosome 13 that renders the Rb1 gene inactive (Friend et al., 1986; Swiss & Casaccia, 2010). In addition to retinoblastoma, deficiencies in Rb1 activity has been documented in a number of other malignant tumors, including most osteosarcomas and small cell lung carcinomas (Classon & Harlow, 2002; Dyson, 2016).

The ability of hypophosphorylated Rb1 to suppress gene transcription and maintain cellular quiescence makes it of interest with respect to peripheral nerve regeneration, a situation in which neurons must upregulate their levels of transcription and protein synthesis in order to meet the demands associated with axonal repair and regrowth. If the growth-suppressive control of Rb1 could be temporarily diminished in the period following a traumatic nerve injury, it may

enhance the expression of pro-growth genes as well as enable the injured neurons to maintain transcriptional upregulation more readily.

1.6 Rb1 in the nervous system

Apart from its ability to control cell proliferation by regulating the progression through cell-cycle checkpoints, Rb1 has also been implicated in processes such as tissue differentiation and morphogenesis, cell migration, and post-mitotic cell survival (Andrusiak et al., 2012; Matsui et al., 2017). All of these cellular processes are necessary to proper development, therefore it is not surprising that a great deal of the literature surrounding Rb1 in the nervous system is focused on its role during neurodevelopment.

During embryogenesis, Rb1 is most prominently expressed in cell lineages and tissues corresponding to the developing nervous system, skeletal muscles, lens, and hematopoietic system (Jiang et al., 1997; Classon & Harlow, 2002). In consequence, these tissues show pronounced defects when Rb1 is deleted or dysfunctional, and it is well established that Rb1 nullizygous mice embryos are not viable, and typically die between days 13 and 16 of gestation due to a combination of neural and hematopoietic abnormalities (Jacks et al., 1992; Lee et al., 1992; Classon & Harlow, 2002). Specifically within the nervous system, examination of $Rb1^{-/-}$ embryos showed widespread cell death throughout the CNS and PNS, especially within the hindbrain, spinal cord, and dorsal root ganglia (Jacks et al., 1992; Lee et al., 1992). Indeed, by embryonic day 15.5, Lee and colleagues (1992) described the dorsal root ganglia (DRG) as "having withered to nothing but small sheath-like structures." In contrast, other areas exhibited increased levels of ectopic mitosis and hyperproliferation due to the failure of differentiating neurons to exit the cell cycle (Lee et al., 1992). To an extent, these contrasting observations are

attributed to the deregulation of E2F transcription factors that occurs in Rb1-deficient conditions (Chau & Wang, 2003). In addition to E2F1, Rb1 has been documented to interact with E2F2, E2F3a, and E2F4 (Classon & Harlow, 2002; Dimova & Dyson, 2005). Of the E2F transcription factors, E2F1, 2, and 3a are potent activators of transcription and are generally considered to promote cell cycle progression, which may account for the aberrant proliferation seen in Rb1 knockout embryos (Dimova & Dyson, 2005; Ertosun, Hapil, & Nidai, 2015). However, depending on the cellular context, these E2Fs can have drastically different roles such as initiating apoptosis (Dimova & Dyson, 2005). For example, binding sites for E2F1, as well as p53, have been found in the promoters of pro-apoptotic genes including apoptosis protease activating factor 1 (Apaf1), a central downstream constituent of the intrinsic mitochondria-dependent apoptotic pathway (Moroni et al., 2001; Chau & Wang, 2003). The E2F1/p53-dependent induction of Apaf1is greatly responsible for the massive amounts of cell death observed in the CNS of $Rb1^{-/-}$ mutant mouse embryos, as it has been shown through terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) analysis that elimination of Apaf1 ameliorates the central apoptotic phenotype in Rb1 nullizygous embryos (Guo et al., 2001; Chau & Wang, 2003). However, this E2F1/p53/Apaf1-dependent pathway of apoptosis in the absence of Rb1 does not apply to peripheral neurons. In contrast to the CNS, TUNEL staining indicated widespread apoptosis throughout the DRGs and trigeminal ganglia within $Rb1^{-/-}$:$Apaf^{-/-}$ double knockout embryos (Guo et at., 2001) and it has also been shown that mutation of p53 in Rb1-null conditions inhibited cell death in central neurons, but not in peripheral neurons (Macleod et al., 1996). Instead, Rb1 deficiency-induced apoptosis in peripheral neurons is reliant upon caspase 3 expression and activation, for deletion of caspase 3 in Rb1-null embryos rescued peripheral neurons but did not affect cell death in the CNS

(Simpson et al., 2001). Overall, the dichotomy of apoptotic signaling under Rb1-null conditions between the CNS and PNS exemplifies the complexity of both Rb1 and E2F signaling networks and emphasizes the need to explore the functions of these proteins across multiple cellular contexts.

Although the actions of Rb1 have been well studied within the fields of cancer and developmental research, little is known about its role in the adult peripheral nervous system, healthy or otherwise. The first article to describe a role for Rb1 in adult peripheral nerves and their regeneration after injury was published by Christie and colleagues in 2014. To begin, the group confirmed the expression of Rb1 in both normal and injured DRGs, as well as in sciatic nerve axons (Christie et al., 2014). Interestingly, although the expression of Rb1 persisted in the cell bodies of injured sensory neurons when analyzed 3 days post-injury, the relative messenger RNA (mRNA) and protein levels were decreased (Christie et al., 2014). This suggests that under normal circumstances, the amount of neuronal Rb1 is reduced after injury to potentially facilitate the de-repression of pro-growth pathways. To assess Rb1's function, the group next evaluated the impact of Rb1 knockdown on neurite outgrowth in dissociated cell cultures by transfecting the neurons with Rb1 siRNA (Christie et al., 2014). In the pre-injured adult rat sensory neuron cultures in which Rb1 expression was suppressed, substantial increases in neurite outgrowth and process length were found in comparison to control cultures (Christie et al., 2014). Furthermore, the addition of an E2F1-targeted siRNA to these cultures eradicated this Rb1 knockdown-induced plasticity, indicating that the initial effect was dependent upon downstream E2F1 activation (Christie et al., 2014).

Looking in *vivo*, the knockdown of Rb1 in the days immediately following sciatic nerve transection resulted in a significant rise in axonal sprouting and outgrowth from the proximal

stump (Christie et al., 2014). This neuronal effect was accompanied by an increase in the number of Schwann cell profiles at the transection zone. Moreover, following a sciatic crush in mice, the positive growth effects produced by Rb1 knockdown translated into an enhanced functional recovery with respect to mechanical and thermal sensitivity, hind paw grip strength, and nerve conduction velocity (Christie et al., 2014). Taken together, these promising in *vitro* and in *vivo* findings suggest a powerful role for Rb1 in hindering early peripheral nerve regeneration following injury.

1.7 Present study: novel non-viral knockdown strategy against Rb1 & its effect on peripheral nerve regeneration

The present study sought to investigate the potential therapeutic benefit of suppressing Rb1 expression in the weeks following peripheral nerve injury through the use of a novel non-viral knockdown method. Specifically, we endeavored to determine the efficacy of electroporation following a local subcutaneous injection of small interfering RNAs (siRNAs) to the mouse hind paw as a means of procuring knockdown. Due to the potentially problematic immunogenic tolerability of human subjects to viral vectors (Thomas, Ehrhardt, & Kay, 2003), we chose to make use of siRNA sequences targeted against Rb1 in order to achieve a transient genetic knockdown, that is, a reduction in the expression of Rb1 by increasing the degradation of its corresponding mRNA transcripts. As summarized above, since experiments have already been carried out by Christie and colleagues (2014) investigating the effects of acute Rb1 knockdown on neurite outgrowth in *vitro* and regeneration at the proximal stump in *vivo*, we were interested in evaluating the impact of a transient knockdown maintained over a chronic time course on the regeneration of distal sensory axons reinnervating the skin.

In the first experiment, the efficacy of electroporation coupled with subcutaneous injection of siRNAs to the hind paw was assessed as a means of reducing Rb1 mRNA expression in the cell bodies of peripheral sensory axons located in the lumbar DRGs of uninjured mice. Since electroporation has never been used on the hind paw to increase the uptake of siRNAs, it was important to ascertain the effectiveness of this protocol before attempting to use it within the context of a nerve injury. Based on evidence from other researchers using electroporation to enhance the absorption of substances into mouse DRGs and embryos (Saijilafu, Hur, & Zhou, 2011; Matsui et al., 2011), we predicted that application of electrical pulses following an injection to the footpad would permeabilize the membranes of nearby axon terminals, allowing greater uptake of the siRNA construct into sensory neurons that would manifest as a knockdown in the DRGs when analyzed with qRT-PCR.

In the ensuing experiments, the effects of chronic electroporation-induced non-viral knockdown of Rb1 on regeneration after injury were assessed through behavioural, electrophysiological, and histological indices. Based on the results demonstrated by Christie et al. (2014), we hypothesized that a delayed disruption of Rb1 expression would enhance axon outgrowth into the epidermis following peripheral nerve injury, and that this would translate into an improvement of functional recovery.

2. Materials & Methods

Animal care. Cohorts of adult male CD1 mice (8-10 weeks) were used for all experiments. All behavioural assays and procedures were carried out according to approved standard operating procedures and in compliance with the University of Alberta Animal Care and Use Committee as well as the University of Alberta Health Sciences Laboratory Animal Services (HSLAS) review, incorporating guidelines from the Canadian Council on Animal Care (CCAC).

Behavioural assays.

Mechanical hypersensitivity. Von Frey monofilaments were used to assess mechanical allodynia in mice. Mice were separated and placed on a wire mesh platform, whereupon the centre of both hind paws were stimulated with Von Frey monofilaments. The bending force of each filament that elicited paw withdrawal and associated pain behaviours at least 75% of the time was recorded as the mechanical pain threshold. Mice were sequentially tested with a set of monofilaments with weights of 0.16, 0.4, 0.6, 1.0, 1.4, 2.0, and 4.0 grams. Testing did not go beyond the 4.0g filament in order to avoid tissue damage and other testing confounds. If the animal did not withdraw in response to stimulation with the 4.0g hair, their score was still recorded as 4.0g. Ipsilateral mechanical sensitivity data is displayed graphically as the raw score for withdrawal threshold (in grams).

Thermal sensitivity. Thermal sensitivity was tested by applying a radiant heat source to the middle of the hind paw and then timing the latency (in seconds) to withdrawal (Christie et al., 2014). Mean withdrawal latency was determined from three separate measurements in each mouse. In order to prevent damage to the animals' paws, each individual trial did not exceed 30

seconds of heat exposure. Ipsilateral thermal sensitivity data is displayed graphically as the latency to paw withdrawal (in seconds).

Electrophysiology. Motor and sensory multi-fiber electrophysiological recordings were carried out under isoflurane anesthesia using platinum subdermal needle electrodes inserted at the appropriate stimulation and recording sites. All measurements were taken using a Cadwell Sierra Ascent base unit, 2-channel amplifier, and single electrical stimulator, together with the NCV feature on the corresponding Sierra® Ascent™ computer software. The velocity and amplitude of compound motor action potentials (CMAPs) from the portion of the sciatic nerve extending between the sciatic notch and knee were determined by supramaximally stimulating first at the sciatic notch and then at the knee and recording from the interosseous foot muscles in the hind paw. Orthodromic sensory nerve action potentials (SNAPs) recorded from the sural nerve were obtained by stimulating the digital nerves and recording at the knee. The sensory velocities and amplitudes were averaged across 10 consecutive recordings. Distances between the recording and stimulating electrodes were manually measured and inputted into the Sierra® Ascent™ software in order to compute the velocities from the collected motor and sensory waveforms. Near nerve temperature was controlled and monitored throughout the duration of the protocol through the use of a heat lamp (37°C) and a thermometer.

Sciatic nerve crush. Mice were anaesthetized using inhaled 2% isoflurane. The right (ipsilateral) hind leg was shaved and disinfected, and a small incision was subsequently made just below the right hip, along the sciatic notch on the anterior thigh. The entire sciatic nerve trunk, approximately halfway down the leg, was then crushed for 15 seconds using forceps. The forceps

were then rotated 90 degrees, and the nerve was crushed for another 15 seconds in this orientation. The wound was then closed by suture and checked twice a day for 3 days post-surgery. At these times, the mice were given 20μL of buprenorphine administered subcutaneously for postoperative pain management.

siRNA administration & electroporation. Rb1 or scrambled siRNA (Qiagen, Germantown, MD) was mixed in HiPerfect Transfection Reagent (Qiagen) for 20 minutes at room temperature, after which saline was added to the solution. While under isoflurane anesthesia, 20uL of the siRNA solution was subcutaneously injected into the footpad of the right hind leg. Immediately following injection, Hank's Balanced Salt Solution (HBSS) was pipetted onto the surface of the footpad to facilitate conduction. Electrodes were then held in contact with the skin, with the negative electrode positioned near the site of injection and the positive electrode positioned further down on the proximal end of the footpad. Five 25V pulses, separated by 1 second and each lasting 50 milliseconds in duration, were then administered using an ECM 830 Electro Square Porator^{TM} unit.

Tissue harvesting. Ipsilateral and contralateral lumbar DRGs (L4-L6), sciatic and sural nerves, and footpad skin biopsies were harvested and used for Western blotting, immunofluorescent imaging, epon semi-thin sectioning, and quantitative real-time polymerase chain reactions (qRT-PCR). Contralateral tissue, which was not associated with any siRNA treatment or injury, was used for comparison against ipsilateral samples in qRT-PCR, immunohistochemistry, and epon sectioning analysis.

Reverse transcription and qRT-PCR. Contralateral and ipsilateral DRGs corresponding to the lumbar spinal sections L4-L6 were collected from mice that received either a negative control scrambled siRNA or a Rb1-targeted siRNA, and then placed directly into TRIzol® reagent (Invitrogen, Waltham, MA). RNA extracted from the samples underwent DNase (Promega, Madison, WI) treatment in order to eradicate any potential genomic DNA contamination. The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was then used to convert the mRNA to its complementary DNA (cDNA). The resulting cDNA was used in the quantitative real-time polymerase chain reactions (qRT-PCR).

The primer sequences corresponding to the primers that were used throughout this project are listed in Table 2. In this project, the cDNA for the housekeeping gene ribosomal protein lateral stalk subunit P0 (RPLP0) was used as an endogenous control to allow for quantification of relative mRNA expression of our gene of interest, Rb1. qRT-PCR was then performed using the ABI PRISM® 7000 Sequence Detection System (ThermoFisher, Waltham, MA) and SYBR Green (Applied Biosystems) as a fluorescent indicator for cDNA amplification.

The cycle threshold (CT) for each well in a qRT-PCR plate is the point at which the fluorescence signal from the amplifying gene product can be distinguished from the background noise (Pfaffl, 2001). Relative fold expression (RFE) values were generated using the comparative CT method ($2^{-\Delta\Delta CT}$), in which the CT values for the target gene of interest is standardized in relation to RPLP0 expression. These values were then statistically compared between cohorts using two-tailed unpaired students' t-tests in GraphPad Prism 7 software.

Gene	GenBank Accession No.	FWD/REV	Primer Sequence (5'-3')
RPLP0	NM_007475.5	FWD	AAGAACACCATGATGCGCAAG
		REV	TTGGTGAACACGAAGCCCA
Rb1	NM_009029.2	FWD	CTTTACTGGCCTGTGCTCTT
		REV	ATCCACGGGAAGGACAAATC

Table 2. *Primers used in qRT-PCR reactions.*

Immunohistochemistry and axon counting. Footpad biopsies fated for immunofluorescent staining and confocal imaging were harvested with a 3-millimeter biopsy punch and placed in PLP solution (paraformaldehyde, L-lysine, and sodium periodate) immediately post-mortem. After 24 hours, samples were rinsed with 0.1M Sorrenson's phosphate buffer and cryoprotected overnight at 4°C in a 20% glycerol solution. The tissue was then frozen in OCT and stored at -80°C until it was ready to be sectioned and mounted onto slides.

Twenty-five micron-thick footpad sections underwent antigen retrieval in 65°C Tris-EDTA buffer solution for 1.5 hours. Following this incubation, they were blocked and stained according to previously established lab protocols using a 1:500 solution of rabbit anti-PGP9.5 (EnCor Biotech. Inc., Gainesville, FL) as the primary antibody. PGP9.5, also known as UCH-L1, is a cytoplasmic protein that is specifically expressed in neurons and neuroendocrine tissue and has been commonly used as a marker for peripheral nerve fibers. It is therefore convenient for visualizing sensory fibers crossing from the dermis into the epidermis.

Footpad sections were imaged using a Leica SP5 confocal microscope. Z-stack images were obtained using a step-size of 0.5µm and 63X/1.2 objective lens. For each skin section, 5 consecutive frames were imaged, and this was repeated in 3 randomly-chosen sections per animal. Using ImageJ software, the number of both horizontal and vertical nerve fibers crossing from the dermis into the epidermis were counted, averaged, and expressed per millimeter of epidermis by an experimenter who was blind to the identities of the treatment groups. Quantification of the number of axons per square millimeter of epidermis included those traversing the dermal-epidermal junction, as well as isolated axon fragments within the epidermis. These various counting approaches were utilized so that comparisons in cutaneous

reinnervation can be made with previously published work as well as with human clinical data, which is conventionally reported as the number of axons crossing the dermal-epidermal junction per millimeter (Toth, Brussee, & Zochodne, 2006; Ebenezer et al., 2007; Beiswenger, Calcutt, & Mizisin, 2008).

Epon semi-thin sections. During tissue harvesting, contralateral and ipsilateral biopsies of the sural nerve were obtained from animals in both cohorts. The samples were then fixed in 2.5% glutaraldehyde buffered by 0.025M cacodylate. They were then post-fixed in 2% osmium tetroxide, dehydrated, and embedded in epon resin. One micron-thick sections from the distal end of the nerve were then sectioned using an ultramicrotome, mounted onto slides, and stained with 0.5% toluidine blue.

Bright-field images of whole nerve sections were taken using oil immersion under a 100X objective lens on a Zeiss Axioskop microscope. The images corresponding to each individual section were then stitched together in Adobe Photoshop CC 2018 and manually counted in order to ascertain the total number of axons per section. This procedure was repeated across 3 sections per animal.

Statistical analysis. All statistical analyses were performed in GraphPad Prism 7 software using two-tailed student's t-tests, one-way analysis of variance (ANOVA), or two-way repeated measures ANOVAs as appropriate. An alpha level of 0.05 was used throughout the study as a threshold for determining statistical significance.

3. Results

3.1 Effect of electroporation on Rb1 expression in uninjured mice.

To determine the efficacy of injection coupled with electroporation as a means of procuring a non-viral knockdown of Rb1 mRNA, Rb1-targeted siRNA was injected into the right (ipsilateral) hind paw of a cohort of mice (n=4), with another cohort (n=3) receiving negative control scrambled siRNA. After a week-long protocol consisting of a total of 4 injection sessions coupled with electroporation, lumbar (L4-L6) DRGs were harvested and analyzed via qRT-PCR. For comparison, in a subsequent experiment a separate set of mice were also treated with either scrambled (n=4) or Rb1 siRNA (n=4) injected into their right hind paw, but without subsequent electroporation (Fig. 3B). Analysis of the relative fold expression (RFE) of Rb1 mRNA showed that its expression was significantly decreased in the ipsilateral DRGs of the cohort receiving Rb1 siRNA with electroporation ($t(5)=3.05$, $P=0.03$; Fig. 3A). Compared to the electroporated mice that received the scrambled siRNA, there was a 50% reduction in the relative fold expression in the Rb1 siRNA-treated cohort, indicating partial knockdown (Fig. 3A). With regards to the mice that received siRNA injections without electroporation, though there was a trend towards a reduction in the RFE of Rb1 mRNA in the group that was treated with Rb1 siRNA, the difference when compared to the negative controls was not statistically significant ($t(6)=2.09$, $P=0.08$; Fig 3B) and there was greater variability in the standard error than in separate measurements with electroporation.

3.2 Effects of delayed Rb1 knockdown following sciatic nerve crush.

In order to investigate the effect of a partial, transient knockdown of Rb1 on the regeneration of epidermal sensory axons and functional recovery after a nerve injury, cohorts of mice (n=5 per cohort) underwent a sciatic nerve crush surgery. Since hind paw injections coupled with electroporation were shown to be efficacious at inducing a partial knockdown of Rb1 mRNA in lumbar DRGs, 14 days following surgery one cohort of mice received Rb1-targeted siRNA and the other a scrambled control siRNA. Treatment continued ever second day over a span of two weeks for a total of 8 sessions. Behavioural and electrophysiological testing were carried out prior to injury, as well as on days 14 and 28 in order to assess outcomes. Two rounds of experiments were carried out, making a total of 10 mice in each group. A schematic of the experimental timeline is illustrated in Figure 4.

3.2-1 Rb1 expression at day 28 post-crush. qRT-PCR analysis of ipsilateral L4-L6 DRGs collected at 28 days post-injury from both cohorts of mice showed a significant 45% knockdown of Rb1 mRNA in the DRGs of Rb1 siRNA-treated animals compared to injured animals receiving a scrambled negative control sequence ($t(8)=2.76$, $P=0.02$; Fig. 5).

In addition to qRT-PCR, western blots using L5/6 DRG samples to analyze the levels of Rb1 protein in scrambled versus Rb1 siRNA-treated mice were carried out to see whether this reduction in the relative expression of Rb1 mRNA translated to a decrease in its protein (data not shown). While we observed trends toward lower Rb1 protein in samples treated with Rb1 siRNA, overall protein levels were low, limiting accurate quantitation.

3.2-2 Behaviour & electrophysiology at days 14 and 28 post-crush. The raw mechanical and thermal sensitivity data are shown in Figure 6A and B. In both Hargreaves and Von Frey behavioural assays, mice showed significant functional recovery between day 14 and 28, regardless of whether they received scrambled or Rb1-targeted siRNA sequences. The only

exception to this was in the data corresponding to thermal sensitivity (Fig. 6A), in which the change in withdrawal latencies between day 14 and day 28 in scrambled-treated control animals was not significant (Tukey's post hoc, $q=1.23$, $P=0.66$), whereas in Rb1 siRNA-treated animals, it was (Tukey's post hoc, $q=4.57$, $P=0.007$). However, the recovery levels between the two cohorts at day 28 did not significantly differ (Bonferroni's post hoc, $t=1.15$, $P=0.77$). With the above caveat the overall results suggest that the partial Rb1 knockdown did not notably enhance functional recovery of thermal sensation. Similarly, the Von Frey and electrophysiological motor and sensory conduction data did not reveal any significant differences in recovery between the two treatment cohorts (Fig. 6B-F), leading us to conclude that by day 28 after injury and after two weeks of siRNA administration, a partial Rb1 knockdown does not confer any notable improvements in behavioural or electrophysiological measures of sensory function over control mice.

3.2-3 Immunohistochemistry of footpad biopsies at day 28. Immunohistochemical analysis of control uninjured footpad biopsy tissue showed that there is an abundance of nerve fibers present in the epidermal layer of the footpad, as denoted by bright PGP9.5 staining of the axon profiles (Fig. 7A). In contrast, the footpad biopsies from animals that had previously undergone a sciatic nerve crush followed by treatment with scrambled siRNA possessed very few epidermal nerve fibers, although some axons did manage to traverse the boundary between the dermis and epidermis (Fig. 7B). This notable lack of skin reinnervation was ameliorated, to an extent, in animals treated with Rb1 siRNA, as more axons penetrated the dermal-epidermal junction (Fig. 7C). Representative sections from control uninjured, injured scrambled siRNA-treated, and injured Rb1 siRNA-treated animals are shown in panels A-C of Figure 7. The increase in the number of axons per millimeter ($t(8)=2.49$, $P=0.04$) and per square millimeter

(t(8)=3.10, P=0.015) in Rb1 siRNA-treated mice as compared to mice that received scrambled sequences is quantified in panels D and E, respectively. In addition to the enhanced intraepidermal nerve fiber density, animals in the Rb1 siRNA-treatment cohort also demonstrated significantly higher numbers of both vertical (>45° trajectory; t(8)=2.44, P=0.04; Fig. 7F) and horizontal (<45° trajectory; t(8)=2.82, P=0.02; Fig. 7G) axons. That being said, both groups, when compared to uninjured footpads from the contralateral side, had significantly fewer axons (P<0.0001), which may account for why no apparent differences in functional recovery between the injured cohorts were detected in the behavioural assays.

Since we were primarily interested in differentiating whether the Rb1 knockdown condition had an effect on epidermal axon outgrowth over the negative control group, unpaired t-tests were used to make statistical comparisons between the two groups across the various axon counting methods (Fig. 7D-G) (O'Brien, Shampo & Dyck, 1989). However, we acknowledge that analysis of changes across the uninjured control, Rb1 siRNA-treated (injured), and scrambled siRNA-treated (injured) samples may instead be carried out through the use of a one-way ANOVA. Re-analysis of the data using this approach showed, similar to the t-test results, that both injured cohorts had significantly fewer epidermal axons than the uninjured contralateral footpads (P<0.0001; data not shown). In contrast, post hoc Tukey's test indicated no significant differences between the two injured groups in the number of axons per millimeter (q=3.62, P=0.06), the number of vertical axons (q=3.64, P=0.06), and the number of horizontal axons (q=3.50, P=0.07), despite there being a trend towards a reduction in the scrambled-treated injured group (analysis not graphically shown). That being said, statistical significance was maintained between the Rb1- and scrambled siRNA-treated groups with respect to the number of

axon profiles per square millimeter, with the scrambled-treated mice possessing substantially lower levels (Tukey's post hoc, q=4.34, P=0.02).

3.2-4 Analysis of distal sural nerve semi-thin sections. A one-way ANOVA to compare the mean number of axons in epon-embedded sural nerve cross-sections taken 28 days post-injury did not reveal any substantial differences between contralateral (uninjured), Rb1 siRNA-treated (injured), and scrambled siRNA-treated (injured) samples ($F_{2,6}$=0.48, P=0.64; Fig. 8). The fact that both injured groups showed significantly impaired SNAP amplitudes relative to their baseline readings suggests, however, that there should be a disparity in the number of distal intact axons compared to uninjured nerves. Nevertheless, it is possible for there to be discrepancies in the electrophysiological and structural properties of regenerating nerves, and it has been previously reported that the morphology of the sural nerve does not necessarily correlate with or forecast the sensory nerve amplitudes and velocities of the distal axons that comprise it (Zochodne, 2008). Further assessment of axon parameters such as g-ratio, axon caliber, and relative quantities of large and small diameter axons making up the nerve may provide better insight into the more subtle aspects of axon regeneration and maturation.

3.3 Long-term effects of delayed Rb1 knockdown following sciatic nerve crush.

Given the promising, albeit subtle, benefits on axon regeneration and skin reinnervation observed in the footpads 28 days post-crush following a partial delayed knockdown of Rb1, we decided to repeat the experiment with the endpoint set at 40 days post-crush. Administration of siRNAs through injection coupled with electroporation to the hind paw remained the same, spanning from day 14 to 28 for a total of 8 treatment sessions. This extended timeline would allow us to determine whether the advantages of Rb1 suppression observed at 28 days persisted

once siRNA administration had ceased, and whether the partial knockdown conferred any longer-term pro-growth benefits over the negative control condition. A schematic of the experimental timeline is illustrated in Figure 9.

3.3-1 Behaviour & electrophysiology at days 28 and 40 post-crush. The raw mechanical and thermal sensitivity data is displayed in Figure 10A and B. On day 28 and 40 post-injury, two-way repeated measures ANOVAs with Bonferroni post hoc analysis indicated that there were no significant changes in the relative withdrawal latencies or relative withdrawal thresholds, as assessed by Hargreaves and Von Frey behavioural assays respectively, between the cohort that received Rb1 siRNA (n=5) and the one that received scrambled siRNA (n=5). When each group's relative performance was statistically analyzed at different time points throughout the experiment, however, there were some discrepancies to be noted. Somewhat unexpectedly, the relative withdrawal latencies of injured mice that were treated with scrambled siRNAs were significantly greater on day 40 than on day 28, indicating a decrease in sensitivity (Tukey's post hoc, $q=4.30$, $P=0.02$; Fig. 10A). This was not the case in the Rb1 siRNA-treated cohort, which did not show major deviations in thermal sensitivity testing between day 28 and 40 (Tukey's post hoc, $q=0.99$, $P=0.77$; Fig 10A). Relative to baseline measurements, at day 40 the mice treated with Rb1 siRNA demonstrated withdrawal latencies near pre-injury levels (Tukey's post hoc, $q=3.40$, $P=0.07$; Fig. 10A), whereas those that were given scrambled siRNA were significantly impaired (Tukey's post hoc, $q=6.15$, $P<0.01$; Fig. 10A). In contrast, with regards to mechanical sensitivity, there were no changes in the two groups' performances during testing between day 28 and 40, nor between baseline and day 40 as analyzed via a two-way repeated measures ANOVA and subsequent Tukey's post hoc test (Fig. 10B).

The overall lack of between-group differences on these two testing days was also seen upon analysis of electrophysiological data (two-way repeated measures ANOVA; Fig. 10C-F). In general, there were no significant improvements in the motor or sensory nerve conduction velocities or amplitudes of Rb1 siRNA-treated mice over scrambled-treated, at either 28 or 40 days post-crush ($P>0.05$; Fig. 10C-F). Also, there were no substantial changes in CMAP amplitudes, CMAP velocities, SNAP velocities, or SNAP amplitudes in either cohort between days 28 and 40. By day 40, both groups' electrophysiological measurements were still considerably lower than their baseline recordings, except for the CMAP velocities, which were ameliorated to pre-injury values in Rb1 siRNA-treated (Tukey's post hoc, $q=0.54$, $P=0.92$) and scrambled-treated mice (Tukey's post hoc, $q=2.68$, $P=0.18$). Overall, these behavioural and electrophysiological indices suggest that the transient and partial knockdown of Rb1 for two weeks following a peripheral nerve injury does not bestow persistent long-term advantages on functional outcomes.

3.3-2 Immunohistochemistry of footpad biopsies at day 40. Footpad biopsies taken at the experimental endpoint on day 40 from both cohorts were analyzed using PGP9.5 immunohistochemical staining. For the sake of comparison, Figure 11 illustrates the day 28 biopsy data previously presented in Figure 7 alongside the data collected from day 40. As expected, in both groups there was an overall increase in the number of epidermal axons from day 28 to day 40 (Fig. 11). However, in contrast to what was observed in the biopsies harvested on day 28, when analyzed with student's t-tests at day 40 there were no significant changes to the epidermal innervation levels in the skin of Rb1 siRNA-treated mice in comparison to mice that were treated with scrambled sequences beyond a nonsignificant trend toward more fibers in the

control group (Fig. 11D-G). The lack of significant differences between the two injured cohorts at 40 days across the various counting approaches was also observed upon re-analysis with one-way ANOVAs and subsequent Tukey's post hoc tests ($P>0.05$; analysis not graphically shown). Overall, apart from the number of horizontal axons, which in scrambled siRNA-treated injured mice were within range of uninjured control levels, the number of axons per millimeter and square millimeter at day 40 in injured animals were still significantly lower than the amount observed in uninjured samples (Fig. 11D-F). Despite the initially encouraging evidence procured from footpads harvested at day 28, an extended timeline reveals that the regenerative benefits observed in Rb1 siRNA-treated mice do not endure.

3.4 Figures

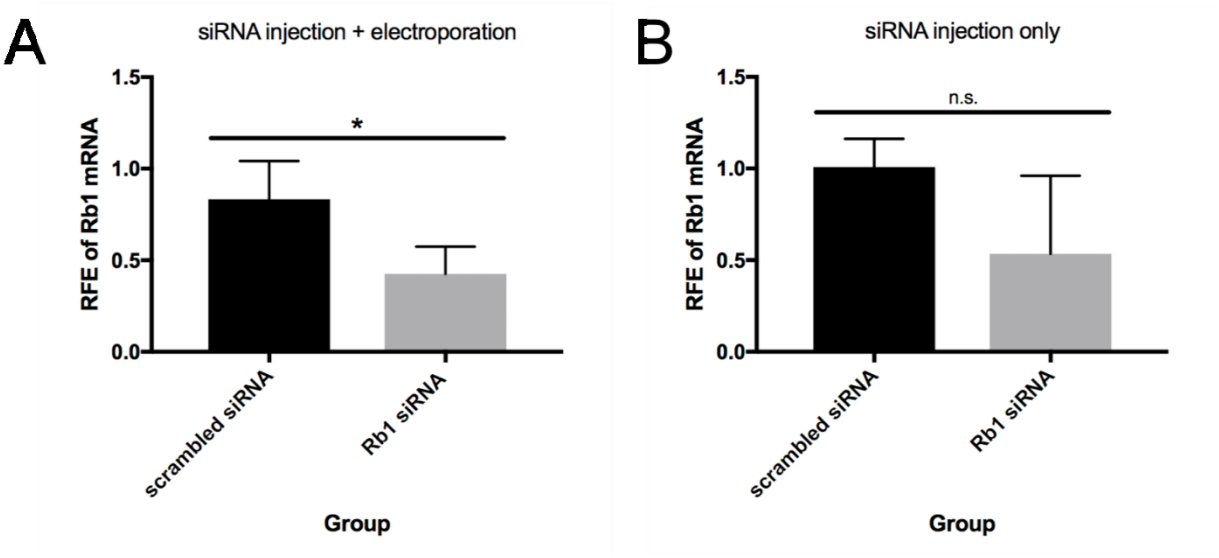

Figure 3. ***mRNA expression of Rb1 following 7-day electroporation protocol.*** Relative fold expression (RFE) of Rb1 mRNA significantly decreases in the ipsilateral DRGs of mice that received Rb1-targeted siRNA followed by electroporation as compared with control mice that were given scrambled control sequences **(A)**. Although, in a separate experiment, there was a trend towards Rb1 knockdown between the siRNA-treated groups that received injection only (without subsequent electroporation), the decrease in RFE in the group that received Rb1 siRNA was not significant compared to the scrambled-treated controls **(B)**. This suggests that electroporation may aid the reliability of transfection. Error bars indicate standard error. Scrambled siRNA+electroporation (n=3); Rb1 siRNA+electroporation (n=4); scrambled siRNA injection only (n=4); Rb1 siRNA injection only (n=4). *P<0.05; unpaired two-tailed student's t-tests.

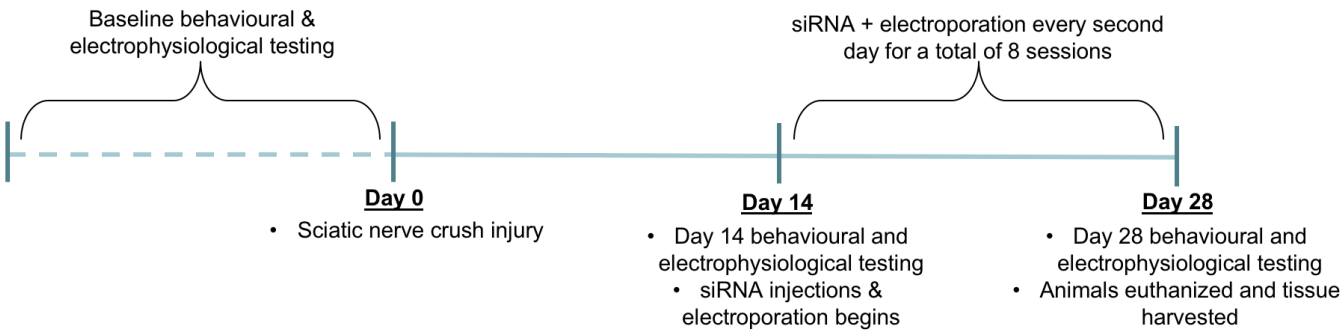

Figure 4. ***Experimental timeline for delayed Rb1 knockdown following sciatic nerve crush.*** Hargreaves, Von Frey, and electrophysiological test measurements were recorded and averaged prior to injury to establish a baseline for later comparison. A sciatic nerve crush injury was then surgically inflicted. Two weeks later, behavioural and electrophysiological recordings were repeated, and the siRNA injection/electroporation protocol began. Mice in both cohorts were treated every other day across a two-week window with either Rb1 or scrambled siRNA. On day 28 post-crush, a final round of behavioural and electrophysiological testing was conducted and animals were euthanized for subsequent tissue analysis.

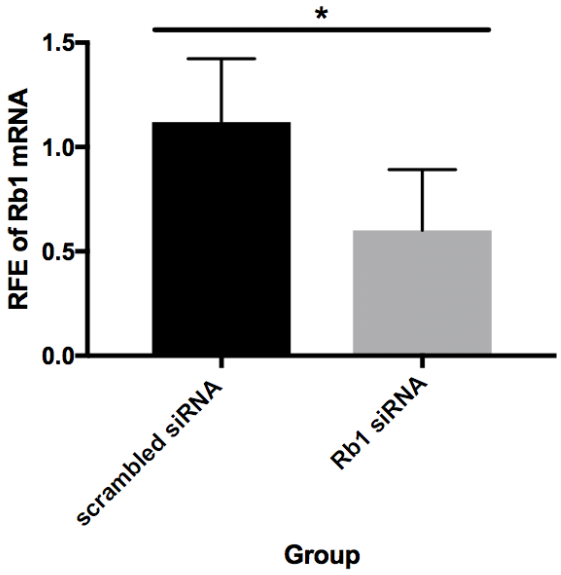

Figure 5. ***mRNA expression of Rb1 on day 28 following sciatic nerve crush and siRNA administration.*** Following 2 weeks of siRNA administration that started on day 14 post-crush, the relative fold expression of Rb1 mRNA in lumbar DRGs was significantly reduced in mice that received Rb1-targeted siRNA with electroporation when compared to mice that were given scrambled negative control siRNA. Error bars indicate standard error. Scrambled (injured) siRNA (n=5); Rb1 (injured) siRNA (n=5). *P<0.05; unpaired two-tailed student's t-tests.

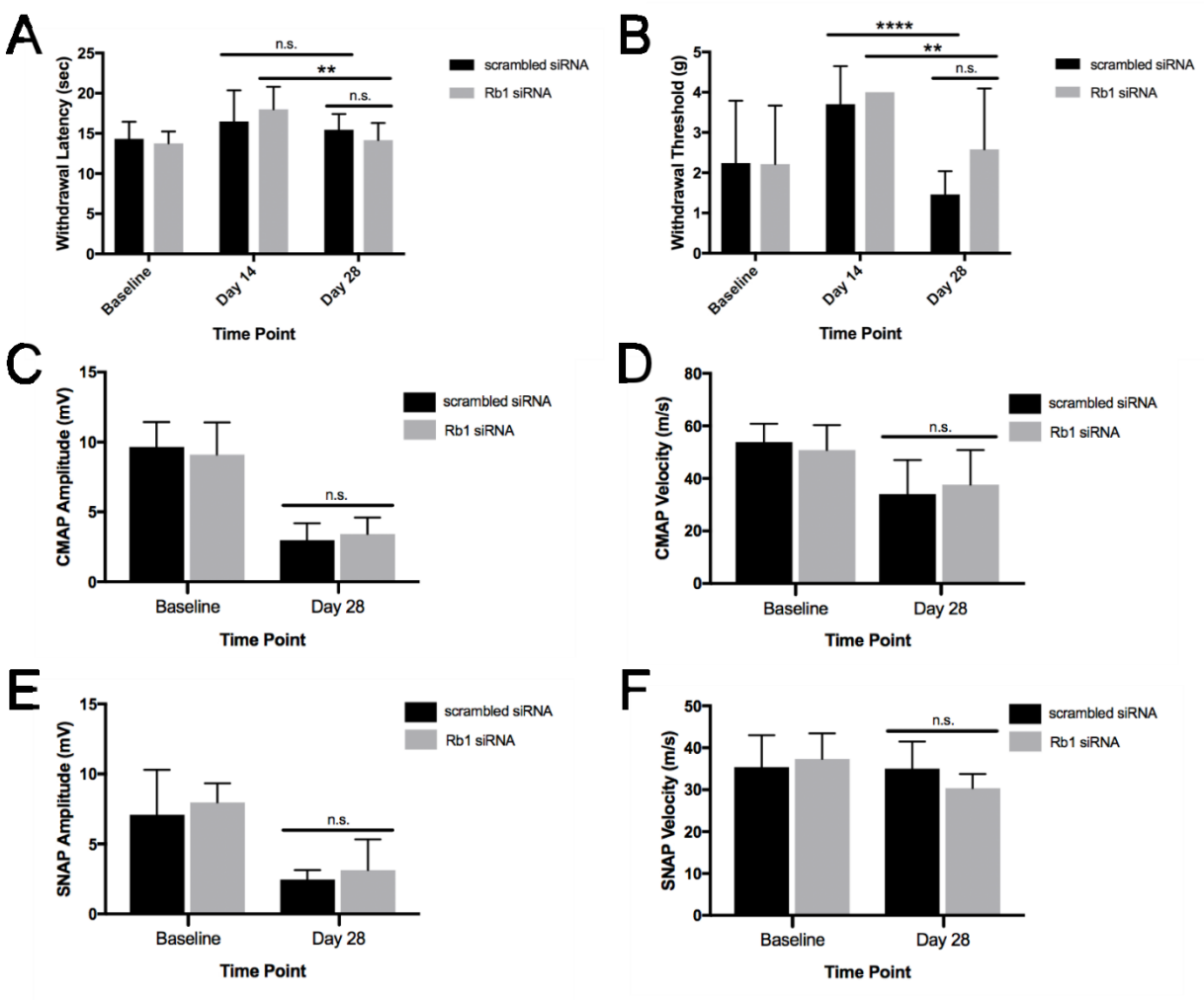

Figure 6. ***Behavioural analysis following sciatic nerve crush and delayed Rb1 knockdown.*** Mice in both cohorts were subjected to a sciatic nerve crush injury to their right (ipsilateral) hind leg. At day 14 post-crush, both cohorts began to receive hind paw siRNA injections (scrambled or Rb1 siRNA) coupled with electroporation. For all behavioural and electrophysiological tests, measurements were taken from the ipsilateral hind paw three separate times prior to injury, to establish a baseline, and at days 14 and 28 after injury. **A)** Shows the thermal sensitivity as assessed via Hargreaves testing and **B)** shows the mechanical sensitivity using Von Frey hair monofilaments. For thermal and mechanical sensitivity, mice generally showed significant recovery between day 14 and day 28, however the recovery levels between the two cohorts at day 28 were not significantly different for either the Hargreaves (Bonferroni's post hoc, t=1.15, P=0.77) or Von Frey (t=2.16, P=0.11) tests. **(C, D)** The average amplitude ($F_{1,18}$=1.15; P=0.30) and velocity ($F_{1,16}$=0.47, P=0.50) of compound motor action potentials (CMAPs) procured for each group throughout the experiment are shown. **(E, F)** Depicts the average amplitude ($F_{1,15}$=0.07, P=0.79) and velocity ($F_{1,15}$=3.16, P=0.10) of sensory nerve action potentials (SNAPs) for each cohort. Taken together, the motor and sensory electrophysiological data do not indicate that there is any significant difference between the two cohorts in terms of conduction velocity or amplitude. Error bars indicate standard error. Scrambled siRNA (n=10); Rb1 siRNA (n=10). **P<0.01, ****P<0.0001; two-way repeated measures ANOVA followed by *post hoc* Tukey or Bonferroni analysis.

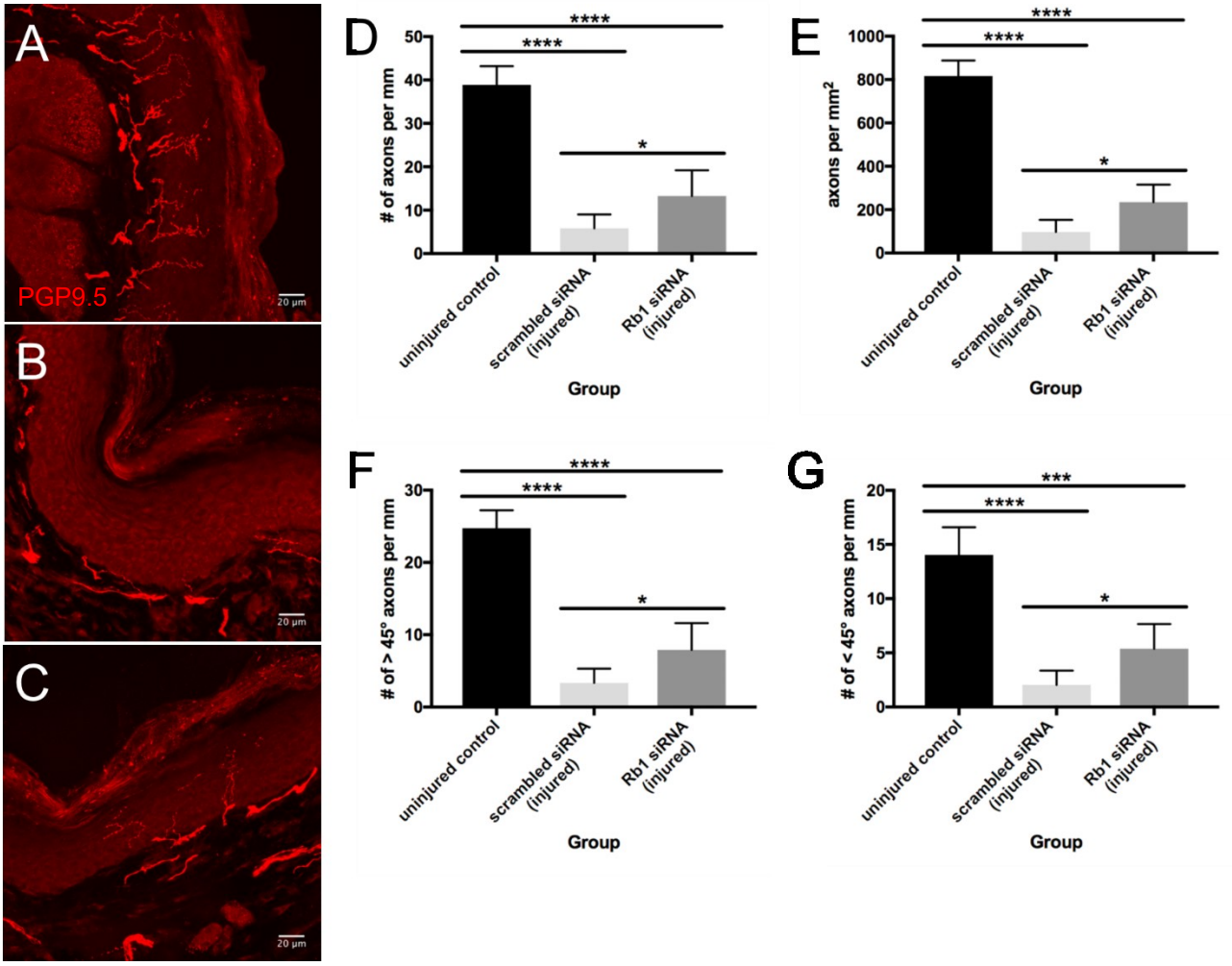

Figure 7. ***Epidermal innervation of the footpad on day 28 following sciatic nerve crush and delayed Rb1 knockdown.*** **(A-C)** Z-stack images depicting typical levels of axon outgrowth into the epidermis in control uninjured **(A)**, injured scrambled siRNA-treated **(B)**, and injured Rb1 siRNA-treated **(C)** mice are shown. Quantification of the total number of axons per millimeter **(D)** and square millimeter **(E)** is shown in addition to a breakdown of the number of vertical (>45°; **F**) and horizontal (<45°; **G**) axons per millimeter. Overall, mice treated with the Rb1 siRNA possess a significantly greater number of axons crossing from the dermis into the epidermis. That being said, regardless of whether they received Rb1 or scrambled siRNAs, the number of epidermal axons in both injured groups was significantly lower than levels found in uninjured, naïve controls. Analysis of the data using one-way ANOVAs (analysis not graphically included) showed that the Rb1-treated cohort only possessed a significantly higher number of axons per millimeter, when compared to the scrambled-treated mice (Tukey's post hoc, q=4.34, P=0.02), however recognizing that the ANOVA may not address specific questions confined to the two injured groups embedded within the larger body of data, we justified comparisons using individual student's t test to answer focused questions on specific data subsets. Error bars indicate standard error. Uninjured, naïve control (n=5); scrambled (injured) siRNA (n=5); Rb1 (injured) siRNA (n=5). *P<0.05, ***P<0.001, ****P<0.0001; unpaired two-tailed student's t-test.

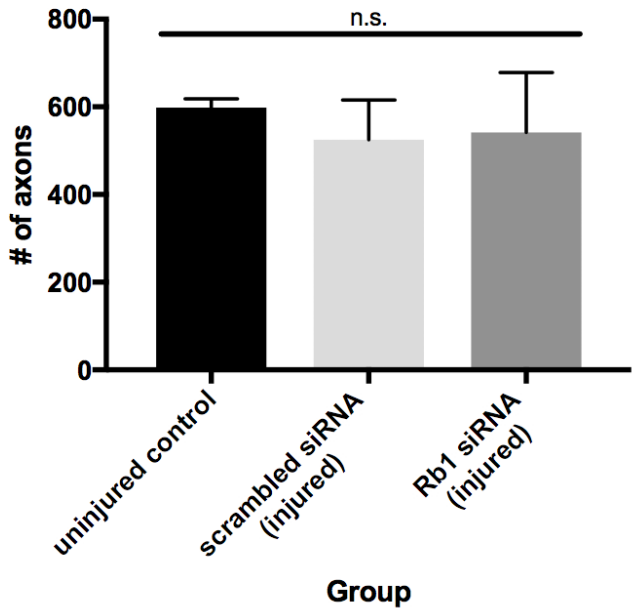

Figure 8. ***Axon count of sural nerve semi-thin sections***. The number of axons in bright-field images of cross-sections taken distally from both injured and uninjured sural nerves were manually counted. When compared using a one-way ANOVA, there were no significant differences in the number of axons contained within the sural nerve sections between any of the groups. Error bars indicate standard error. Uninjured, naïve control (n=3); scrambled (injured) siRNA (n=3); Rb1 (injured) siRNA (n=3). One-way ANOVA.

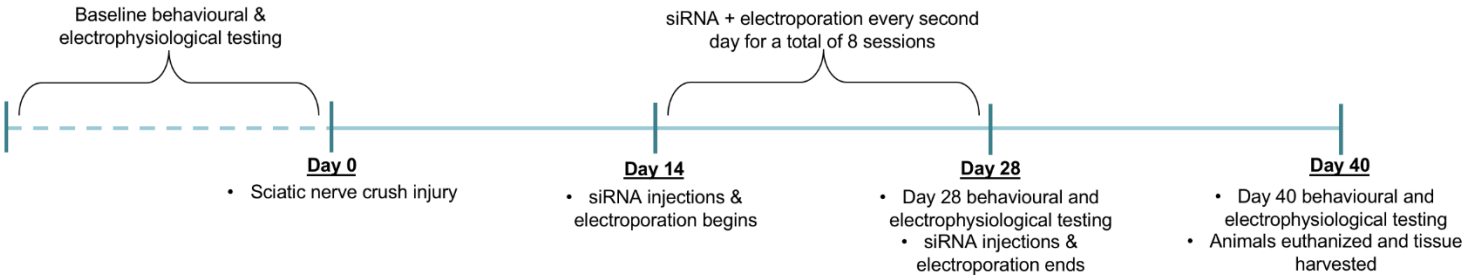

Figure 9. ***Extended 40-day experimental timeline for delayed Rb1 knockdown following sciatic nerve crush.*** Hargreaves, Von Frey, and electrophysiological test measurements were recorded and averaged prior to injury to establish a baseline for later comparison. A sciatic nerve crush injury was then surgically inflicted. Two weeks later, the siRNA injection/electroporation protocol began. Mice in both cohorts were treated every other day across a two-week window with either Rb1 or scrambled siRNA. On day 28 post-crush, behavioural and electrophysiological testing was conducted and then reassessed on day 40. Animals were subsequently euthanized for tissue analysis.

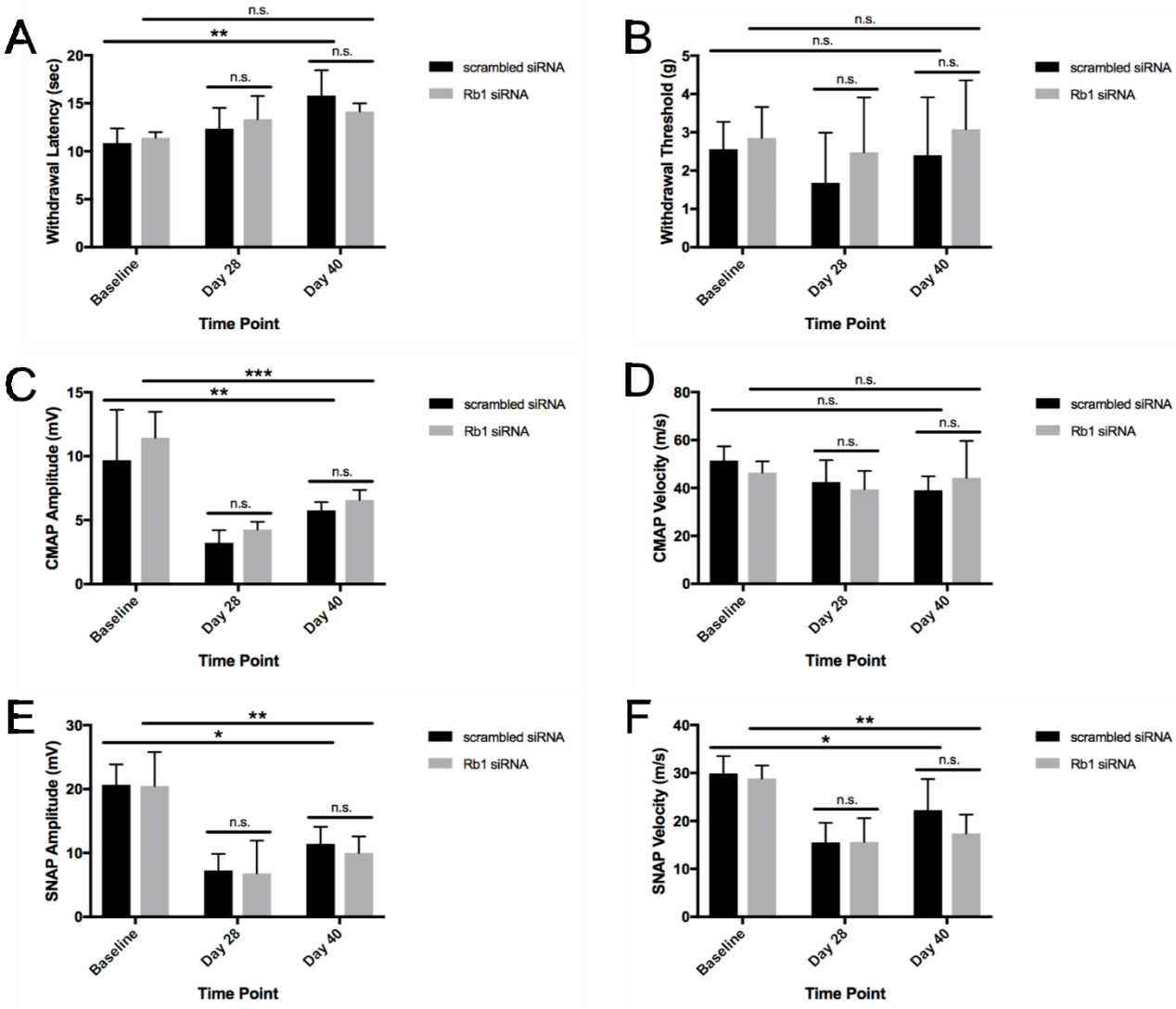

Figure 10. ***Behavioural analysis 40 days following sciatic nerve crush.*** As illustrated in Figure 9, mice in both cohorts underwent a sciatic nerve crush injury to their right (ipsilateral) hind leg, and then were treated with either Rb1 or scrambled siRNA between days 14 and 28 post-crush via injection coupled with electroporation. For all behavioural and electrophysiological tests, measurements were taken from the ipsilateral hind paw three separate times prior to injury, to establish a baseline, and at days 28 and 40 after injury. **A)** Shows the thermal sensitivity as assessed via Hargreaves testing and **B)** shows the mechanical sensitivity using Von Frey hair filaments. **(C, D)** The average amplitude and velocity of CMAPs procured for each group throughout the experiment are shown. **(E, F)** Depicts the average amplitude and velocity of SNAPs for each cohort. Statistical analysis of the groups' raw data does not reveal a significant interaction between siRNA treatment and testing day for either Hargreaves ($F_{2,16}$=1.57, P=0.24) or Von Frey ($F_{2,16}$=0.28, P=0.76) tests. Likewise, the motor and sensory electrophysiological data do not indicate that there is any significant difference post-injury between the two cohorts in terms of conduction velocity or amplitude. Error bars indicate standard error. Scrambled siRNA (n=5); Rb1 siRNA (n=5). *P<0.05, **P<0.01, ***P<0.001; two-way repeated measures ANOVA followed by *post hoc* Tukey or Bonferroni analysis.

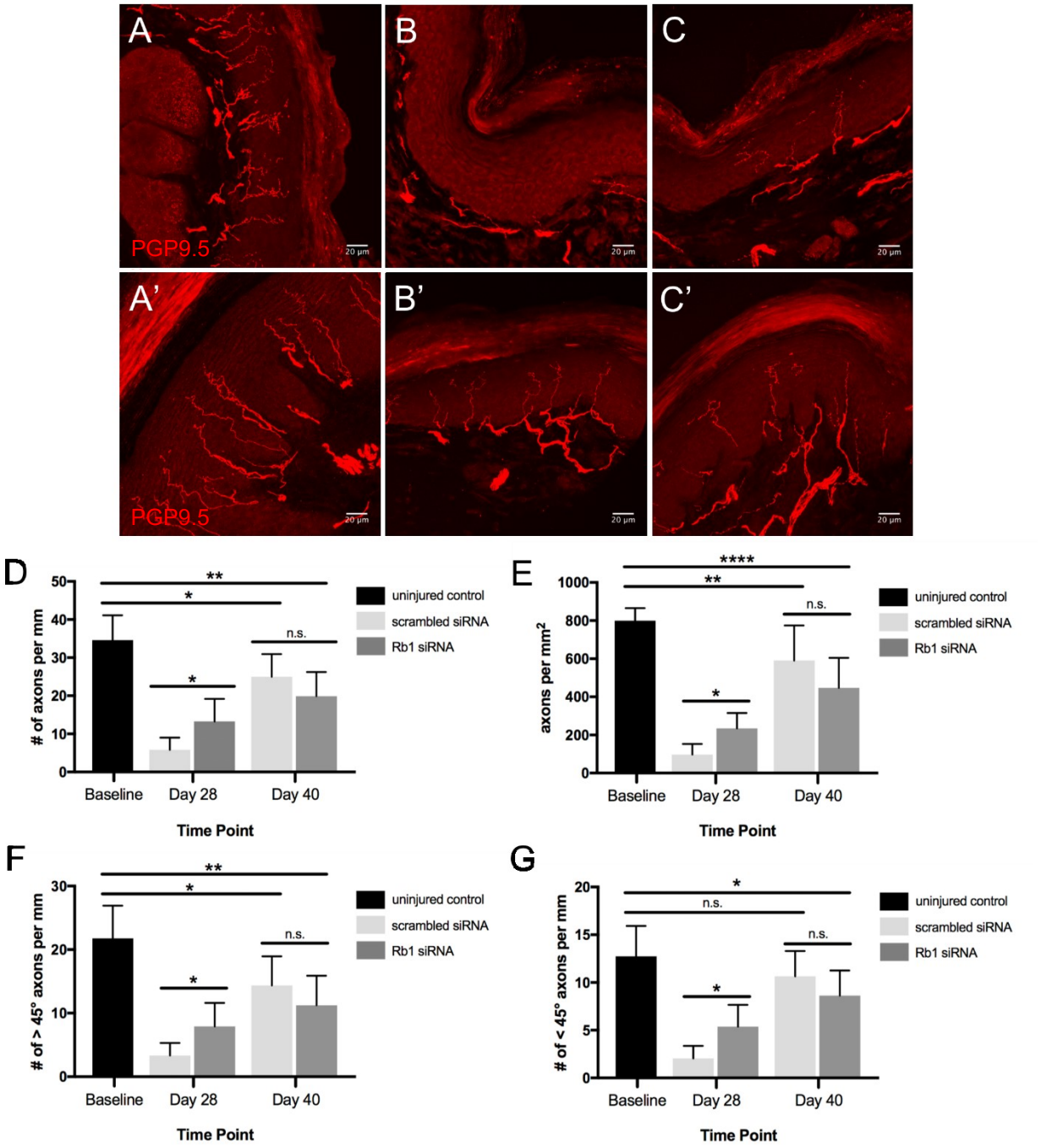

Figure 11. ***Epidermal innervation of the footpad on day 40 following sciatic nerve crush and delayed Rb1 knockdown.*** Axon profiles in footpad biopsies taken at the experimental endpoint on day 40 from both cohorts were quantified through PGP9.5 immunohistochemical staining. For comparison's sake, day 28 biopsy data previously presented in figure 7 is shown alongside the data collected at day 40. In the top row, Z-stack images depicting typical levels of axon outgrowth into the epidermis at day 28 in control uninjured **(A)**, injured scrambled siRNA-treated **(B)**, and injured Rb1 siRNA-treated **(C)** mice are shown. Representative images corresponding to footpads harvested on day 40 are displayed below for control uninjured **(A')**, injured scrambled siRNA-treated **(B')**, and injured Rb1 siRNA-treated **(C')** cohorts.

Quantification of the total number of axons per millimeter **(D)** and square millimeter **(E)** is shown in addition to a breakdown of the number of vertical (>45°; **F**) and horizontal (<45°; **G**) axons per millimeter. At day 40, although as expected there was a general improvement in the number of axons compared to day 28, there was no longer a significant difference in the epidermal innervation levels between the Rb1 and scrambled siRNA-treated cohorts. Overall, the number of epidermal axons per millimeter and square millimeter at day 40 in injured animals remained significantly lower than the amount observed in uninjured, naïve samples taken from the contralateral side. Axon measurements from day 28 and 40 for uninjured control animals were pooled. Error bars indicate standard error. Uninjured, naïve control (n=9); scrambled (injured) siRNA (n=5); Rb1 (injured) siRNA (n=5). *P<0.05, ***P<0.001, ****P<0.0001; unpaired two-tailed student's t-test.

4. Discussion

The present study sought to investigate the potential therapeutic benefits of suppressing Rb1 expression in the weeks following a peripheral nerve injury through the use of a novel non-viral knockdown method. First, the efficacy of hind paw siRNA injection and electroporation was assessed in intact mice, and then this technique was utilized within the context of a surgically inflicted sciatic nerve crush in order to ascertain the effect of Rb1 knockdown on the regeneration of distal sensory neurons and subsequent skin reinnervation. Functional indices of recovery were evaluated throughout the experimental time courses using electrophysiological recordings and assessments of mechanical and thermal sensitivity. Histology to examine axon outgrowth and epidermal reinnervation was conducted upon treatment cessation at 28 days post-injury, as well as on samples taken at a later time point on day 40 to determine the short- and long-term repercussions of delayed Rb1 knockdown.

4.1 Electroporation

In the past, our lab and others have made use of subcutaneous microinjection ports (MIPs) in order to administer siRNAs, drugs, and growth factors to surgically transected sciatic nerves (McDonald & Zochodne, 2003; Christie et al., 2014; Law et al., 2016). This method of administration is both invasive and technically challenging, as the port must be surgically implanted, with the proximal and distal nerve stumps secured within a T-tube chamber. In the case of a sciatic nerve crush, to induce Rb1 knockdown, Christie et al. (2014) bathed the crushed nerve in siRNA solution for 20 minutes immediately following the crush, and then administered injections of siRNAs through the sutures of the crush site and into the hind paw for 5 days post-

injury. Although this strategy was efficacious in terms of procuring knockdown, it too is invasive and potentially damaging. It is also not a practical technique for administration beyond the point of wound healing so that the effects of long-term or delayed knockdown may be studied.

Since this project was concerned with investigating the effects of Rb1 knockdown on the regeneration of epidermal nerve fibers, we were interested in developing a new protocol for achieving transient knockdown that was efficient, less invasive, and conducive to a longer time course of siRNA treatment. We were also interested in developing an approach that could be used to target outgrowth that was already underway, with an appropriate delay awaiting axon re-entry into the skin. Electroporation is a technique that is used to permeabilize cell membranes to drugs, chemicals, or genetic constructs through the application of brief pulses of electrical current (Neumann et al., 1982). These electric pulses create small, temporary, water-filled pores in the phospholipid bilayer of cells, allowing charged substances, such as DNA, to traverse the hydrophobic membrane more easily (Neumann et al., 1982). In previous studies conducted by other research groups, electroporation has been used for the purposes of increasing the absorption of genetic constructs into DRGs and the brains of developing mouse embryos (Saijilafu et al., 2011; Matsui et al., 2011). However, to our knowledge, electroporation has never been coupled with siRNA injections to the hind paw in order to achieve genetic suppression in lumbar DRGs. It was our hope that by applying electrical stimulation to the surface of the hind paw following an siRNA injection, the current would increase the extent to which the negatively-charged RNA sequences were absorbed by nearby sensory nerve terminals. Ideally, these sequences would then be retrogradely transported up the axons to the neuronal cell bodies, where they would interfere with the expression of Rb1. A previous study conducted by Singh and colleagues (2014) supports the notion of retrograde transport of siRNA in peripheral

nerve axons. Following a sciatic nerve crush, the group ligated the nerve proximal to the site of injury and then injected 3'-Alexa Fluor 488-labelled siRNA sequences into the crush zone and hind paw (Singh et al., 2014). They subsequently observed an accumulation of the fluorescently-labelled siRNA just distal to the ligation site, suggesting that the siRNAs from the hind paw and crush site were being transported back toward the neuronal cell bodies in the DRG but were prevented from successfully reaching their destination by the disruption created by the ligature (Singh et al., 2014). Knockdown of the target gene of interest was also confirmed in un-ligated nerves and DRGs through a reduction of both the corresponding mRNA transcripts and protein levels (Singh et al., 2014).

The qRT-PCR results procured in both intact (Fig. 3) and injured (Fig. 5) Rb1 siRNA-treated mice suggest that injection followed by electroporation of siRNAs to the hind paw is indeed capable of inducing a significant, albeit partial, knockdown of the target mRNA in lumbar dorsal root ganglia. Although in uninjured mice injection alone did reduce the RFE of Rb1 mRNA (Fig. 3B), the decrease was not significant in comparison to the scrambled-treated control mice, which supports the notion that carrying out electroporation after an siRNA injection augments the degree of transfection. For the purposes of this study and its experiments, we deemed the 45-50% level of knockdown to be sufficient, however since this represents a novel application of electroporation-induced transfection, it would be beneficial for future studies to further develop and optimize the hind paw injection/electroporation protocol, as well as to investigate the extent to which injected substances diffuse into the surrounding non-neuronal cells, such as the keratinocytes in the skin, or Schwann cells associated with the peripheral nerve axons.

4.2 Behavioural results at days 14, 28, and 40 following sciatic nerve injury

4.2-1 Thermal & mechanical sensitivity. Bearing in mind that the data displayed in figures 6 and 10 above were collected from different cohorts of mice in different experiments, when considered together in order to discern a pattern of recovery across the different testing time points, it appears that in both treatment groups there was a decrease in thermal sensitivity relative to baseline measurements two weeks after sciatic nerve crush prior to siRNA treatment onset (Fig. 6A). By day 28, which coincides with the end of the siRNA treatment period, relative withdrawal latencies in the Hargreaves assay normalized to around baseline (Fig. 6A & 10A). Surprisingly, however, at day 40 post-crush the withdrawal latencies in the scrambled-treated control group once again increased, indicating a decline in sensitivity (Fig. 10A). That being said, overall, when analyzing the relative withdrawal latencies between the groups that received Rb1-targeted siRNA and those that received scrambled sequences, there were no significant differences in the groups' responses across the various testing time points ($P>0.05$). This suggests that a delayed, partial knockdown of Rb1 through hind paw siRNA injection and electroporation neither worsens nor enhances functional recovery of thermal sensation in the long-term following peripheral nerve injury compared to scrambled-treated controls.

These findings differ from data reported by Christie and colleagues (2014). As briefly described above, to induce Rb1 knockdown in the in *vivo* regeneration experiment conducted by Christie et al. (2014), the sciatic nerve was continuously bathed in siRNA solution for 20 minutes immediately following nerve crush, and the siRNA was also injected transdermally into the hind paw on the injured side. Administration of the siRNA solution (Rb1 or scrambled) continued for five consecutive days post-injury, through injections given through the sutures at the site of nerve injury, as well as into the hind paw (Christie et al., 2014). As a result of these

efforts, the group reported a greater degree of Rb1 mRNA suppression than what was achieved through our use of hind paw injection coupled with electroporation in the present study (Fig. 5). However, that approach, as discussed above, was more invasive than that studied here. When the researchers then tested thermal sensitivity in these mice on day 14, those that received scrambled siRNA displayed injury-related hyperalgesia, or increased sensitivity, whereas the paw withdrawal latencies in the Rb1 siRNA cohort were near baseline (Christie et al., 2014). These results differ from the observed outcomes of the present study. Significant between-group differences were noticed on day 14 by Christie et al., and further analysis suggested that Rb1 siRNA had a positive effect by preventing hyperalgesia. However, our siRNA transfection protocol did not begin until day 14 after behavioural testing had been carried out, therefore it is not surprising that no significant behavioural differences were found between the two cohorts at this time. Rather than being hyperalgesic at day 14, both cohorts in the present study trended towards being hypoalgesic (Fig. 6A). By day 28 when siRNA treatment had ended, and we had hypothesized improved recovery in the Rb1 siRNA-treated mice, no significant differences were discerned between the two groups in terms of withdrawal latency (Fig. 6A). Given that Christie et al. (2014) saw significant between-group differences following their acute knockdown whereas our delayed treatment paradigm did not significantly alter withdrawal latencies between the two treatment groups suggests that the timing of Rb1 knockdown following injury has an impact on subsequent recovery.

When analyzing recovery across days 14, 28, and 40 with respect to mechanical sensitivity (again taking into account that this data is being combined from different groups of mice across different experiments), both groups were significantly hyposensitive when tested at day 14, as indicated by heightened withdrawal thresholds relative to baseline (Fig. 6B). In both

Rb1 and scrambled siRNA-treated groups, withdrawal responses returned to near baseline levels by day 28 and did not show significant fluctuation from these baseline values when re-tested on day 40 (Fig. 6B & 10B). In accordance with the thermal sensitivity data described above, there were no significant between-group differences in mechanical sensitivity on any of the testing days between the Rb1 and scrambled-treated cohorts, thus implying that a delayed, partial knockdown of Rb1 following nerve injury did not impart any notable advantages on recovery of mechanical sensation over the scrambled group.

Similar to the thermal findings, there were discrepancies between the mechanical sensitivity results obtained during the current study and those presented by Christie and colleagues (2014). Although the group used an automated Von Frey apparatus instead of a range of differentially weighted monofilaments, and so expressed responses as time until paw withdrawal (in seconds), on days 14 and 28 following injury the cohort that received scrambled siRNAs had significantly longer withdrawal times whereas the responses in mice exposed to Rb1 siRNA were near baseline levels (Christie et al., 2014). These reported differences on day 28 between treatment groups were not detected in the present study, nor were the patterns of hyposensitivity.

As a result of their promising mechanical sensitivity findings, taken in conjunction with improved hind paw grip strength and prevention of thermal hyperalgesia, Christie and colleagues (2014) concluded that local knockdown of Rb1 induced by siRNA delivered immediately following injury results in improved recovery. Since similar findings were not recapitulated following a delayed, prolonged time course of siRNA-induced knockdown, we were unable to draw the same conclusions regarding Rb1 suppression. This suggests that the onset and

subsequent timeline of siRNA administration is an important factor when assessing the therapeutic benefit of Rb1 knockdown following nerve injury.

As alluded to above, another factor that could have contributed to the distinct experimental outcomes were the relative levels of Rb1 mRNA knockdown achieved in each experiment as a result of the transfection protocol that was used. Due to their more direct approach of siRNA delivery, Christie and colleagues (2014) attained a more potent in *vivo* knockdown of Rb1 siRNA 7 days following injury than what was measured in the present study 4 weeks after injury and 2 weeks after the initiation of the hind paw injection/electroporation protocol. Assuming that the reduction of genetic expression in both of these experiments corresponded with a decrease in functional Rb1 protein, the differences in behaviour and recovery may be partially attributed to the respective levels of Rb1 suppression.

4.2-2 Electrophysiology. Consistent with the behavioural results, there were no significant differences in the motor or sensory conduction velocities and amplitudes between the groups treated with Rb1 siRNA and those treated with scrambled siRNA at either 28 or 40 days after injury (Fig. 6D-F & 10D-F). In contrast to these findings, when tested on day 28 post-crush Christie and colleagues (2014) found that mice acutely treated with Rb1 siRNA after injury showed a significant improvement in sensory but not motor conduction velocity compared to scrambled siRNA-treated controls. These discrepancies once again highlight the likelihood of the Rb1 siRNA transfection protocol onset being an important factor to determining the overall prognosis following a peripheral nerve injury.

4.2-3 Epidermal innervation. Although no obvious variations in the quantity of axons in distal sural nerve sections were discerned between the samples analyzed from contralateral uninjured, injured scrambled siRNA-treated, and injured Rb1 siRNA-treated mice (Fig. 8), there were significant changes in the epidermal innervation levels between the cohorts in footpad skin samples harvested on day 28 post-crush. Across the various axon counting methods, the group exposed to Rb1 siRNA exhibited a significant increase in the number of nerve fibers in the epidermis compared to scrambled-treated controls (Fig. 7). Compared to contralateral samples however, the ipsilateral innervation levels in both injured groups, regardless of having received Rb1 or scrambled siRNA, were still substantially reduced (Fig. 7). This may account for why no significant between-group differences on day 28 were observed in any of the behavioural assessments; the Hargreaves and Von Frey testing apparatuses and paradigms may have simply lacked the resolution necessary to distinguish the two treatment groups' recovery levels in a manner that corresponded with their respective differences in the extent of epidermal reinnervation. Despite these early promising results, by day 40 the treatment effect was lost and although there was a general increase in both groups in the number of epidermal nerve fibers, as was to be expected, there were no longer notable differences between the cohort that received Rb1 siRNA and the one that received scrambled (Fig. 11). This indicates that the effect observed at day 28 was transient, and perhaps could have been maintained if the transfection protocol had continued.

In the study conducted by Christie et al. (2014), the effects of in *vivo* Rb1 knockdown on peripheral nerve regeneration were histologically evaluated by quantifying the degree of outgrowth from the proximal stump after sciatic nerve transection. When assessed 7 days after siRNA treatment, the group found through labelling with neurofilament 200 (NF200) that the

number and length of regenerating axon profiles were significantly increased in the cohort that was given Rb1 siRNA (Christie et al., 2014). However, since the group only collected measurements at one time point, it would have been interesting to see whether this regenerative advantage in the Rb1 siRNA treatment group was preserved at a later point, or whether the effect was temporary as it was in the present study. If indeed the treatment effect diminished over time and the number and length of axon profiles in the scrambled control mice came within range of the levels observed in the Rb1 siRNA-treated group, we may hypothesize, in conjunction with the findings in the current study, that transient Rb1 knockdown only temporarily improves the extent of axonal regeneration, but does not affect long term outcomes.

4.3 Limitations & Future Directions

4.3-1 RNAi technique optimization. As alluded to above, since siRNA injection coupled with electroporation to the mouse hind paw is a new approach for attempting the knockdown of a target gene in peripheral neurons, further investigation into the application and optimization of this RNAi technique is warranted. The present study did not fully develop this technique, and the conclusions that can be made are restricted in their power due to small sample sizes. Therefore, experiments aimed at determining treatment parameters such as the optimal concentration of siRNA to be administered (by creating a dose-response curve), the timing of treatments, or the ideal strength and duration of electrical stimulation during electroporation should be carried out in order to utilize this transfection method to its full potential.

4.3-2 Sensitivity of behavioural assays. There are several limitations and caveats in the methodology of this study that may have bearing on the conclusions that can be drawn. One major limitation exists in the behavioural tests that were used to collect mechanical and thermal

sensitivity data. The resolution of the Hargreaves and Von Frey tests that were conducted is limited, which may account for why no significant functional changes were observed between cohorts on day 28 when there was indeed a significant difference in the degree of footpad reinnervation as assessed with PGP9.5 immunofluorescent staining. Behavioural assays are inherently much more variable in their output as it is challenging to control all aspects of the status of a given animal and the testing conditions, despite careful attempts made here. Blinding of the experimenter to the treatment arm of the animals is important, and was employed throughout the experiments, but is unable to eliminate all inherent variability.

4.3-3 Collateral sprouting of the saphenous nerve. Another technical limitation within this project is the footpad harvesting method. The sensory innervation of the plantar surface of a mouse's hind paw may be divided into three main sections: medial, central, and lateral (Cobianchi, de Cruz, & Navarro, 2014). Under normal conditions, there is a degree of overlap in these innervation territories. The central section is innervated by the tibial nerve and the lateral section by the sural nerve. Both of these nerves originate from the sciatic nerve, and therefore undergo degeneration following a sciatic nerve crush. The medial portion of the hind paw however, is innervated by the saphenous nerve, which is not associated with the sciatic nerve and remains intact throughout the duration of the experiment. The three-millimeter biopsy punches that were used to take circular cross sections of the footpad during tissue harvesting are large enough that a biopsy contains some degree of territory belonging to the saphenous and sural nerves in addition to that associated with the tibial nerve, which predominates. It is the intact saphenous nerve that poses a potential source of testing confound within this study.

Following peripheral nerve trauma, there are two sources of axons by which a denervated target, be it a muscle, organ, or skin, can be reinnervated (Cobianchi, de Cruz, & Navarro, 2014).

Either it can be reinnervated by regenerating axons from the damaged nerve that was originally associated with the specific target, or it can be reinnervated by collateral sprouting from intact, adjacent nerve fibers (Zochodne, 2008; Cobianchi, de Cruz, & Navarro, 2014). Although in a clinical setting collateral sprouting is a beneficial compensatory mechanism that can contribute to recovery, within the context of this experiment it is problematic as it confounds our ability to ascribe behavioural and histological conclusions to sciatic regeneration alone. To ameliorate this source of error, future experiments may be carried out in which the saphenous nerve is transected prior to tissue harvesting so that any fibers left in the epidermis can be confidently attributed to regenerating sciatic nerve afferents. Targeted behavioural testing of the three specific hind paw regions could also be conducted and measurements kept separate so that recovery of sensory function can be more accurately accredited to regeneration of the tibial and sural nerves (Cobianchi, de Cruz, & Navarro, 2014). Despite this concern, the clear early differences in regrowth from low baseline values makes large scale collateral sprouting as the route of reinnervation debatable. It would also likely not explain differences between the knockdown and control groups.

4.3-4 Rb1 function and potential mechanisms of knockdown-induced plasticity. Lastly, a challenge that is not so much a technical limitation but a hurdle moving forward is the complexity of Rb1, as well as E2F1, function. Since Rb1 is stationed as a 'master regulator' of a hierarchy of transcription factors, which each affect the expression of a multitude of gene products including other transcription factors, it is difficult to pinpoint a single pathway or the key pathways that are activated to promote growth when Rb1 is partially knocked down.

Although we did not conduct any experiments within the present study to elucidate the molecular pathways that are influenced by Rb1 knockdown and that may contribute to peripheral

neural plasticity, Christie et al. (2014) went so far as to demonstrate that the outgrowth mediated by Rb1 knockdown in *vitro* was dependent upon E2F1 expression. They then went on to examine whether certain proteins that are known to interact with Rb1 could be involved in facilitating the enhanced neurite outgrowth and branching that had been observed in their dissociated adult DRG neuron cultures exposed to Rb1 siRNA (Christie et al., 2014). Of these candidate proteins, peroxisome proliferator-activated receptor γ (PPARγ), showed a marked and consistent upregulation in protein and mRNA content within sensory neurons following Rb1 knockdown (Christie et al., 2014). When the action of PPARγ was pharmacologically inhibited through the addition of GW9662, a selective competitive antagonist, the Rb1 knockdown-induced neurite outgrowth was significantly dampened (Christie et al., 2014). When instead an agonist of PPARγ, 15PGJ2, was added to the cultures that had been treated with Rb1 siRNA, it further increased the length and outgrowth of neurites (Christie et al., 2014). Although PPARγ may not account for all the changes observed, these experimental findings led to the conclusion that PPARγ acts downstream of Rb1 to promote growth when the expression of Rb1 is repressed (Christie et al., 2014).

PPARγ is itself a transcription factor that is involved in fatty acid storage, lipid and metabolic homeostasis, and adipocyte differentiation (Lezana et al., 2016). It has been demonstrated that there is a consensus E2F binding site within the PPARγ promoter, and that E2F1 and E2F3 can stimulate adipogenesis by driving the expression of PPARγ (Fajas et al., 2002b). In addition to this, it has been found that Rb1 can inhibit PPARγ activity, both through direct binding as well as through recruitment of and complex formation with histone deacetylase 3 (HDAC3) at PPARγ-dependent promoters (Fajas et al., 2002a). Although these interaction pathways have not been so well characterized in peripheral neurons, given the previously

mentioned expressional patterns of PPARγ with relation to Rb1 knockdown described by Christie et al. (2014), it is reasonable to hypothesize that the activation of PPARγ following Rb1 knockdown in adult DRG neuronal cultures is due to a combination of reduced Rb1 inhibition and enhanced E2F1 activity.

Within the nervous system, the induction of PPARγ by pharmacological agonists has been shown to possess therapeutic potential within neurodegenerative disorders such as Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease (Sundararajan et al., 2006). Benefits associated with PPARγ expression have also been reported with regards to peripheral nerve injury (Lezana et al., 2016). In rats that had previously undergone a sciatic nerve crush, it was demonstrated that the levels of PPARγ in the axons and cell bodies of sensory neurons increased and that inhibition of axonal PPARγ with GW9962 hindered neurite outgrowth in pre-conditioned L4-L6 DRG cell cultures, as well as in axotomized human neurons (differentiated from NP1 human neural precursors) that had been re-plated (Lezana et al., 2016). Additionally, the expression of PPARγ has been localized to Schwann cells in rat peripheral nerves, and this expression is also upregulated in response to a crush injury, although its role in this cellular context has not been well characterized (Cao et al., 2012; Lezana et al., 2016). The expression of PPARγ in Schwann cells is interesting however, as Christie et al. (2014) reported increased numbers of Schwann cell profiles at the site of injury in rats given Rb1 siRNA. This suggests that there could possibly be an additional anti-regenerative role for Rb1 in glial cells that is repressed upon siRNA-induced knockdown.

Are the pro-regenerative effects of Rb1 knockdown described by Christie and colleagues (2014) exclusively mediated through molecular changes in neurons or is there also an effect in Schwann cells? How does PPARγ activation following Rb1 knockdown promote growth in

peripheral sensory neurons? Are there other E2F-dependent pathways involved? To answer these questions and others, future studies should be conducted to further elucidate the Rb1-E2F-PPARγ pathway and its molecular constituents. A better knowledge of the relative expression and action of proteins acting downstream of Rb1, E2F1, and PPARγ following a peripheral nerve injury may enhance our understanding of why an acute, immediate post-crush knockdown of Rb1 seems to induce a greater regenerative effect and augmented functional outcomes (Christie et al., 2014) over the delayed, prolonged knockdown protocol that was utilized in the present study. What the present study does show is exciting however, and potentially therapeutically more important – that an intervention, staged well after neurons have reprogrammed and regrowth has begun, can yet influence the reinnervation of the skin suggests that chronic treatment strategies, which are more clinically achievable, may yet be able to aid regeneration. The differences in approaches could in turn have implications on the onset and subsequent timing of future clinical treatment protocols. Although clinical interventions using RNAi have yet to be established for use in humans that have suffered peripheral nerve trauma, knowledge of the post-injury time points at which such interventions would be most efficacious would be beneficial so that precious windows of plasticity are not inadvertently squandered.

Conclusion

The findings presented in this thesis support the overall conclusion that i) injection of siRNAs coupled with electroporation to the hind paw presents a novel means of inducing partial knockdown in ipsilateral lumbar DRGs, and ii) delayed Rb1 knockdown following a nerve crush influences the capacity of regrowing axons to infiltrate the skin within a two-week time frame; while these early benefits did not persist to 40 days, it is possible that ongoing interventions to maintain or enhance this effect are required. These results are important and relevant for they demonstrate that a delayed, prolonged timeline of treatment following a peripheral nerve injury, although more clinically feasible, does not necessarily result in optimal recovery. Not surprisingly, earlier intervention seems to confer more robust regenerative advantages (Christie et al., 2014), potentially because the treatment coincides with the natural upregulation of regeneration-associated genes that occurs after injury, thereby allowing for potential synergistic effects and a 'window' for enhanced neural plasticity.

Given the prevalence of traumatic nerve injury and the current lack of molecular treatment options, it is worthwhile to continue to explore the roles and interactions of tumor suppressor proteins, more classical regeneration pathways, and their downstream targets in modulating peripheral nerve regeneration and skin reinnervation. A thorough understanding of their cellular function and downstream molecular mediators may open the door to the development of pharmacological interventions or non-viral gene therapies that could accompany existing surgical techniques in order to augment regenerative outcome and improve the quality of life for patients.

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