Studies on vertebrate nervous system myelination
the role of Cdc42, Rac1, and profilin 1 signaling in
oligodendrocyte and Schwann cell biology

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Studies on vertebrate nervous system myelination: The role of Cdc42, Rac1, and profilin 1 signaling in oligodendrocyte and Schwann cell biology

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<tr>
<td>ABP</td>
<td>Actin binding proteins</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Bluogal</td>
<td>5-Bromo-3-indolyl-D-galactopyranoside</td>
</tr>
<tr>
<td>CA</td>
<td>Constitutive active</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Cell division cycle 42</td>
</tr>
<tr>
<td>CMT</td>
<td>Charcot-Mary-Tooth disease</td>
</tr>
<tr>
<td>CNP</td>
<td>2',3'-cyclic nucleotide 3'-phosphodiesterase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-phenylindole dihydrochloride</td>
</tr>
<tr>
<td>Dhh</td>
<td>Desert hedgehog</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxygenin</td>
</tr>
<tr>
<td>DIGE</td>
<td>Differential gel electrophoresis</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Desoxyribonucleid acid</td>
</tr>
<tr>
<td>e.g.</td>
<td>Exempli gratia</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscope</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>GA</td>
<td>Glutaraldehyde</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDI</td>
<td>Guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
</tr>
<tr>
<td>ICAT</td>
<td>Isotope-coded affinity tagging</td>
</tr>
<tr>
<td>IF</td>
<td>Intermediate filaments</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin linked kinase</td>
</tr>
<tr>
<td>IPL</td>
<td>Intraperiod line</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>Lysolecithin</td>
<td>L-α-Lysophosphatidylcholine</td>
</tr>
<tr>
<td>MAG</td>
<td>Myelin associated glycoprotein</td>
</tr>
<tr>
<td>MAP</td>
<td>Microtubule associated protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>MDL</td>
<td>Major dense line</td>
</tr>
<tr>
<td>MF</td>
<td>Microfilaments</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MOG</td>
<td>Myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubules</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule organization center</td>
</tr>
<tr>
<td>NG2</td>
<td>Chondroitin sulfate proteoglycan</td>
</tr>
<tr>
<td>OPC</td>
<td>Oligodendrocyte precursor cell</td>
</tr>
<tr>
<td>PAK</td>
<td>p21 activated kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>Platelet derived growth factor receptor α</td>
</tr>
<tr>
<td>PDL</td>
<td>Poly-D-lysine</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>Pfn</td>
<td>Profilin</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3’ kinase</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol (4,5)-bisphosphate (=PtdIns(4,5)P₂)</td>
</tr>
<tr>
<td>PLP</td>
<td>Proteolipid protein</td>
</tr>
<tr>
<td>PMP-22</td>
<td>Peripheral myelin protein 22</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PO</td>
<td>Protein zero</td>
</tr>
<tr>
<td>PtdIns(4,5)P₂</td>
<td>Phosphatidylinositol (4,5)-bisphosphate (=PIP₂)</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidiene fluoride</td>
</tr>
<tr>
<td>Rac1</td>
<td>Ras-related C3 botulinum toxin substate 1</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homologous member A</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SCP</td>
<td>Schwann cell precursor</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase UTP nick end labeling</td>
</tr>
<tr>
<td>vs.</td>
<td>versus</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-Aldrich syndrome protein</td>
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<tr>
<td>WAVE</td>
<td>WASP family verprolin homologous protein</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot (SDS-PAGE)</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-Bromo-4-chloro-3-indolyl-D-galactopyranoside</td>
</tr>
</tbody>
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1. SUMMARY

My doctoral thesis has focused on the characterization of cellular and molecular mechanisms regulating myelination in the vertebrate nervous system. Myelination is carried out by oligodendrocytes in the central nervous system (CNS) and by Schwann cells in the peripheral nervous system (PNS). These cells extend processes that repeatedly wrap around axons to produce myelin, a lipid-rich biological membrane. The resulting myelin sheaths act as insulators, enabling the rapid, saltatory conduction of electrical signals along the axons. The importance of the myelination process is illustrated by the neurological deficits caused by demyelinating diseases, such as Multiple Sclerosis (MS) in the CNS and peripheral neuropathies in the PNS.

To sense their environment and to regulate their developmental program accordingly, oligodendrocytes and Schwann cells need to interpret instructive cues originating within the extracellular environment, among which growth factors and proteins of the extracellular matrix (ECM) are essential components. GTPases of the Rho subfamily integrate many extracellular signals and activate a variety of signaling pathways to regulate the actin and microtubule cytoskeleton, vesicle transport, and gene expression. Therefore, they are good candidate molecules to regulate several aspects of oligodendrocyte and Schwann cell biology including those associated with the ensheathment and myelination of axons.

In the first part of this thesis, we examine the role of the small Rho GTPases Cdc42 and Rac1 during myelination of the developing CNS using tissue-specific conditional gene ablation in a mouse model. Our results reveal an essential role for these proteins in myelination of the CNS. Ablation of Cdc42 or Rac1 produces a unique and stage-specific phenotype characterized by the extraordinary enlargement of the inner tongue of the oligodendrocyte process as a result of abnormal accumulation of large amounts of cytoplasm in this region. Moreover, we show that Cdc42 and Rac1 synergize to regulate the late stages of myelination, when oligodendrocyte processes ensheath axons and form compact myelin sheaths.

In the second part of this thesis we show that Cdc42 and Rac1 are also essential for myelination of the PNS. Sciatic nerves of Cdc42 and Rac1 mutant mice contain large
bundles of unsorted axons throughout development, indicating that Cdc42 and Rac1 are important for the radial sorting of axons. Our data also indicate that Cdc42 is necessary for Schwann cell proliferation and later in development for the proper myelination of axons.

The mechanisms controlling oligodendrocyte and Schwann cell proliferation, migration, axon ensheathment, and myelination are likely to involve a dynamic reorganization of the actin cytoskeleton. Profilin 1 is a G-actin-binding protein necessary for the dynamic reorganization of the actin cytoskeleton. Therefore, we investigate the function of profilin 1 in myelination of the nervous system, again using a conditional gene ablation strategy in a mouse model. We demonstrate that profilin 1 is required for proper myelination of the PNS but is dispensable for CNS myelination. Schwann cells lacking profilin 1 migrate and proliferate normally, but their differentiation is impaired. Our results indicate that the requirements for profilin 1 signaling in CNS myelination are different from those in PNS myelination.

Overall, our studies reveal an essential role for Cdc42, Rac1 and profilin 1 in the myelination of the nervous system. In addition, the phenotype observed in the CNS of Cdc42 and Rac1 mutant mice challenges the commonly held view that the formation of the spiral multilamellar myelin sheath implicates the continuous progression of the inner tongue over the axon surface. An important task for the future will be to identify the intracellular signaling pathways involved in the formation of the different phenotypes described here.
2. ZUSAMMENFASSUNG

Das Hauptziel dieser Doktorarbeit war die Charakterisierung von zellulären und molekularen Mechanismen, welche die Myelinisierung des Vertebraten-Nervensystems regulieren. Oligodendrozyten sind die myelin-bildenden Zellen des Zentralnervensystems (ZNS), während das periphere Nervensystem (PNS) von den Schwann’schen Zellen myelinisiert wird. Myelin ist eine lipidreiche Memran, die sich sprialformig um die Axone windet und die schnelle Übertragung von Nervenimpulsen ermöglicht. Der Verlust dieser Myelinscheiden führt zur Entstehung von schweren Krankheiten wie der Multiplen Sklerose im ZNS und peripheren Neuropathien im PNS.


Im ersten Teil dieser Doktorarbeit haben wir die Funktion von Cdc42 und Rac1 während der Myelinisierung des Zentralnervensystems mittels konditioneller Gen-Inaktivierung im Mausmodell untersucht. Wir zeigen, dass Cdc42 und Rac1 für die Bildung von Myelinscheiden im Zentralnervensystem wichtig sind. Der Verlust von Cdc42 oder Rac1 manifestiert sich in einem neuartigen Phänotyp, der sich durch eine Vergrösserung der inneren Schlaufe der Myelinscheide auszeichnet. Diese wird wahrscheinlich durch eine Ansammlung von Zytoplasma hervorgerufen und führt im Verlauf der Myelinisierung zu grossen fokalen Ausstülplungen der Myelinscheide. Im Gegensatz zur abnormalen Veränderung der Myelinscheide hat der Verlust von Cdc42 keinen Einfluss auf die Zellteilung, die gerichtete Migration und die morphologische Entwicklung von Oligodendrozyten in vitro. Schliesslich zeigt die Analyse verschiedener Cdc42/Rac1 Doppelmutanten eine positive Korrelation zwischen der Anzahl rekombinierter Allele und der Intensität des Phänotyps.

Im zweiten Teil dieser Arbeit beweisen wir, dass Cdc42 und Rac1 auch die normale
2. Zusammenfassung

Myelinisierung des peripheren Nervensystems regulieren. Unsere Resultate zeigen, dass Cdc42 und Rac1 unterschiedliche Vorgänge während der Entwicklung der Schwann’schen Zelle beeinflussen. Während Cdc42 die Teilung der Schwann’schen Zellen und die Ausbildung von Myelinscheiden reguliert, ist Rac1 unerlässlich für das Sortieren einzelner Axone aus embryonalen Axonbündeln.

Mehrere Studien weisen darauf hin, dass die Myelinisierung, die Migration und die Zellteilung der Oligodendrozyten und Schwann’schen Zellen eine Veränderung des Aktin Zytoskeletts erfordern. Profilin 1 ist ein Aktin-bindendes Protein und übernimmt eine wichtige Funktion in der Regulation des Aktin Zytoskeletts. Mittels konditioneller Gen-Inaktivierung im Mausmodell haben wir die Rolle von Profilin 1 während der Myelinisierung des Nervensystems untersucht. Unsere Resultate zeigen, dass Profilin 1 die Myelinisierung des peripheren Nervensystems reguliert, nicht jedoch die Bildung von Myelin im Zentralnervensystem. Der Verlust von Profilin 1 in Schwann’schen Zellen hat keinen Einfluss auf die Teilung oder die Migration dieser Zellen, beeinträchtigt aber deren Entwicklung zu myelin-bildenden Zellen.

Zusammengefasst zeigen unsere Resultate die zentrale Bedeutung von Cdc42, Rac1 und Profilin 1 für die Myelinisierung des Nervensystems. Ausserdem stellt der Phänotyp, den wir im ZNS von Cdc42 und Rac1 Mutanten beobachten, eine vorherrschende Hypothese in Frage, die besagt, dass die Rotation der inneren Schlaufe um das Axon zur Bildung der spiralförmigen Myelinscheide führt. Eine wichtige Aufgabe wird nun die Identifikation der beteiligten Signalübertragungswege sein.
3. AIMS OF THE THESIS

Myelination represents one of the most remarkable examples of cellular differentiation. During development of the nervous system, glial cells proliferate and migrate extensively before undergoing the extraordinary morphological changes associated with the ensheathment and myelination of axons. Over the past years, substantial insights were obtained concerning the embryonic origin and cell lineages of oligodendrocytes and Schwann cells, as well as the biochemical and biophysical properties of the myelin membrane. However, little is known about the cellular mechanisms that determine how the myelin sheath is extended and stabilized around the axon.

The aim of this study is to gain new insights into the fundamental processes taking place in glial cells during myelination of the nervous system. Dynamic changes in the cytoskeleton are essential for many key cellular functions such as migration, cytokinesis, shape changes, and polarity. As these processes are also crucial for myelination, I wanted to analyze the importance of cytoskeletal rearrangements in glial cells during myelination by the specific ablation of several key regulators of cytoskeletal dynamics. Rho GTPases are major regulators of microfilaments and microtubule organization, and they also can regulate a variety of other cellular functions. A more specific regulator is profilin, which directly interacts with actin monomers during microfilament turnover.

I have chosen a genetic route to selectively ablate Cdc42, Rac1, and profilin1 in cells of the glial lineage during mouse development. This approach provides the most stringent test system currently available, circumventing both early embryonic lethality caused by the homozygous inactivation of these proteins and the potential lack of specificity entailed by the use of dominant-negative mutant forms of the proteins. In conjunction with established in vitro models of differentiation, migration and cell survival, I was able to dissect the potential effects of these molecules on oligodendrocyte and Schwann cell morphology and on the outcome of myelination.

Increasing our knowledge about the cellular and molecular mechanisms involved in myelination during normal development may help us to better understand the pathological processes occurring in demyelinating diseases, and to design new approaches for their treatment.
4. INTRODUCTION

4.1. Organization of the vertebrate nervous system

During evolution vertebrates developed a highly complex nervous system that is crucial for functions ranging from sensory perception and motor coordination to cognitive features such as learning and memory. It can be subdivided into the central nervous system (CNS), consisting of brain and spinal cord, which processes information, and the peripheral nervous system (PNS), formed by the peripheral nerves that distribute the information. The major cellular components of nervous tissue are the neurons and neuroglia. While neurons conduct electrical signals, glial cells, which are much more abundant than neurons, fulfill supportive and trophic roles critical for the normal function of the nervous tissue. There are three major types of supporting cells in the CNS: oligodendrocytes, astrocytes and microglia. In the PNS, the Schwann cell is the main neuroglial component. Glia have important developmental roles, guiding migration of neurons in early development and regulating neuronal survival and differentiation. Two types of glial cells (oligodendrocytes and Schwann cells) produce myelin sheaths used to insulate nerve axons, thereby allowing the fast conduction of electrical signals essential for nervous system function (see Chapter 1.2). Other glial cells (astrocytes) form a selective filter around the brain capillaries, the blood-brain barrier, that prevents toxic substances and pathogens in the blood from entering the brain, while other substances are allowed to enter freely. Some glia promote efficient signaling between neurons by maintaining appropriate concentrations of ions and neurotransmitters in the neuronal environment. Recent findings indicate a role for glial cells in the formation, maintenance and function of synapses. Following injury, glial cells are major regulators of neuronal repair.
4.2. Myelination

4.2.1. Structure and Function of the myelin sheath

Myelination is achieved by oligodendrocytes in the CNS (Bunge et al., 1962), whereas Schwann cells are the myelinating cells in the PNS (Geren and Schmitt, 1954) (Figure 1A). Although the myelin sheaths of the CNS and PNS differ in their cellular origins, anatomical details and molecular constituents, they are thought to function similarly (Berthold and Rydmark, 1995; Peters et al., 1991).

Myelin is a multilamellar spiral of specialized membrane that ensheathes axons larger than 1 \( \mu m \) in diameter. It arises from glial cells as a flattened cell process that is elaborated around the axons and later becomes compacted by withdrawal of cytoplasm to form a tightly wound, membranous sheath comprising a number of lamellae (Figure 1B). Its unique composition (richness in lipids and low water content, allowing electrical insulation of axons) and segmental structure is responsible for the saltatory conduction of nerve impulses and allows the myelin sheath to support fast nerve conduction in the thin fibres of the vertebrate nervous system. Thus, in contrast to the invertebrate nervous system where rapid conduction is achieved by an increase in the axon diameter, myelination provides a remarkable saving of space and energy. Other roles of myelin comprise the regulation of axonal maturation as well as the maintenance and survival of axons (Griffiths et al., 1998; Waxman, 1997). The importance of the myelination process is illustrated by the neurological deficits caused by demyelinating diseases such as leukodystrophies and Multiple Sclerosis (MS) in the CNS and peripheral neuropathies in the PNS.

Myelin can be divided into two domains, compact and noncompact myelin, each of which contains a non-overlapping set of proteins. Most of the myelin consists of compact myelin that appears in electron microscope as a periodic structure with alternating electron-dense and light layers. The major dense line (dark layer) forms as the cytoplasmic leaflets fuse, while extracellular leaflets of adjacent lamellae become closely apposed to form the intraperiod line (Figure 4A). Abundant proteins include P0 protein, the myelin basic protein (MBP), P2 protein, and PMP-22 in PNS myelin (Greenfield et al., 1973), and proteolipid protein (PLP) and MBP in CNS myelin (Lees and Brostoff, 1984). PLP and MBP are required for normal spacing of compact CNS myelin (Duncan et al., 1987), while P0 is essential for normal spacing of PNS compact myelin (Giese et al., 1992). Large areas
of uncompacted myelin containing cytoplasm are present in immature myelin sheaths. During maturation, noncompact myelin becomes restricted to distinct sites such as the paranodal loops (the lateral borders of the myelin sheath), abaxonal and adaxonal compartments (inner and outer collar of the myelin sheath), and in Schmidt-Lanterman incisures (the funnel-like interruptions in the compact myelin; rare in the CNS).
Figure 1: Myelination of the central and peripheral nervous system

(A) Schematic representation of the organization of the myelin sheaths in the CNS and PNS. In the PNS, Schwann cells myelinate one segment of the axons (right half of scheme), whereas in the CNS (left half of scheme), the oligodendrocyte forms multiple myelin internodes. A cutaway view of a nodal region illustrates the terminal ends of adjacent myelin internodes. One internode is unrolled and schematically shows compact myelin and the cytoplasmic domains of the paranodal loops and inner and outer tongue process. P: paranodes; JP: juxtaparanodes (B) Schematic representation myelin sheath formation. A glial process wraps spirally around an axon, and during myelin compaction, the cytoplasm is excluded. Cytoplasmic leaflets fuse to from the major dense line (MDL), while extracellular leaflets become closely apposed to form the intraperiod lines (IPL).

Myelin sheaths do not cover the entire length of an axon but are separated by spaces where the axolemma is exposed to the extracellular milieu, the node of Ranvier. The stretch of axon between two nodes is called an internode. Structurally and functionally, the nodal region is organized into three distinct domains: node, paranode and juxtaparanode. At the node, the presence of voltage-gated sodium channels and the accessibility of the axolemma for currents allows for the fast saltatory conduction of the nervous impulse along the axon. In the PNS, the nodal axolemma is surrounded by Schwann cell microvilli, whereas processes from astrocytes and possibly oligodendrocyte precursor cells (OPCs) cover the nodes in the CNS. At the paranodes, myelin lamellae end as cytoplasmic pockets, the so-called paranodal loops. They are tightly tethered to the axon and to each other by junctional complexes. The juxtaparanodal region is found in the terminal 10-15 nm of the myelin internode that is adjacent to the paranodal loops. This region harbours voltage-dependent K⁺ channels thought to play a role in saltatory conduction by providing for repolarization after an action potential.
4. Introduction

4.2.2. Mechanisms of myelin growth

Little is known about the cellular mechanisms that determine how the myelin sheath is extended and stabilized around the axon. Glial cells are able to recognize the “future” caliber of an axon, as they only myelinate axons with a minimal diameter of 1 μm. How this is achieved is not understood, but proteins expressed on the axonal surface might be involved. Adhesion molecules, such as L1 and polysialylated NCAM (neural cell adhesion molecule) are known to be expressed on unmyelinated axons and to be downregulated during axonal myelination (Charles et al., 2002; Haney et al., 1999). However, no obvious myelination defects are observed in the L1 “knock-out” mouse (Cohen et al., 1998; Dahme et al., 1997). Other neuronal candidate molecules that influence glia to myelinate include secreted molecules such as neuregulins and neurotrophins (Chan et al., 2004). Another factor that was suggested to be required for myelination is electrical activity (Demerens et al., 1996). Recent studies showed that the cytokine leukaemia inhibitory factor (LIF) acts on mature oligodendrocytes to promote myelination (Ishibashi et al., 2006). LIF is released by astrocytes in response to ATP liberated from axons firing action potentials. These findings uncover a novel role for astrocytes in mediating the intricate communication between axons and myelinating glial cells.

In 1954, Betty Ben Geren showed that myelin is not an inanimate wax but a living cell process that elongates and spirally wraps around the axon (Geren and Schmitt, 1954). It is still not established whether spiral extension occurs by rotation of one or both “leading edges”. Early in vitro and in vivo studies suggest that the myelin sheath forms by progressive wrapping of the inner tongue of the Schwann cell around the axon (Bunge et al., 1989; Geren and Schmitt, 1954). Oligodendrocytes extend a filopodia along the axon and later, a ruffling lamellipodia spirals around the axon by a mechanism comparable to an inner tongue rotation (Asou et al., 1995). The sheath is initially loosely spiralled but compacts after a few spiral turns are formed by the extrusion of the cytoplasm. Subsequently, the sheath further expands in the radial and longitudinal directions (Peters et al., 1991). These dramatic morphogenetic events are likely to reflect major changes in the organization of the glial cell actin cytoskeleton.

The thickness of the myelin sheath is dependent on the axon caliber, with bigger axons having thicker myelin. Therefore, the ratio of the axon diameter divided by the axon and its
myelin sheath, the so-called growth-ratio (g-ratio), is a constant value, which is usually between 0.6-0.7. A key axonal signal for the regulation of myelin sheath thickness is the growth factor neuregulin 1 type III (Nrg1) (Michailov et al., 2004). Mice haploinsufficient for the \( \text{Nrg1} \) gene have thinner peripheral myelin sheaths than do wild-type mice, whereas Nrg1 overexpression results in the hypermyelination of axons. Recently, BDNF and the neurotrophin p75 receptor have also been implicated in regulating later stages of myelination, including myelin thickness (Tolwani et al., 2004). Nrg1 did not affect the internodal length. Therefore, the myelin thickness and elongation are regulated by different molecular mechanisms. Elongation of Schwann cells internodes requires intact L-periaxin-DRP2-dystroglycan (PDG) complexes and Cajal bands (Court et al., 2004). These are cytoplasmic channels beneath the surface of the plasma membrane that are important for the mRNA transport from the nucleus to distal sites.

### 4.2.3. CNS Myelination

Myelination of the CNS is carried out by oligodendrocytes. These cells extend many processes to form several myelin sheaths, and in the optic nerve one oligodendrocyte can myelinate up to 30-50 axon segments (Bunge, 1968; Peters et al., 1991). Oligodendrocyte progenitor cells (OPCs) are derived from neuroectodermal cells within ventricular areas of both spinal cord and brain. In the spinal cord, OPCs are mainly born in a tightly restricted area of the ventricular zone in a process that is regulated by the transcription factors Olig 1 and 2, and indirectly by the signaling molecule sonic hedgehog (Nave and Trapp, 2000). OPCs then migrate extensively throughout the CNS, proliferate to increase their numbers, and finally mature into post-mitotic myelin-producing cells. Oligodendrocyte generation in more anterior parts of the neural tube is not as well understood. Only recently, it was found that different waves of OPCs originate from several parts of the telencephalic ventricular zone and compete for space in the developing brain (Kessaris et al., 2006). The mature CNS retains a population of adult OPCs that are thought to be activated later for repair of demyelinated axons. The sequential expression of developmental markers, identified by a panel of cell-specific antibodies, divides the oligodendrocyte lineage into distinct phenotypic stages characterized by proliferative capacities, migratory abilities and changes in cell morphology (Figure 2).
4. Introduction

Figure 2: Oligodendrocyte differentiation in vivo

OPCs are born in the subventricular zones (SVZ) and migrate to future white matter areas as early progenitors. Late oligodendrocyte progenitor cells give rise to premyelinating oligodendrocytes and adult progenitors. Finally, oligodendrocytes either myelinate axons or die through programmed cell death. Some characteristic markers during oligodendrocyte lineage development are listed. Figure taken from Lazzarini et al., 2004.

Early OPCs express the platelet derived growth factor receptor α (PDGFrαR) and the sulphated proteoglycan NG2 (Nishiyama et al., 1999; Nishiyama et al., 1991). They differentiate into a late OPC stage that appears to be committed to oligodendrogenesis, and express the tetraspan protein CD9 and the POA antigen recognized by the monoclonal antibody O4 (Terada et al., 2002). Late OPCs are post-migratory but still proliferate (Warrington et al., 1993). They develop into premyelinating oligodendrocytes, which extend multiple processes.

In the CNS, the direction of myelin formation is caudal to rostral in the brain and rostral to caudal in the spinal cord (Warrington and Pfeiffer, 1992). Rat and mouse, which I have used in my experiments, are the most commonly used animals to study myelination. In these species, myelination starts perinatally, peaks at postnatal week three, and decreases to a low level thereafter. In humans, myelination is also postnatal and continues for several years after birth. Several aspects of oligodendrocyte development can be studied in vitro, as purified oligodendrocytes express myelin proteins (see Table 1), extend multiple processes, and form myelin-like membranes. When cultured together with neurons these cells extend flattened processes that wrap around the axons and form compact myelin.
### Table 1: Myelin proteins of the CNS

<table>
<thead>
<tr>
<th>Protein</th>
<th>Structure</th>
<th>Localization in myelin</th>
<th>Function</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP</td>
<td>Membrane protein with 4 TM-domains</td>
<td>Compact myelin</td>
<td>Maintains spacing of extracellular leaflets</td>
<td>May be required for long-term survival of some axons</td>
</tr>
<tr>
<td>MBP</td>
<td>Cytoplasmic, membrane-associated protein</td>
<td>Compact myelin</td>
<td>Maintenance of MDL</td>
<td>MT-based transport of MBP mRNA</td>
</tr>
<tr>
<td>CNP</td>
<td>Intrinsic membrane protein</td>
<td>Non-compact myelin</td>
<td>Targeting of MBP to compact myelin? Role in membrane expansion?</td>
<td>No physiological substrate for its enzymatic activity known</td>
</tr>
<tr>
<td>MAG</td>
<td>Adhesion molecule with 5 Ig-like domains</td>
<td>Non-compact myelin</td>
<td>Maintenance of myelin and axonal integrity</td>
<td>Role in myelin formation?</td>
</tr>
<tr>
<td>MOG</td>
<td>Membrane protein with 1 TM-domain</td>
<td>Preferentially at myelin surfaces</td>
<td>Receptor for signal transduction?</td>
<td></td>
</tr>
</tbody>
</table>

For more detailed information see Lazzarini 2004. Abbreviations: CNP, 2'3'-cyclic nucleotide 3'-phosphodiesterase; Ig, immune-globuline; MAG, myelin associated glycoprotein; MBP, myelin basic protein; MDL, major dense line; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein; TM, transmembrane.

### 4.2.4. CNS remyelination

The loss or destruction of myelin is a hallmark of MS and a characteristic of numerous demyelinating pathologies. Remyelination is the process by which myelin sheaths are restored to axons. The newly formed myelin sheaths protect axons from degeneration and restore the saltatory conduction of action potentials. A characteristic feature of remyelination is the formation of myelin sheaths that are thinner and shorter than expected for the diameter of the axons. Nevertheless, the composition of the myelin and the manner in which it is formed are essentially similar to those in developmental myelination.

In the CNS, remyelination may occur in a variety of clinical and experimental situations. However, it stands out as a rare regenerative phenomenon. Remyelination might fail due to an inadequate provision of OPCs (recruitment failure) or because of a failure of recruited OPCs to differentiate into remyelinating oligodendrocytes (differentiation failure) (for summary see Franklin, 2002). The reasons for failure are not yet clearly understood, but are likely to be many. According to the “dysregulation hypothesis”, remyelination fails because the signaling environment becomes inappropriately regulated or “dysregulated” (Franklin, 2002). A pro-recruitment environment must be maintained long enough to allow...
a lesion to be repopulated to an extent adequate for complete remyelination, and only when repopulation is complete should the environment shift to one that supports differentiation. Therefore, remyelination might fail because the complex and finely tuned mechanism by which it proceeds loses its precise coordination.

There are two principal strategies envisaged to promoting remyelination. The first involves the transplantation of exogenous cells with a repair enhancing or myelinogenic capacity. A second avenue is to design methods by which the endogenous process of remyelination can be enhanced or reactivated. This requires a better understanding of the molecular pathways regulating oligodendrocyte differentiation and myelination.

4.2.5. PNS Myelination

Schwann cells are the myelin-forming cells of the PNS. Development of the Schwann cell lineage differs in several ways from that of oligodendrocytes (Figure 3).

![Figure 3: The Schwann cell lineage](image)

Schwann cell precursors are neural crest cells derivatives. They differentiate into immature Schwann cells, with the same developmental potential. Envelopment of large caliber axons leads to the formation of myelinating Schwann cells, whereas small diameter axons are associated with non-myelinating Schwann cells. Dashed arrows indicate potential reversibility of different stages of development. Figure taken from Jessen and Mirsky 2005.

Schwann cells originate from the neural crest, and travel along axon bundles to peripheral locations. These Schwann cell precursors (SCPs) are devoid of a basal lamina and lack the ability to survive in the absence of axons. Once they reach their final destination, Schwann cell start to form a basal lamina and extend processes that interact with axons, separating them into large bundles. At this stage, such immature Schwann cells are able to survive
independently from axons due to an autocrine survival mechanism. The immature Schwann cell segregates a single axon, divides, and its daughter cell establishes a 1:1 relationship with the segregated axon and myelinates it (Webster, 1971). This process of “radial sorting” continues until only small caliber axons remain together with a nonmyelinating Schwann cell. All immature Schwann cells are considered to have the same developmental potential, and their fate is determined by the axons with which they associate. Only those Schwann cells that by chance are engaged with perspective large caliber axons will form a myelin sheath. Although mature Schwann cells dramatically change their morphology, they respond to nerve injury by reverting to a phenotype similar to that of immature Schwann cells.

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**Figure 4: Some characteristic markers of the Schwann cell lineage**

Schematic representation of protein expression during Schwann cell development. As most proteins are expressed in more than one differentiation stage (grouped in similarly coloured boxes), a combination of several markers is needed to define a specific developmental stage. For more detailed information see Jessen and Mirsky 2005.

Different stages of the Schwann cell lineage can be distinguished by a partially overlapping set of molecular markers (reviewed by Jessen and Mirsky, 2005) (Figure 4). PNS myelination occurs perinatally in rodents and humans. Schwann cell myelination
differs from oligodendrocyte myelination in several ways. First, Schwann cells myelinate a single axon, whereas oligodendrocytes can myelinate several axons. Second, only Schwann cells generate a basal lamina, a crucial requirement for the myelination of the PNS. This has first become evident in classic *in vitro* myelination studies where the lack of a proper basal lamina prevented myelination and the accompanying expression of myelin proteins (Bunge, 1993). Third, the expression of myelin proteins in Schwann cells (see Table 2) requires axonal contact whereas cultured oligodendrocytes can produce myelin-like membranes in the absence of axons.

Table 2: Myelin proteins of the PNS

<table>
<thead>
<tr>
<th>Protein</th>
<th>Structure</th>
<th>Localization in myelin</th>
<th>Function</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>Adhesion molecule with one Ig-like domain</td>
<td>Compact myelin</td>
<td>Extra- and intracellular compaction, promotion of myelination</td>
<td>Mutations lead to inherited myelin disorders</td>
</tr>
<tr>
<td>PMP-22</td>
<td>Membrane protein with 4 TM-domains</td>
<td>Compact myelin</td>
<td>Cell cycle proliferation, apoptosis, spreading, regulation of myelination</td>
<td>Mutations lead to inherited myelin disorders</td>
</tr>
<tr>
<td>MBP</td>
<td>Cytoplasmic, membrane-associated protein</td>
<td>Compact myelin</td>
<td>Supports P0 in intracellular compaction</td>
<td>Lack of MBP leads to minor abnormalities in PNS</td>
</tr>
<tr>
<td>MAG</td>
<td>Adhesion molecule with 5 Ig-like domains</td>
<td>Non-compact myelin</td>
<td>Maintenance of myelin and axonal integrity</td>
<td>Role in myelin formation?</td>
</tr>
<tr>
<td>P2</td>
<td>Extrinsic membrane protein</td>
<td>Compact myelin</td>
<td>May participate in intracellular compaction</td>
<td></td>
</tr>
</tbody>
</table>

For more detailed information see Lazzarini 2004. Abbreviations: Ig, immune-globuline; MAG, myelin associated glycoprotein; MBP, myelin basic protein; P0, protein zero; PMP22, peripheral myelin protein 22; TM, transmembrane.
4.2.6. PNS Remyelination

Remyelination in the PNS is efficient, quick, and consistently found in all demyelinating diseases. The transection of a peripheral nerve causes degeneration of the distal segment, a process that is known as "Wallerian degeneration" in honour of Augustus Waller. While distal axons degenerate, Schwann cells in the distal part of the injured nerve lose their myelin sheaths, de-differentiate, and re-enter the cell cycle. Macrophages invade the area to remove the myelin and axonal debris. During this process, the basal lamina, which surrounds the axon and Schwann cell, remains intact. Schwann cells line up in the basal lamina tube and synthesize growth factors, which attract axonal sprouts formed at the terminal of the proximal segment of the severed axon. The basal lamina tubes provide pathways for the regenerating axons to follow to muscles and skin. The Schwann cells then remyelinate the newly formed axons; however, these myelin sheaths are thinner than normal and the newly formed internodes are shorter than normal.

Pathologically, the axon degeneration and regeneration that occur in human and experimental peripheral neuropathies is quite similar to Wallerian degeneration, and is appropriately described as "Wallerian-like". This similarity has allowed researchers to use experimental Wallerian degeneration as a tool for studying the pathogenesis and potential treatments of peripheral neuropathies.
4. Introduction

4.3. Role of the cytoskeleton in myelination

4.3.1. Microfilaments and microtubules

The dynamic reorganization of the cytoskeleton is central to the control of key cellular processes such as migration, cytokinesis, shape changes, and the maintenance of polarity. The cytoskeleton is composed of three distinct fibrillar elements: actin microfilaments (MF), microtubules (MT) and intermediate filaments (IF). MF are thought to generate protrusive and contractile forces, whereas a polarized network of MT is crucial for the transport and localization of proteins and organelles. Unlike MF and MT, IF are very stable and their main function seems to be to maintain cell integrity. These cytoskeletal systems do not act independently but are highly coordinated to fulfil complex cellular functions. Cytoskeletal crosstalk is both directly and indirectly mediated by signaling molecules such as small Rho GTPases (Etienne-Manneville, 2004).

Actin is a highly conserved, abundant protein and exists in a monomeric, globular (G-actin) or filamentous form (F-actin). Actin assembly into MF is associated with the hydrolysis of ATP, resulting in polar structures with a rapidly growing end (barbed- or plus-end) and a slowly growing end (pointed- or minus-end). Treadmilling is a direct consequence of the irreversible hydrolysis of ATP, and describes the translocation of actin monomer form one end to the other without an overall change in the length of the polymer. This directional actin filament growth is the driving force behind cell locomotion, morphogenetic movements, and many intracellular transport events (Pollard et al., 2000). In vitro, actin filaments treadmill very slowly and a variety of regulatory actin binding proteins (ABPs) are required to explain the physiological behaviour e.g. why cells can advance quickly. Pollard developed the dendritic-nucleation hypothesis of assembly and disassembly of actin at the leading edge to explain the molecular mechanisms regulating shape changes and locomotion (Pollard et al., 2000) (Figure 5). In this model, the activation of membrane receptors by an appropriate extracellular signal triggers intracellular signaling molecules such as Rho family GTPases. These GTPases activate WASP/Scar family proteins by freeing them from autoinhibition. Active WASP/Scar initiates the binding of an actin monomer to the heptameric Arp2/3 complex. This complex then binds to the side of an older actin filament and induces the formation of a new branch. The addition of profilin-actin to the barbed end leads to the rapid growth of the new
filament and pushes the membrane forward. After a few seconds, elongation is halted by
the binding of capping proteins. Filaments age by the fast hydrolysis of ATP and the
subsequent, slow release of the phosphate. ADF/cofilin can bind to ADP-actin and is able
to sever and depolymerize ADP-actin filaments. Profilin binds the released ADP-actin
monomers and catalyzes the exchange of ADP for ATP thereby replenishing the pool of
ATP-actin-profilin monomers ready for another cycle of assembly. Beside the initiation of
new filaments, Rho family GTPases also regulate the rate of filament turnover by
activating p21-activated protein kinase (PAK).

Figure 5: Dendritic nucleation/array treadmilling model for protrusion of the leading edge
Extracellular signals trigger intracellular signaling pathways (1), which lead to the activation of Rho-family
GTPases (2). They in turn stimulate WASP/Scar proteins (3) to bring together the Arp2/3 complex and an
actin monomer on the side of a pre-existing filament to form a new branch (4). Filaments grow by the
addition of ATP-actin to their barbed ends (5), thereby pushing the plasma membrane forward (6). Binding of
capping proteins stops elongation of the filaments (7). They age by hydrolysis of ATP bound to the actin
monomers (8) and are subsequently severed and depolymerised by ADF/cofilin (9). Profilin binds the
released ADP-actin monomers and catalyzes the exchange of ADP for ATP (10), returning the monomers to
the pool of ATP-actin, ready for another round of polymerization (11). Rho GTPases also activate PAK and
LIM kinase, which phosphorylates ADF/cofilin (12). This tends to slow down filament turnover. Figure taken
from Pollard and Borisy, 2003.
MT consist of heterodimers of α and β tubulin, which polymerize into cylindrical filaments. MT are polar structures with a free plus end and a minus end which, in most cells, is associated with a MT organizing centre (MTOC). The plus end of MT exhibit 'dynamic instability', oscillating between persistent phases of growth and shortening. Slowly growing MT are especially unstable and collapse rapidly as the hydrolysis of GTP weakens the binding between individual tubulin dimers. To stabilize MT selectively, tubulin subunits are modified by acetylation and detyrosinylation and form platforms for specific MT-associated proteins (MAPs), which further protect them from collapsing. An important group of MAPs are the motor-proteins kinesin and dynein which translocate organelles and vesicles towards the plus and minus ends of MT, respectively.

4.3.2. Role for the oligodendrocyte cytoskeleton in myelination

The size and the complex morphology of oligodendrocytes requires specialized mechanisms for the spatial and temporal regulation of the transport of lipids, proteins and mRNAs essential for myelin synthesis and maintenance. The cytoskeleton is crucial to organize the synthetic machinery, and provides the tracks for intracellular translocation of myelin gene products (Amur-Umarjee et al., 1990; Colman et al., 1982; Trapp et al., 1987). Oligodendrocytes contain an extensive cytoskeletal network of MT and MF, but are devoid of IF (Pfeiffer et al., 1993; Wilson and Brophy, 1989).

MF are present in all regions of oligodendrocytes throughout their development (Song et al., 2001). They are enriched in the peripheral regions beneath the plasma membrane, particularly at the leading edges of processes and in areas where new branches are being formed. Different myosin motors might be involved in the local reorganization of MF in response to specific environmental cues. In oligodendrocytes, myosin IIB is enriched in the leading edge and at branch sites while myosin IIA tends to be more localized in the perinuclear area and proximal regions of the processes (Song et al., 2001).

MT are present in the cell body and in the oligodendrocyte processes, and splay before extending into the leading edge and branches, following the tracks laid by MF (Song et al., 2001). This cytoskeletal organization is reminiscent of that found during the growth of an axon (Baas, 1999), and is consistent with the idea that MF guide the local reorganization of MT for the elongation of oligodendrocyte processes and the formation of new branches.

The two major groups of neuronal microtubule-associated proteins (MAPs), MAP2 and
tau, are expressed in oligodendrocytes and might be involved in the regulation of MT stability and organization.

In contrast to Schwann cells that form only one process, oligodendrocytes can extend several processes. Therefore, it is likely that oligodendrocytes contain specialized forms of MT organizations not found in Schwann cells. This hypothesis is supported by an oligodendrocyte-specific MT-related gene defect in the taiep rat (Duncan et al., 1992). Taiep rats develop progressive neurological phenotypes due to CNS-specific dysmyelination. Morphological phenotypes include accumulation and bundling of MT in oligodendrocytes, and reversal of MT polarity in oligodendrocyte processes. This phenotype might arise due to a genetic defect in an oligodendrocyte-specific MAP, which indirectly reduces myelin protein processing, myelin formation and myelin internode maintenance. Besides MAPs, myelin-specific proteins, such as MBP and CNP, interact with the cytoskeleton. CNP mediates MT and MF reorganization, and promotes MT assembly for process outgrowth in oligodendrocytes (Lee et al., 2006).

4.3.3. Role for the Schwann cell cytoskeleton in myelination

A central role for the Schwann cell cytoskeleton in the process of peripheral nerve myelination has long been suggested (Fernandez-Valle et al., 1997; Trapp et al., 1989; Trapp et al., 1995). First molecular evidences to support this hypothesis came from the analysis of dystonia musculorum (dt) mice, which carry mutations in dystonin, a cytoskeletal crosslinker protein. These mice display hypo/amyelinated peripheral nerves and morphological analysis revealed that Schwann cells were arrested in the promyelinating stage and had multiple myelinating lips (Bernier et al., 1998).

Unlike oligodendrocytes, Schwann cells express several IF proteins during development. Vimentin is expressed in immature Schwann cells and in differentiated cells (Autilio-Gambetti et al., 1982). GFAP is present in immature Schwann cells and non-myelinating Schwann cells (Jessen and Mirsky, 1991), but not in myelin-forming Schwann cells. Myelinating Schwann cells express neurofilament-M (NF-M), showing that IF proteins of the neurofilament-type are not restricted to neurons in the vertebrate nervous system (Kelly et al., 1992).

During Schwann cell differentiation actin plays a critical in the regulation of cell shape, and influences the expression of myelin genes (Fernandez-Valle et al., 1997). Disruption of
MF by low concentrations of cytochalasin D prevented spiralling of the myelin process around the axon and the expression of genes encoding myelin proteins in myelinating Schwann cell-neuron cocultures. Localization studies have demonstrated that actin and ERM (ezrin, radixin and moesin) proteins are initially diffusely distributed in the Schwann cell cytoplasm but concentrate at either end of the Schwann cell just before myelination (Melendez-Vasquez et al., 2001; Pedraza et al., 2001). The distribution of Schwann cell MT is influenced by the underlying axon, which regulates the location and the number of MT-nucleation sites (Kidd et al., 1996). In Schwann cells without axonal contact, MT are nucleated from a single MTOC. Physical contact with appropriate axons initiates a myelin-forming phenotype that disperses MT minus ends and induces multiple MT-nucleating sites in Schwann cell perinuclear cytoplasm. The axonal signal that initiates myelin breakdown during Wallerian degeneration induces multiple MTOCs and MT bundles in Schwann cell perinuclear cytoplasm and in cytoplasm between degenerating myelin ovoids. These specialized MT distributions and configurations are essential for the dispersal of the major membrane synthesizing organelles (RER and Golgi apparatus) as well as for the site-specific targeting of membrane proteins (Trapp et al., 1995).

4.4. Rho family GTPases, central coordinators in the cell

4.4.1. Structure and Function of Rho GTPases
The Rho-family of small guanosine triphosphatases (Rho GTPases) forms a large subgroup of the Ras superfamily of 20-30 kDa GTP-binding proteins. They are defined by the presence of a Rho-type GTPase domain, which contains a special Rho insert loop between the fifth β strand and the fourth α helix that distinguishes them from other small GTPases. Rho family proteins are ubiquitously expressed from yeast to man. To date, 22 human genes encoding at least 25 proteins have been described. These proteins share >50% sequence identity and are divided into six subfamilies: the RhoA-related subfamily (RhoA, RhoB, RhoC), the Rac1-related subfamily (Rac1, Rac2, Rac3, RhoG), the Cdc42-related subfamily (Cdc42, TC10, TCL, Chp/Wrch-2, Wrch1), the Rnd subfamily (Rnd1, Rnd2, RhoE/Rnd3), the RhoBTB subfamily, and the recently described Miro subfamily. In addition, RhoD, Rif and TTF/RhoH do not obviously fall into any of these families. Among all Rho GTPases, Cdc42 (cell division cycle 42), Rac1 (Ras-related C3 botulinum
toxin substrate 1) and RhoA (Ras homologous member A) have been studied most extensively.

Rho GTPases are ubiquitous binary molecular switches that cycle between an inactive GDP-bound and an active GTP-bound state in response to a variety of extracellular stimuli (Figure 6A). In the active state they can bind to downstream effectors to elicit different biological responses (Luo, 2000; Moon and Zheng, 2003). Each Rho-family protein activates multiple effectors, and some effectors are recognized by multiple family members. The GDP/GTP cycling is controlled by three classes of regulatory proteins: (1) ~60 GEFs (GTPase exchange factors), which promotes the exchange of the bound GDP for GTP and thus activate Rho GTPases (Schmidt and Hall, 2002); (2) ~80 GAPs (GTPase activating proteins), which stimulate the intrinsic GTP hydrolysis rate of Rho-family proteins, thereby inactivating them (Moon and Zheng, 2003); and ~4 GDIs (guanine dissociation inhibitors), which inhibit binding of Rho proteins to membranes and prevent nucleotide exchange (Olofsson, 1999). Recent findings have indicated that the activities of Rho GTPases, especially of many of the less studied family members, are regulated in several additional ways (Wennnerberg and Der, 2004). Rho protein activity is also influenced by C-terminal modifications (prenylation, palmitoylation) and sequences (polybasic K/R rich sequences), which are responsible for their subcellular localization and association with specific membranes (Figure 6B). In addition, many Rho GTPases seem to be highly regulated at the level of their expression.

For a long period, Rho GTPases were believed to be involved primarily in the regulation of cytoskeletal organization in response to extracellular signals. However, over the past few years, Rho GTPases have been found to participate in a multitude of other fundamental processes such as polarization, transcriptional regulation, cell cycle progression, membrane transport pathways, regulation of transport factor activity, and cell adhesion (Etienne-Manneville and Hall, 2002).
4. Introduction

Effectors

Rac1, Rac2, Rac3, RhoA, RhoC, RhoG, Cdc42, RhoH/TTF

Rnd1, Rnd2, Rnd3

TC10, (TCL)

RhoB

RhoBTB

Miro

Figure 6: Regulation of Rho GTPases

(A) Rho GTPases cycle between an inactive (GDP-bound) and an active (GTP-bound) conformation. Active Rho proteins interact with a variety of effector proteins. The cycle is tightly regulated: guanine nucleotide exchange factors (GEFs) catalyze nucleotide exchange; GTPase-activating proteins (GAPs) stimulate GTP hydrolysis; and guanine dissociation inhibitors (GDIs) extract the inactive GTPase from membranes. (B) Representation of different C-termini, the post-translational lipid modification and additional membrane targeting signals. Cysteine residues in the C-terminal CAAX motif can be modified by either farnesyl (F) or geranylgeranyl (GG) isoprenoid modification, followed by proteolytical removal of the AAX residues, and carboxymethylation (OMe) of the now-terminal cysteine residue. Additional modification by palmitate (P) fatty acid is observed in TC10 and RhoB. RhoBTB, which does not undergo post-translational modifications, contains two BTB domains. Miro contains two EF-hand (EFH) motifs and one additional GTPase domain but no C-terminal CAAX motif. Figure adapted from Etienne-Manneville and Hall 2002 (A) and Wennerberg and Der 2004 (B).
4.4.2. Rho GTPase signaling pathways

Rho GTPases are implicated in a multitude of cellular processes, which might lie in their ability to interact with a number of downstream targets so that they can coordinately activate several molecular processes required for a particular cellular response. Unfortunately, target proteins do not contain a single recognizable sequence motif useful in database searches, but for Cdc42, Rac, and Rho over 60 targets have so far been identified experimentally (for detailed reviews for these effector pathways see Bishop and Hall, 2000; Bokoch, 2003; Riento and Ridley, 2003; Symons and Settleman, 2000). It is still unclear which of these are responsible for the diverse biological effects of Rho GTPases. Furthermore, established signaling pathways cannot simply be transferred to every system, but rather seem to be cell type and context dependent.

Most progress has been made in the cytoskeleton field, and several biochemical links have now been established between GTPases and the assembly of filamentous actin (Figure 7). Cdc42 and phosphatidylinositol-4,5-bisphosphate (PIP2) stimulate Arp2/3-complex-induced actin polymerization by interacting with N-WASP (Prehoda et al., 2000). Regulation of Scar activity is less well understood, but appears to involve Rac1 rather than Cdc42 (Miki et al., 1998). The Rac effector phosphatidylinositol-4-phosphate 5-kinase (PI4P5K), which generates PIP2, is a likely mediator between Rac and both Scar and N-WASP. PIP2 also promotes the extension of existing filaments by inhibiting barbed end capping proteins. Cdc42 and Rac1 can also interact with p21-activated kinase (PAK) to stimulate the serine/threonine kinase LIM (LIMK, acronym of three gene products Lin-11, Isl-1, Mec-3), which inhibits cofilin-induced actin depolymerization. In addition, myosin light chain kinase (MLCK) is inhibited by PAK, resulting in decreased phosphorylation of myosin light chain and hence decreased actomyosin contractility. By blocking the retrograde flow of actin filaments but not actin assembly, protrusive forces are exerted at the leading edge. The serine/threonine kinase Rho-kinase (ROCK) is probably the most important Rho effector in many cell types. ROCK phosphorylates myosin light chain phosphatase, as well as myosin light chain itself, both of which lead to increased contraction of the actomyosin network (Amano et al., 1996; Kimura et al., 1996). Similar to PAK, ROCK also activates LIMK to inhibit cofilin (Maekawa et al., 1999).
4. Introduction

RhoGEFs RhoGAPs
Rho Rac Cdc42
ROCK Dock Pak P4PSK
MLC phosphatase LIM kinase MLCK PIP2

Figure 7: Signal transduction pathways that link Rho GTPases and the actin cytoskeleton
The pathways shown are based primarily on studies of fibroblast motility. Rho GTPases regulate actin dynamics at several points, including filament nucleation and branching (Arp2/3 complex), filament extension (capping proteins), retrograde flow (myosin) and actin recycling (cofilin). Figure adapted from Dickson, 2001.

4.4.3. Effects of Rho GTPases on the cytoskeleton
Rho GTPases are key regulatory molecules that link surface receptors to the organization of the actin cytoskeleton (Nobes and Hall, 1995; Ridley and Hall, 1992; Ridley et al., 1992) (Figure 8). Activation of RhoA in fibroblasts has been shown to cause the bundling of actin filaments into contractile stress fibres and the clustering of integrins and associated proteins into focal adhesion complexes. Rac1 activation promotes de novo actin polymerization at the cell periphery to form lamellipodial extensions, and active Cdc42
triggers actin polymerization to form filopodia. Actin filaments found in lamellipodia and filopodia are, like stress fibres, associated with integrin adhesion complexes, although the function of these complexes is not clear (Machesky et al., 1997; Nobes and Hall, 1995). In addition to their downstream effect on the actin cytoskeleton, Rho family proteins affect each other’s activity, and a signal-transduction pathway from Cdc42 to Rac1 to RhoA was proposed (Bradke and Dotti, 2000). In particular, Cdc42 is a strong activator of Rac, such that filopodial extensions are usually seen associated with lamellipodial protrusions (Kozma et al., 1995; Nobes and Hall, 1995).

Figure 8: Rho GTPases organize the actin cytoskeleton
(A) Actin filaments are evenly distributed in the cytoplasm of quiescent, serum-starved Swiss 3T3 fibroblasts. Activation of Rho by lysophosphatidic acid induces the formation of stress fibres (B). Microinjection of constitutively active Rac leads to the formation of lamellipodia (C), while activation of Cdc42 by microinjection of a Cdc42-GEF leads to filopodia formation (D). Actin filaments are visualized with rhodamin phalloidin. Figure adapted from Hall 1998.

Reorganization of the actin cytoskeleton is required in a variety of cellular processes such as cell movement, axon guidance, cytokinesis, and morphogenetic processes involving changes in cell shape and polarity (Hall, 1998). As expected, Rho, Rac and Cdc42 participate in the regulation of these processes in a variety of different cell types.

There is also increasing evidence that Rho GTPases affect the organization of the microtubule and intermediate filament networks (Rodriguez et al., 2003; Waterman-Storer and Salmon, 1999; Wittmann and Waterman-Storer, 2001). In migrating cells, the MT cytoskeleton is highly polarized. RhoA (acting through p160Rho kinase and mDia) promotes the accumulation of detyrosinated MT in the vicinity of the leading edge (Ishizaki et al., 2001) and Rac1 and Cdc42 (acting through p65Pak) inactivates the MT destabilizing protein stathmin (Daub et al., 2001; Kuntziger et al., 2001). In migrating astrocytes, Cdc42 regulates the reorientation of the MT and centrosome by activating a complex of Par6 and PKCζ (Etienne-Manneville and Hall, 2001). PKCζ then
phosphorylates and inactivates GSK-3, inducing the association of APC with the plus ends of MT at the leading edge. MT reorganization and centrosome reorientation then takes place through a dynein- or dynactin-dependent mechanism (Raftopoulou and Hall, 2004). Inhibition of Cdc42 in neurons leads to axonal and dendritic outgrowth defects and random orientation of the MTOC (Luo et al., 1994; Scott et al., 2003).

Maintenance of cell polarity also requires reorganization of the MT cytoskeleton and Rho GTPases (particular cdc42) play an important role in this process (Raftopoulou and Hall, 2004; Wittmann and Waterman-Storer, 2001). Cdc42 controls polarity of the MF and MT cytoskeleton through two distinct signaling pathways (Cau and Hall, 2005). A direct interaction with Par6 leads to activation of the associated atypical PKC and polarization of the MT, while a distinct interaction with PAK activates this kinase, leading to the localization of active βPIX/ Rac and polarized actin polymerization.

Rho family proteins affect cytoskeletal dynamics and in turn, the activity of Rho proteins is regulated by microtubules and actin. Microtubule or actin disassembly activates RhoA (Ren et al., 1999), whereas microtubule assembly promotes Rac1 activation (Waterman-Storer and Salmon, 1999).

### 4.4.4. Rho GTPases in CNS myelination

Many cellular process that are regulated by Rho GTPases are also crucially involved during myelination of the nervous system. Glial cells proliferate and migrate extensively before undergoing dramatic morphological changes and sending out processes to wrap axons.

Oligodendrocytes express the major Rho GTPases (Erschbamer et al., 2005), and their expression and activity is developmentally regulated (Liang et al., 2004). During differentiation of primary oligodendrocyte cultures, the activity of RhoA is downregulated, whereas the expression and activity of Cdc42 and Rac1 increases. Rho GTPases are thought to be important for the morphological differentiation of oligodendrocytes *in vitro* (Liang et al., 2004). While expression of dominant negative (DN) Rho, constitutively active (CA) Rac1, or CA Cdc42 induced outgrowth of oligodendrocyte processes, introduction of CA Rho, DN Rac1, or DN Cdc42 inhibited process extension and oligodendrocyte differentiation. This is similar to what happens in neurons, where Rac and Cdc42 act as positive regulators of neurite extension and RhoA mediates neurite retraction.
(Katoh et al., 2000; Kozma et al., 1997). Moreover, a linear signaling pathway from integrins to Fyn to Rho family GTPases is suggested to control the morphological differentiation of oligodendrocytes. In addition, CNS myelination is negatively regulated by LINGO-1, a transmembrane protein of oligodendrocytes that activates RhoA, thereby inhibiting oligodendrocyte differentiation and myelination (Mi et al., 2005).

4.4.5. Rho GTPases in PNS myelination

Rho GTPases are expressed in Schwann cells (Terashima et al., 2001) and their active forms are targeted to different subcellular sites. In cultured rat Schwann cells, Cdc42 and Rac1 are localized in the cytoplasm and after IGF-I treatment, Cdc42 re-localizes from the cytoplasm into filopodia, while Rac1 accumulates in membrane ruffles (Cheng et al., 2000). RhoA is diffusely expressed in the cytoplasm of primary rat Schwann cell cultures and in teased fibres it localizes to the abaxonal membrane, the nodes of Ranvier, and Schmidt-Lanterman incisures (Melendez-Vasquez et al., 2004). In primary rat Schwann cell cultures, Cdc42 and Rac1 affect IGF-I-induced cell motility (Cheng et al., 2000), while RhoA activity influences cell morphology (Brancolini et al., 1999). Cdc42 and Rac1 also have an effect on Schwann cell migration induced by neurotrophin-3 (Yamauchi et al., 2003). Abnormal myelination occurs in Schwann cell-neuron cocultures after the inhibition of Rho kinase, a major downstream effector of Rho (Melendez-Vasquez et al., 2004). Treated Schwann cells branch aberrantly and form multiple, small myelin segments along the axons.
4.5. Profilin, an ubiquitous actin binding protein

4.5.1. The profilin family

Profilins (Pfn) are small (molecular weight =12-16 kDa) actin-binding proteins and are among the most highly expressed (20-100 μM) cytoplasmic proteins (Buss et al., 1992). Although profilin was initially isolated from calf thymus, it has since been found in all organisms examined to date including amoebae (Reichstein and Korn, 1979), yeast (Oechsner et al., 1987), flies (Cooley et al., 1992) and plants (Valenta et al., 1991). While lower eukaryotes possess just one profilin gene, mammals encode four different profilin proteins (profilin 1-4). Expression studies in mice showed that profilin 1, the major profilin isoform, is found throughout development and in nearly all tissues in adult mice except skeletal muscle (Witke et al., 1998). The profilin 2-gene is alternatively spliced, resulting in two isoforms (designated 2a and 2b) that are differentially expressed and have distinct biochemical properties (Lambrechts et al., 2000). Profilin 2a is expressed in the developing nervous system and in differentiated neurons, and profilin 2b constitutes a minor form present in a restricted range of tissues (Lambrechts et al., 2000; Witke et al., 2001). The more recently discovered profilin 3 and profilin 4 are both characterized as testicular isoforms; however, the timing of their expression is distinct (Braun et al., 2002; Obermann et al., 2005). The necessity for different profilin isoforms might have arisen by the acquisition of additional functions of profilins besides the regulation of actin assembly. This is supported by the fact that, in mouse brain, profilin 1 and profilin 2 bind to different sets of ligands (Witke et al., 1998).

4.5.2. Profilin functions

To assure proper profilin function, the preservation of their secondary and tertiary structures seems to be more important than their amino acid sequence, which is only poorly conserved. Profilin is a globular protein with a central, seven-stranded β sheet flanked by four helices (Figure 9). This profilin domain enables the interaction with monomeric actin, poly-L-proline containing proteins, and phosphoinositides. Whereas the binding sites for actin and poly-L-proline are distinct, the PtdIns(4,5)P₂-binding area is more spread out over the surface of the molecule.
4. Introduction

Profilin consists of a seven-stranded β-sheet flanked by four helices. Profilin binds to actin, proline-rich ligands and PtdIns(4,5)P$_2$. While binding sites for actin (red) and poly-L-proline (yellow) are distinct, the PtdIns(4,5)P$_2$-binding area is more spread over the surface of the molecule (light green) and can affect both actin binding and poly-L-proline binding. Figure adapted from Witke 2004.

One of the first functions assigned to profilin after its discovery was its 1:1 binding to G-actin monomer, thus regulating the availability of actin monomers (Carlsson et al., 1977). This sequestration allows a cell to exceed its critical concentration of ATP-actin (0.1 μM) without the occurrence of spontaneous filament nucleation or polymerization. A large pool of ATP-actin is important for the rapid elongation of microfilaments. However, in vivo, the main G-actin sequestering protein is thymosin-β which is much more abundant than profilin (560 μM versus 50 μM) and binds actin with a much higher affinity (K$_d$ ~ 0.4-0.7 μM versus ~ 0.4-10 μM for profilin) (Safer et al., 1991). Thus, sequestration might be a limited function of profilin's interaction with the actin cytoskeleton. Another way in which profilin influences actin dynamics is by regulating the nucleotide status of actin monomers. Binding of profilin to G-actin accelerates the ADP-ATP exchange rate 1000-fold, thereby replenishing the pool of ATP-actin needed for rapid microfilament polymerization (Goldschmidt-Clermont et al., 1992; Mockrin and Korn, 1980). Once ATP is bound to G-
actin, profilin completely inhibits its hydrolysis and maintains actin monomers in a high affinity state for microfilament barbed ends (Tobacman and Korn, 1982). Following cell stimulation, capping proteins are dissociated from the barbed ends and profilin-actin complexes associate with the free filaments. The profilin-bound actin is released at the filament’s barbed end to become a subunit of F-actin. *In vivo* support for profilin’s role in promotion of actin polymerization is provided by the bacteria *Listeria monocytogenes* where depletion of profilin completely blocks actin-based motility (Theriot et al., 1994). Taking together all the ways in which these proteins can influence the actin cytoskeleton, a key role emerged for profilin in promoting actin dynamics at the plasma membrane to drive cell motility and other actin-linked processes.

A decade after the discovery of profilin, it was found that it can bind to membrane phospholipids – mainly PtdIns (4,5)P_2 (phosphatidylinositol (4,5)-bisphosphate) and PtdIns (3,4,5)P_3 (Lassing and Lindberg, 1985). *In vitro*, this interaction was shown to induce the rapid and efficient dissociation of profilin-actin complexes, leading under certain conditions to actin polymerization. These findings provided a new, exciting link between lipid based signaling pathways and the actin cytoskeleton. In addition to the regulation of the binding of profilin to actin, PtdIns(4,5)P_2 also influences the binding of poly-L-proline to profilin (Lambrechts et al., 1997). It is thought that through binding of different proteins with proline-rich domains, profilin is subcellularly localized to sites where actin polymerization is induced. This hypothesis is strengthened by the fact that many of these proteins are intimately involved in filament elongation, as for example VASP, N-WASP and palladin (Boukhelifa et al., 2006; Reinhard et al., 1995; Suetsugu et al., 1998). Profilin interacts through its poly-L-proline domain with a plethora of proteins that will here be referred to as profilin ligands. More than 50 ligands from different organisms have been characterized to date, and these might reflect only a small fraction of all profilin-binding partners that exist. The list includes not only ligands that link intracellular pathways to the actin cytoskeleton as initially expected, but also regulators of endocytosis, putative transcription factors, nuclear export receptors, and Rho GTPase effector molecules (Witke, 2004) (Table 3).
### Table 3: Profilin ligands identified in mammalian cells

<table>
<thead>
<tr>
<th>Focal contacts</th>
<th>Specificity</th>
<th>Cellular pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>VASP</td>
<td>Pfn1&lt; Pfn2</td>
<td>Platelet activation</td>
</tr>
<tr>
<td>Mena</td>
<td>Pfn1&lt; Pfn2</td>
<td>Axon pathfinding</td>
</tr>
<tr>
<td>EVL</td>
<td>Pfn1, Pfn2</td>
<td>Focal contacts</td>
</tr>
<tr>
<td>Palladin</td>
<td>(Pfn1, Pfn2)</td>
<td>Focal contacts</td>
</tr>
<tr>
<td>Synaptic scaffold</td>
<td></td>
<td></td>
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<tr>
<td>Gephyrin</td>
<td>(Pfn1, Pfn2)</td>
<td>Receptor clustering</td>
</tr>
<tr>
<td>Dreglin</td>
<td>(Pfn1, Pfn2)</td>
<td>Dendritic spines</td>
</tr>
<tr>
<td>Aczolin</td>
<td>Pfn1&lt; Pfn2</td>
<td>Vesicle trafficking</td>
</tr>
<tr>
<td>Delphlin</td>
<td>Pfn1, Pfn2</td>
<td>Glutamate receptor</td>
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<tr>
<td>Membrane trafficking</td>
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<td></td>
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<tr>
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<td>Pfn2</td>
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<tr>
<td>Huntingtin</td>
<td>Pfn2</td>
<td>Vesicle trafficking</td>
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<tr>
<td>Synaptojanin</td>
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<td>Endocytosis</td>
</tr>
<tr>
<td>Annexin1</td>
<td>(Pfn1, Pfn2)</td>
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<td>Rho GTPase signaling</td>
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<td></td>
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<td>POP 130 or CYFIP</td>
<td>Pfn2</td>
<td>Rac1, fragile X syndrome</td>
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<td>Pfn2</td>
<td>RhoA, neurite outgrowth</td>
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<td>MDiaphanosus</td>
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<td>FRL</td>
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<td>Rac1, lymphocyte function</td>
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<tr>
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<td>SMN</td>
<td>Pfn1&lt; Pfn2</td>
<td>Splicing, muscular atrophy</td>
</tr>
</tbody>
</table>

For more detailed information see Witke 2004. Abbreviations: AF-6, All-1 fusion partner from chromosome 6; CYFIP, cytoplasmic FMRP-interacting protein; EVL, Ena VASP like; FMRP, fragile X mental retardation protein; FRL, forming-related gene in leukocytes; Mena, mouse homolog of Drosophila enabled; Nap, nck-associated protein; POP, partner of profilin; Pfn, profilin; ROCK, rho-dependent coiled-coil kinase; SMN, survival of motor neuron; VASP, vasodilatory-stimulated phosphoprotein; WASP, Wiskott-Aldrich syndrome protein; WAVE, WASP-family verprolin-homologous protein; WIP, WASP-interacting protein; VCP, valosine-containing protein.

Although the exact functions of these profilin-ligand interactions are not completely understood, it was recognized that these ligands often interact with each other or are part of different complexes. Therefore, a novel picture emerges of large platforms that can exchange or share components and integrate diverse signaling pathways. Profilins seem to be a common denominator in these signaling platforms, and the challenge for the future is to determine their role therein. One possibility might be that profilins directly regulate the activity of their ligands, as is thought to be the case for WASP and diaphanos activation,
where profilin might be involved in the release of their autoinhibition (Yang et al., 2000).

Profilins might also function as central hubs that control the composition of the complexes by enabling certain ligands to enter or leave.

Interestingly, the *in vivo* function of profilin was shown to be dependent on the cell type or organism studied. Deletion of profilin in *Schizosaccharomyces pombe* and *Drosophila melanogaster* is lethal (Balasubramanian et al., 1994; Verheyen and Cooley, 1994), whereas profilin ablation in *Saccharomyces cerevisiae* results in severe growth reduction (Haarer et al., 1990). In the amoebae *Dictyostelium discoideum* the loss of both profilin genes leads to defects in cytokinesis (Haugwitz et al., 1994), and profilin-deficient *Dictyostelium* cells show increased F-actin levels.

Activities of profilin have been studied extensively *in vitro* but rarely *in vivo*. Gene inactivation of profilin 1 in mice causes early embryonic lethality due to a defect in cell division (Witke et al., 2001). Profilin 2-null mice are viable but display severe neurological deficits (A.Di Nardo and W.Witke, unpublished). To circumvent this early lethality, the use of tissue-specific conditional gene ablation might provide a valuable tool for the further analysis of profilin function *in vivo*.

In this work, I have used this methodology to study the role of profilin 1 and the small RhoGTPases Cdc42 and Rac1 in CNS and PNS myelination.
4.6. References


4. Introduction


4. Introduction


4. Introduction


5. PART I, Rho GTPases in PNS myelination

5. PART I

Cdc42 and Rac1 signaling are both required for and act synergistically in the correct formation of myelin sheaths in the central nervous system

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5.1. Abstract

The formation of myelin sheaths in the CNS is the result of a complex series of events involving oligodendrocyte progenitor cell (OPC) proliferation, directed migration and the morphological changes associated with axon ensheathment and myelination. To examine the role of Rho GTPases in oligodendrocyte biology we have used a conditional tissue-specific gene targeting approach. Ablation of Cdc42 in cells of the oligodendrocyte lineage did not affect OPC proliferation, directed migration, or in vitro differentiation, but led to the formation of a unique and stage-specific myelination phenotype. This was characterized by the extraordinary enlargement of the inner tongue of the oligodendrocyte process as a result of abnormal accumulation of cytoplasm in this region. The fact that myelination continued in the presence of such a phenotype challenges the commonly held view that the formation of the multilamellar myelin sheath requires the continuous progression of the inner tongue over the axon surface. Ablation of Rac1 also resulted in the abnormal accumulation of cytoplasm in the inner tongue of the oligodendrocyte process and we provide genetic evidence that Rac1 synergizes with Cdc42 in a gene-dosage dependent way to regulate myelination.

Key words: Cdc42, Rac1, Rho GTPase, myelin, CNS, oligodendrocyte
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5.2. Introduction

During oligodendrocyte development, the coordinated interpretation of extracellular cues, such as integrin, growth factor or chemokine receptor activation, allows oligodendrocytes to sense their environment and regulate their intrinsic developmental program (Baron et al., 2005; Miller, 2002). This process is likely to involve the GTPases of the Rho subfamily of which Cdc42, Rac1 and RhoA are the most well studied members. These molecules, best known for their roles in regulating signaling pathways linking extracellular stimuli to the assembly and organization of the actin cytoskeleton (Hall, 1998), also control microtubule dynamics, cell polarity, membrane trafficking and gene transcription (Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005). In the context of CNS myelination, pathways known to influence oligodendrocyte development such as those initiated by the activation of integrin receptors (Liang et al., 2004) or the transmembrane protein Lingo-1 (Mi et al., 2005) can modulate the activities of Cdc42, Rac1 and RhoA. These GTPases are expressed by oligodendrocytes (Erschbamer et al., 2005) and, in primary oligodendrocyte cultures, their expression and activity is developmentally regulated (Liang et al., 2004). While RhoA is expressed and active during early progenitor stages, the expression and activity of Cdc42 and Rac1 increases as differentiation proceeds (Liang et al., 2004). Perturbation of the activities of these GTPases by expression of corresponding dominant-negative or constitutively-active mutant molecules, led to the conclusion that Cdc42 and Rac1 act as positive regulators of morphological differentiation, inducing process extension and branching, while RhoA acts as a negative regulator inhibiting process extension (Liang et al., 2004). Cdc42 itself is thought to play a key role in establishing eukaryotic cell polarity, a fundamental requirement for cell proliferation, migration and morphogenesis (Etienne-Manneville, 2004). This makes Cdc42 an attractive candidate molecule to regulate several other aspects of oligodendrocyte biology including those associated with the ensheathment and myelination of axons.

In this study, we examine the role of Cdc42 and Rac1 signaling during myelination of the developing CNS using tissue-specific conditional gene ablation in the oligodendrocyte lineage. This approach circumvents potential problems associated with the use of dominant-negative Rho GTPase mutants (Braga et al., 2000; Czuchra et al., 2005) and examines rigorously the requirements for these GTPases under physiological conditions.
Our data indicate a stage-specific and critical role for Cdc42 and Rac1 in myelination. Furthermore, Cdc42 synergizes with Rac1 in the regulation of this crucial process.

5.3. Material and methods

5.3.1. Antibodies
The following primary antibodies were used for this study: monoclonal antibodies against MAG (Chemicon 1:1000), MBP (Serotec 1:75), CNPase (Santa Cruz 1:500), MOG (R&D Systems 1:1000), β-actin (Sigma 1:1000), Rac1 (BD Biosciences 1:1000), phospho-ERK1,2 (Cell signaling 1:1000), phospho-JNK (Cell signalling 1:1000), phospho-p38 (Cell signalling 1:1000); polyclonal antibodies against PLP (A341, K.A. Nave 1:500), Cdc42 (Santa Cruz 1:1000), ERK1,2 (Cell signaling 1:1000), JNK (Cell signalling 1:1000), p38 (Cell signalling 1:1000). Secondary antibodies for Western blots were obtained from Pierce and Santa Cruz and for immunocytochemistry from Jackson Immunoresearch Laboratories.

5.3.2. Generation of conditional knockout mice
The generation of conditional Cdc42 mutant mice has been described previously (Wu et al., 2006). Mice homozygous for the Cdc42 floxed allele (Cdc42<sup>lox/lox</sup>), were crossed with mice heterozygous for the Cdc42 floxed allele, which expressed the Cre recombinase under the control of the 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (CNP-Cre<sup>Cdc42<sup>lox/lox</sup></sup>) (Genoud et al., 2002; Lappe-Siefke et al., 2003; Saher et al., 2005), to obtain single Cdc42 homozygous mutants (CNP-Cre<sup>+</sup> Cdc42<sup>lox/lox</sup>) and single Cdc42 heterozygous mutant (CNP-Cre<sup>+</sup> Cdc42<sup>lox/wt</sup>) mice. To generate double transgenic mice with different allelic combinations, double heterozygous mutants (CNP-Cre<sup>+</sup> Cdc42<sup>lox/wt</sup> Rac1<sup>lox/wt</sup>) were crossed with mice homozygous for both the Cdc42 and the Rac1 floxed alleles (Cdc42<sup>lox/lox</sup> Rac1<sup>lox/lox</sup>). To follow the fate of the recombined cells by detection of β-galactosidase expression, we bred the conditional LacZ allele from the ROSA26 reporter mouse strain (Soriano, 1999) into control and mutant mice. CNP-Cre is active in over 70% of all oligodendroglial cells (our own unpublished results). Genotypes were determined by carrying out PCR on genomic DNA. The generation of conditional Rac1 mice will be described elsewhere. The breeding strategy to produce mutant and control Rac1 mice was the same as described for Cdc42 mice.
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5.3.3. Electron microscopy
Mice were deeply anesthetized and then perfused with 0.1M phosphate buffer (pH 7.4) followed by buffer containing 3% glutaraldehyde (GA) and 4% paraformaldehyde (PFA). Fixed tissues were post-fixed in 2% osmium tetroxide, dehydrated through a graded acetone series, and embedded in Spurrs resin (Electron Microscopy Sciences). Semithin sections were stained with toluidine blue for analysis at the light microscope and ultrathin sections with 3% uranyl acetate and 1% lead citrate before observation in a Hitachi H-600 TEM at 75kV. For g-ratio calculations at least 150 myelinated axons were measured with MCID software (Imaging research Inc.).

5.3.4. Immunofluorescence, TUNEL staining
Frozen tissue sections and oligodendrocyte cultures were fixed in 4% PFA/PBS, incubated overnight with primary antibodies in 0.1% Triton X-100/PBS, washed in PBS and incubated with the appropriate secondary antibodies. Apoptotic cell death was analyzed by TUNEL staining using biotin-labeled UTP and FITC-conjugated steptavidin complex according to the manufacturer’s instructions (Roche Diagnostics). Images were acquired using a Zeiss fluorescence microscope equipped with a Zeiss Axiocam CCD camera, and Axio Vision V4.5 (Zeiss) acquisition software. Pictures were prepared using Adobe Photoshop version 8.0.

5.3.5. In situ hybridisation
Twenty micrometer frozen tissue sections were collected and hybridized with digoxigenin-labeled RNA probes overnight at 72°C in buffer containing 50% formamide. The bound probe was detected using an anti-DIG-AP antibody (Roche Diagnostics). The MBP plasmid was a kind gift of Dr W.D. Richardson.

5.3.6. Primary cell culture
OPCs were purified from mixed glial cultures (McCarthy and de Vellis, 1980) and differentiated on ECM substrates (Relvas et al., 2001). To obtain immature OPCs, cells were cultured for 1 day in proliferation media (SATO with 10ng/ml PDGF, 10ng/ml FGF-2; SATO: 5μg/ml insulin, 50μg/ml human transferring, 100 g/ml BSA, 6.2ng/ml Progesterone, 16 μg/ml Putrescine, 5ng/ml sodium selenite, 400ng/ml T3, 400ng/ml T4, 1% P/S). Mature oligodendrocytes were obtained after culturing the cells for 3 days in
differentiation media (SATO with 1% horse serum). For X-gal staining, cells were fixed in 2% formaldehyde and 0.2% GA in PBS for 7 minutes and stained in X-gal staining solution (5mM K₃[Fe(CN)₆], 5mM K₄[Fe(CN)₆], 2mM MgCl₂, and 1mg/ml X-gal (Calbiochem) in PBS). Proliferation was measured using the 5-Bromo-2’-deoxyuridine Labeling and Detection Kit I (Roche) according to the manufacturer’s instructions. Apoptotic cell death was analyzed by TUNEL according to the manufacturer’s instructions (Roche). To stain the cytoskeleton, cultures were fixed in 4%PFA in MP buffer (65mM PIPES, 25mM HEPES, 10mM EGTA, 3mM MgCl₂, pH 6.9) for 10 min at room temperature. The cells were permeabilized with 0.2% Triton X-100 in MP buffer for 5 min at room temperature. Primary antibody against α-tubulin (Sigma 1:100) and Alexa Fluor 488 phalloidin (Molecular probes 1:100) were incubated in 1 mg/ml BSA in PBS overnight at 4°C, followed by incubation with secondary antibody at room temperature. Supplements were from Sigma unless stated otherwise.

5.3.7. Western blot
Purified oligodendrocytes and spinal cord tissue were homogenized in lysis buffer (0.1% SDS, 10 mM TrisHCl, 150 mM NaCl, 50 mM NaF, 1mM NaVO₄, 1 mM EDTA, 0.5% sodium-deoxycholate, protease inhibitor cocktail (Sigma)). Proteins were separated using standard SDS-PAGE and electroblotted onto PVDF membranes (Hybond-C, Pharmacia) using standard protocols. Densitometry of the relative levels of protein expression were carried out using Quantity one software (Biorad).

5.3.8. Rho GTPase activity assay
The GST-PAK-CD construct was a kind gift of Dr. J. Collard. Rac1 activity was measured as described (Sander et al., 1998). Following lysis, supernatants were incubated with GST-PAK-CD fusion protein bound to glutathione-coupled Sepharose beads (Amersham Biosciences) at 4°C for 30 min. The beads were then washed and the protein eluted in Laemmli sample buffer and analyzed by Western blotting.

5.3.9. Cell migration assay
Directed OPC migration was assayed using a 24-well Boyden chamber according to the instructions of the manufacturer (BD Biosciences). Filters were coated with 5 μg/ml PDL and 5x 10⁴ OPCs were loaded into each well. To induce migration, different concentrations
of PDGF or netrin-1 (Preprotec) were added to either the upper or lower chambers. Cells were allowed to migrate for 10-12h at 37°C, fixed and the number of DAPI-positive migrated cells quantified on the lower surface of the filter.

5.4. Results

5.4.1. Recombination of the conditional Cdc42 allele in the CNS of mutant mice
To study the role of Cdc42 signaling in oligodendrocytes, we conditionally ablated Cdc42 by expressing Cre-recombinase (Cre) under the control of 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNP) gene regulatory sequences (Lappe-Siefke et al., 2003; Saher et al., 2005) (Figure 10A). In this setting, Cre is active in pre-myelinating oligodendrocytes and in some spinal motoneurones from embryonic day (E) 12 (Genoud et al., 2002). To identify the recombined cells, we bred the conditional LacZ allele from the ROSA26 reporter mouse (Soriano, 1999), into control and mutant mice. Recombination of the conditional Cdc42 allele (Figure 10C) led to the loss of Cdc42 mRNA signal in the white matter and in spinal motoneurons of the mutant spinal cord (Figure 10D) and to a strong reduction in Cdc42 protein in mutant OPC cultures (Figure 10B). The low Cdc42 level of expression detected in the mutant lysates was probably due to the presence of a small percentage of contaminating astrocytes and unrecombined, Cdc42-positive, OPCs in the mutant cultures. In these cultures, 76% of mutant OPCs were recombined as assessed by X-gal staining.
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**A**

![Schematic representation of the CNP-Cre knock in allele, the conditional Cdc42 allele and the reporter LacZ allele. Upon CNP-Cre-mediated recombination the genomic region between the two LoxP sites is excised, inactivating the Cdc42 gene and triggering the expression of the enzyme β-galactosidase in recombined oligodendrocyte cells.](image)

**B**

![Western blot analysis shows a marked decrease of Cdc42 protein expression in mutant OPC cultures. The remaining Cdc42 signal detected is likely due to some contaminating astrocytes and non-recombined OPCs in these cultures. Values in D are shown as the mean ± SD of 3 independent experiments. Student's t-tests were used to determine significance (** p < 0.005).](image)

**C**

![PCR shows the recombination of the conditional Cdc42 allele on genomic DNA isolated from 1 day-old control (**p**-Cdc42 flox w/t), Cdc42 floxed (flox) and Cdc42 mutant (KO) alleles are indicated.](image)

**D**

![In situ hybridization using a riboprobe complementary to the full-length Cdc42 cDNA, failed to detect a Cdc42 signal in spinal motorneurons (arrowheads) or in the ventral developing white matter of P2 mutant mice (area between dotted lines). Scale bar represents 50μm.](image)

Figure 10: Recombination of the conditional Cdc42 allele in the CNS of mutant mice

(A) Schematic representation of the CNP-Cre knock in allele, the conditional Cdc42 allele and the reporter LacZ allele. Upon CNP-Cre-mediated recombination the genomic region between the two LoxP sites is excised, inactivating the Cdc42 gene and triggering the expression of the enzyme β-galactosidase in recombined oligodendrocyte cells. (B) Western blot analysis shows a marked decrease of Cdc42 protein expression in mutant OPC cultures. The remaining Cdc42 signal detected is likely due to some contaminating astrocytes and non-recombined OPCs in these cultures. Values in D are shown as the mean ± SD of 3 independent experiments. Student's t-tests were used to determine significance (** p < 0.005). (C) PCR shows the recombination of the conditional Cdc42 allele on genomic DNA isolated from 1 day-old control (CNP-Cre - Cdc42 flox w/t) and mutant (CNP-Cre - Cdc42 flox KO) brain and spinal cord. The fragment sizes for wildtype (wt), Cdc42 floxed (flox) and Cdc42 mutant (KO) alleles are indicated. (D) In situ hybridization using a riboprobe complementary to the full-length Cdc42 cDNA, failed to detect a Cdc42 signal in spinal motorneurons (arrowheads) or in the ventral developing white matter of P2 mutant mice (area between dotted lines). Scale bar represents 50μm.
5.4.2. *Cdc42* mutant mice display several neurological deficits

*Cdc42* mutant mice are born with an expected Mendelian frequency but die around 4 weeks postnatally. Three-week-old *Cdc42* mutant mice display neurological symptoms that include ataxia, severe hind limb paresis and muscle atrophy (Figure 11).

![Figure 11: Phenotype of *Cdc42* mutant mice](image)
P24 mutant mice display hind limb weakness (a) and when lifted at the tail, they clamp their hind limbs (b).

5.4.3. *Cdc42* is required for proper ensheathment and myelination of axons

Examination of cross sections of spinal cord fibre tracts, optic nerve and midsagittal corpus callosum of postnatal day 24 (P24) *Cdc42* mutant mice revealed the presence of abnormal myelin outfoldings, here defined as areas of the internode where the myelin sheath protrudes away from the axon surface (Figure 12A:d, f and h). These abnormal structures were already identifiable in the mutant spinal cord at P5 (Figure 12A:b). At P24, with the addition of further myelin wraps, the outfoldings grew larger (Figure 12A:d).
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Figure 12: Cross sections of spinal cord, optic nerve and midsagittal corpus callosum of control and Cdc42 mutant mice

(A) At P5, fiber profiles with excess cytoplasm (black arrows) and myelin sheath outfoldings (white arrows) are present in mutant (b) but not in control (a) spinal cord. At P24, the mutant spinal cord (d), optic nerve (f) and corpus callosum (h) contain profiles with large myelin sheath outfoldings. Such defects are found only very rarely in P24 control spinal cord (c), optic nerve (e) or corpus callosum (g). Scale bars represent 2μm.

(B) Excess cytoplasm in the innermost loops and inner tongue of mutant profiles in the spinal cord. (a) In P5 control profiles the compacted myelin sheath is apposed to the axolemma and there are only moderate amounts of cytoplasm in the inner loop (black arrowheads) and inner tongue (henceforth labeled by an asterisk). (b) In many mutant profiles excess cytoplasm is present in the innermost loops (pound signs) and inner tongue. (c) The inner tongue occasionally contains large vesicles (open arrowheads). At P5 (d), P14 (e) and especially at P24 (f) the myelin outfoldings can be large and complex. Scale bars represent 1μm.

During early stages of myelination, excess cytoplasm was found in the innermost loops and the inner tongue of a large number of profiles (Figure 12A and 12B). In profiles with larger outfoldings the innermost loops became gradually compacted while the inner tongue grew in size, possibly as a consequence of the extrusion of the cytoplasm from the innermost loops into the inner tongue (Figure 12B).

Examination of longitudinal sections of the spinal cord showed that at early stages of myelination the excess cytoplasm occupied relatively large areas of the internodes (Figure 13A). With ongoing myelination the excessive cytoplasm became confined to the concomitantly growing myelin sheath outfolding (Figures 12B and 13). These abnormalities were randomly distributed along the internode and only occasionally paranodal regions affected.
Figure 13: Internodal profiles of myelinated fibers in control and mutant spinal cords
(A) (a) In the control P5 fiber, the myelin sheath (henceforth labeled by black arrowheads) is apposed to the axolemma. (b) In P5 mutant fibers, excess cytoplasm is found in uncompacted lamella (white arrows) and in the inner tongue (henceforth represented by an asterisk). On the opposite side of the fibers, the myelin sheath is compacted and apposed to the axolemma. With ongoing myelination, the excess cytoplasm accumulates in the inner tongue (c) and progressively becomes confined to a restricted region (d). Over time, this region will presumably give rise to a growing myelin sheath outfolding (e), which will become large and complex (f). The outfoldings contain inner tongue cytoplasm (black arrows in e and f). Large cytoplasmic vesicles (open arrowheads in c, d and f) are occasionally observed. Scale bars represent 2 μm. (B) Hypothetical model for the pathogenesis of myelin sheath outfoldings. Mutant internodal profiles (a,c and e) and their corresponding cross sectional profiles (b, d and f). The red arrows indicate flow of the excess cytoplasm during the formation of the outfoldings.
At P24, measurements carried out in transverse profiles, excluding myelin outfoldings, showed that the myelin sheaths in the mutant spinal cord (Figure 12A:d) were significantly thinner (Figure 14A) than those in control spinal cords (Figure 12A:c). It is likely that the formation of the outfoldings expended myelin that would otherwise have been used to form additional myelin wrappings, thereby causing a reduction in myelin thickness. The loss of Cdc42 did not alter the relative amounts of myelin-associated glycoprotein (MAG), proteolipid protein (PLP), CNPase, myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) in the spinal cord of mutant mice (Figure 14B). The periodicity of mutant myelin sheaths was normal (Figure 14C).

Figure 14: Mutant myelin sheaths are significantly thinner than control, but the specific protein composition of the myelin in mutant mice is not affected
(A) The graph shows the linear regressions of fiber measurements carried out in the spinal cord for each control (straight lines) and mutant (dashed lines) mice. Control and mutant linear regressions are significantly different (p=0.013) and the average g-ratio in mutant spinal cords is significantly higher (0.8) than in controls (0.75) (p=0.039). For the gratio/ linear regression analysis, the Welch two sample t-test and the Wilcoxon rank sum test were applied. (B) Western blot analysis of P14 and P24 mutant and control spinal cord lysates shows no differences in the relative amounts of the myelin proteins proteolipid protein (PLP), CNP, myelin basic protein (MBP) myelin associated glycoprotein (MAG) and Myelin oligodendrocyte glycoprotein (MOG). Actin levels confirm equal protein loading. Western blots were performed at least twice. (C) The periodicity of the compact myelin sheath in the mutant spinal cord is not altered.
5.4.4. Cdc42 is not required for OPC morphological differentiation in vitro

To investigate whether the myelin abnormalities observed in the Cdc42 mutant CNS could be directly attributed to impairment of oligodendrocyte process extension and branching (Liang et al., 2004), control and mutant OPCs were allowed to differentiate in culture (Buttery and ffrench-Constant, 1999; Relvas et al., 2001). OPC differentiation in culture involves process extension and formation of myelin membranes.

After three days of differentiation, cells were fixed, stained for X-gal, and randomly chosen mutant and control recombined (X-gal positive) oligodendrocytes were scored and assigned to five pre-established categories according to their morphological complexity (Liang et al., 2004). Category one was used to describe cells with the lowest degree of morphological complexity and five to describe cells with the highest. In each experiment,
over 250 mutant and control recombined oligodendrocytes were examined and assigned to the different categories. The degree of morphological complexity of mutant cells, as a measure of their differentiation status, was not significantly different from control cells (Figure 15A,B). In addition, no significant differences were found in the numbers of MBP-positive cells in mutant or control differentiated cell cultures. Furthermore, no difference was found in the area of the myelin membrane formed by mutant and control cells that had acquired MBP expression when plated on different ECM substrates (Figure 15C). We conclude that Cdc42 is not required for OPC differentiation in culture.

5.4.5. Oligodendrocytes lacking Cdc42 disperse and colonize the spinal cord normally

Rho GTPase family members, and Cdc42 in particular, are thought to be widely involved in the directed migration of cells in response to tropic cues (Ridley et al., 2003). If Cdc42 were involved in the migration of OPCs away from their germinal zones, one would predict a reduced dispersion of OPCs and oligodendrocytes throughout the developing CNS, including the spinal cord. However, at P1 and P5 there was no significant difference in the distribution of oligodendrocytes positive for myelin basic protein (MBP) mRNA in the ventral and dorsal fiber tracts of mutant and control mouse thoracic spinal cord (Figure 16A), suggesting that Cdc42 is largely dispensable for OPC dispersion. This conclusion was also supported by standard in vitro assays where, in the absence of Cdc42, freshly isolated mutant OPCs were able to migrate normally in response to PDGF, an OPC chemoattractant secreted along OPC migratory pathways (McKinnon et al., 1993) or netrin-1, an OPC chemorepellent expressed at the ventricular zone during OPC emigration (Jarjour et al., 2003) (Figure 16B). OPCs were also able to proliferate and survive normally in the absence of Cdc42 (Figure 16C,D).
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Figure 16: Loss of Cdc42 does not affect the dispersion and the distribution of oligodendrocytes in the spinal cord

(A) At P1 and P5 MBP mRNA-positive OPCs start to accumulate in large numbers in the developing white matter. Their distribution in the ventral and dorsal fiber tracts of mutant and control mouse thoracic spinal cord is not significantly different. (B) In Boyden chamber assays, no significant differences are found in directed migration of mutant and control OPCs in response to 1 and 10 ng/ml of the chemoattractant PDGF and to 25 and 100 ng/ml of the chemorepellent netrin-1. PB- PDGF added to bottom chamber; PT- PDGF added to top chamber; PTB- PDGF added to top and bottom chambers; NB- netrin-1 added to bottom chamber; NT- netrin-1 added to top chamber. (C) In response to different concentrations of the OPC mitogen PDGF, the numbers of proliferating, BrdU-positive, mutant and control OPCs are not significantly different. (D) Mutant OPC survival is not differentially affected in response to PDGF. Values are shown as mean ± SD of at least 3 independent experiments. Student’s t-test was used to determine significance. Scale bars represent 100µm.
5.4.6. Cdc42-deficient OPCs display decreased activation of ERK

To assess if Cdc42 ablation affected the levels of mitogen-activated protein kinases (MAP kinases) family members, we measured their activation in control and mutant OPC cultures. The MAP kinase family includes the extracellular signal-regulated protein kinase (ERK), the stress-activated c-Jun N-terminal kinase (JNK), and the 38kDa high osmolarity glycerol response kinase (p38). While ERK activation has been shown to play a role in proliferation, process extension and survival of oligodendrocytes, JNK and/or p38 activation is thought to mediate oligodendrocyte cell death (Stariha and Kim, 2001). Levels of phosphorylated ERK (P-ERK) were significantly decreased in mutant OPCs whereas the amounts of phosphorylated JNK (P-JNK) and phosphorylated p38 (P-p38) were not significantly different in control and mutant cultures (Figure 17).
Figure 17: Phosphorylation of ERK is decreased in Cdc42-deficient OPCs
Western blot analysis shows a significant decrease of phosphorylated ERK (P-ERK) in mutant OPC cultures. No significant difference was found for P-JNK (* unspecific band) and P-p38. Note that total protein levels of ERK, JNK and p38 were not altered. Values are shown as mean ± SD of 3 independent experiments. Student’s t-test was used to determine significance (* p < 0.05).

5.4.7. Ablation of Cdc42 did not affect the cytoskeleton of cultured oligodendrocytes
Rho GTPases are key regulators of the actin cytoskeleton and there is increasing evidence that they also influence the dynamics of microtubules (Nobes and Hall, 1995; Rodriguez et al., 2003). In many cell types, Cdc42 promotes the formation of actin-rich, finger-like membrane extensions (filopodia). To analyze if the cytoskeleton was affected by the lack of Cdc42, we prepared primary oligodendrocyte cultures from neonatal control and mutant mice. The organization of the cytoskeleton was not severely altered in Cdc42 mutant oligodendrocytes (Figure 18). Actin microfilaments were prominent at the surface of the plasma membrane, especially at the leading edge of the processes and within newly formed connections between branches. Small actin-rich microspikes/filopodia were present along the processes of Cdc42-deficient oligodendrocytes, and microtubules were found in the cell body and in the processes of these cells.
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Figure 18: Loss of Cdc42 does not affect the organization of the oligodendrocyte cytoskeleton

Organization of the cytoskeleton in developing (A) and mature oligodendrocytes (B). The actin cytoskeleton (green) and MT (red) were visualized by phalloidin and tubulin antibodies, respectively. A single process or the branched network (right panels) of an oligodendrocyte (left panel, white box) is magnified for a more detailed view. (A) The organization of the cytoskeleton is not affected in mutant immature oligodendrocytes. These cells display strong actin staining in the peripheral regions beneath the plasma membrane, particularly at the leading edges of processes and microtubules are present in the cell body and in processes. (B). In the branched network of mature oligodendrocytes, microfilaments are prominent within newly forming connections between branches and at the leading edge of the processes in both control and mutant cultures. Scale bars represent 50μm.
5.4.8. Rac1 is required for proper axon ensheathment and myelination

Cdc42 and Rac1 cooperate in pathways regulating important cellular functions (Cotteret and Chernoff, 2002). During OPC differentiation, Cdc42 and Rac1 show a similar pattern of activity and expression (Liang et al., 2004), raising the possibility that these two molecules act synergistically to regulate different stages of oligodendrocyte development. Therefore, having shown that Cdc42 is required for the normal ensheathment and myelination of axons, we asked whether Rac1 also regulates this process.

**Figure 19: Rac1 is required for correct myelination**

(A) Schematic representation of the CNP-Cre knock in allele, the conditional Rac1 allele and the reporter LacZ allele. Upon CNP-Cre-mediated recombination the genomic region between the two LoxP sites is excised, inactivating the Rac1 gene and triggering the expression of the enzyme β-galactosidase in recombined oligodendrocyte cells. (B) Western blot analysis shows a marked decrease of rac1 protein expression in mutant OPC cultures. The remaining Rac1 signal detected is likely due to some contaminating astrocytes and non-recombined OPCs in these cultures. Values in D are shown as mean ± SD of 3 independent experiments. Student’s t-tests were used to determine significance (** p < 0.005). (C) P24 Rac1 mutant spinal cords contain myelin sheath outfoldings (white arrows) (a, b). Excess cytoplasm is confined to the inner tongue, which contains large vesicles (arrowhead). Scale bars represent 2 μm (a) and 1 μm (b).
To address this question, we targeted the recombination and ablation of a Rac1 conditional allele to oligodendrocytes as in Cdc42 mutants (see Material and Methods). Recombination of the conditional Rac1 allele (Figure 19A) to a strong reduction in Rac1 protein in mutant OPC cultures (Figure 19B). The low Rac1 level of expression detected in the mutant lysates was probably due to the presence of a small percentage of contaminating astrocytes and unrecombined, Rac1-positive, OPCs in the mutant cultures. Examination of P24 spinal cord of Rac1 mutants revealed the presence of myelin outfoldings similar to these observed in the Cdc42 mutants (Figure 19C), showing that also Rac1 is required for proper myelination.

Several cell biological and biochemical data point to different modes of crosstalk between Rho GTPases and it was shown that Cdc42 can activate Rac (Nobes and Hall, 1995). Pulldown assays carried out in mutant Cdc42 OPCs showed that the levels of both Rac1 activity and expression were not significantly regulated (Figure 20).

![Figure 20: The activity of Rac1 is not regulated in the absence of Cdc42](image)

Active Rac1 levels were measured in a pull down assay using GST-PAK-CD constructs. No significant differences were found in the amounts of active and total Rac1 between control and Cdc42 mutant OPC cultures. Values are shown as mean ± SD of 3 independent experiments. Student's t-test was used to determine significance (* p < 0.05).

5.4.9. Cdc42 and Rac1 synergize to regulate pathways controlling CNS myelination

Although the ablation of either Cdc42 or Rac1 led to the formation of myelin outfoldings, this phenotype could still be caused by impairment of different, independent, Rac1- and Cdc42-regulated pathways. To address this point, we generated mice carrying at least one recombined Cdc42 and one recombined Rac1 allele (see Material and Methods). Then we compared the number of myelin outfoldings present on semithin sections obtained from
P24 spinal cords of double and single transgenic mice (Figure 21A,B). We hypothesized that if Cdc42 and Rac1 synergize in pathways regulating axon ensheathment and myelination, the myelin outfoldings should, to a large extent, reflect the number of recombined alleles present in each transgenic mouse, i.e., should be “gene dosage”-dependent. The results (Figure 21B) show that: (1) the numbers of outfoldings in wild type, single heterozygous Cdc42 and single heterozygous Rac1 were very low and not significantly different; (2) the numbers of outfoldings in double heterozygous mutant mice were significantly higher than those in single Cdc42 or Rac1 heterozygous mutant mice; (3) the numbers of outfoldings in single Cdc42 homozygous mutant mice were significantly higher than those in single Rac1 homozygous mutant mice; (4) the numbers of outfoldings in Rac1 homozygous/ Cdc42 heterozygous mutant mice were significantly higher than those in Rac1 homozygous mutant mice; (5) the double homozygous mutant mice contained the highest number of outfoldings of all mice analyzed. Our results suggest that Cdc42 and Rac1 cooperate, in a non-exchangeable manner, in regulating common effectors involved in the process of myelination.
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Figure 21: Cdc42 and Rac1 synergize to regulate myelination

(A) Toluidine Bluestained semithin cross sections of wild type (a), single Rac1 homozygous mutant (b), single Cdc42 homozygous mutant (c), and double homozygous mutant (d) spinal cords. Myelin sheath outfoldings (arrowheads in b) are easily recognizable (b-d). Note their gradual increase in frequency and size from Rac1 mutants (b) over Cdc42 mutants (c) to double homozygous mutants (d). Scale bar represents 20 μm. (B) Cdc42 and Rac1 cooperate to regulate myelination in a “gene-dosage” dependent manner. The graph shows the number of myelin outfoldings per area unit counted on cross sections of different Cdc42 and Rac1 mutant spinal cords. Values are given as mean ± SD of 3 animals per genotype. Statistical significance was determined using the Student’s t-test (* p < 0.05; ** p < 0.005).
5.5. Discussion

We have used a conditional tissue specific gene ablation approach to examine the roles of the small Rho GTPases Cdc42 and Rac1 in CNS myelination. This approach revealed that Cdc42 and Rac1 are both necessary and synergize in a "gene-dosage" dependent way to regulate myelination when oligodendrocyte processes ensheath axons and form compacted myelin sheaths. Ablation of \textit{Cdc42} or \textit{Rac1} leads to a unique myelin phenotype characterized by abnormal accumulation of large amounts of cytoplasm in the inner tongue and innermost loops of the ensheathing oligodendrocyte process.

5.5.1. \textit{Cdc42} is not required for OPC proliferation, directed migration or morphological differentiation

Our results do not support a requirement for Cdc42 in OPC proliferation, directed migration or \textit{in vitro} morphological differentiation. However, there is a significant body of evidence showing a key role for Cdc42 in the regulation of directed cell migration and cell morphology (Etienne-Manneville and Hall, 2002; Govek et al., 2005). In oligodendrocytes, Cdc42 and Rac1 are thought to act as positive regulators of OPC differentiation, promoting oligodendrocyte process extension and branching (Liang et al., 2004). How can we reconcile these results with our own data? Many key cellular functions ascribed in the literature to Rho GTPases rely on experiments where their function is blocked by expression of dominant-negative mutants (Feig, 1999). Such mutants bind the corresponding activating guanine nucleotide exchange factors (GEFs) with higher affinity than do the endogenous Rho GTPases, but do not interact with their downstream targets. Therefore, by sequestering the GEFs, they prevent the activation of endogenous Rho GTPases. A disadvantage of such an approach is the fact that GEFs activating Cdc42, for example, also act on other Rho GTPases. Therefore dominant-negative mutants of Cdc42 may, especially if expressed at high levels (Braga et al., 2000), inhibit the activation of other Rho GTPases, making it nearly impossible to discriminate between the specific contributions from different Rho GTPases. Recent work by some of us (Czuchra et al., 2005) shows that the transfection of \textit{Cdc42}-null fibroblastoid cells with a \textit{Cdc42} dominant-negative mutant (N17 Cdc42) elicits additional phenotypes that are not present in untransfected \textit{Cdc42}-null fibroblasts. This observation raises important questions about the specificity of Rho GTPase dominant-negative approaches and is in line with previous
reports where dominant-negative mutant expression induced more severe phenotypes than those produced by genetic loss-of-function approaches on identical cell types or organisms (Luo et al., 1994; Scott et al., 2003). Therefore, the use of conditional tissue-specific gene ablation techniques provides a stringent system to study the roles of Rho GTPases at the cellular, tissue and organism level.

5.5.2. Ablation of Cdc42 results in the formation of aberrant myelin outfoldings

The ablation of Cdc42 in oligodendrocytes led to widespread formation of aberrant myelin outfoldings, likely as a consequence of abnormal accumulation of cytoplasm in the inner tongue of the oligodendrocyte process. Such aberrant myelin outfoldings were occasionally also found in control CNS white matter. The sporadic presence of such structures during normal development has been reported previously in amphibian cerebellum (Rosenbluth, 1966). Their genesis is unclear, but may be related to the deregulation of molecular events that accompany the early stages of axon ensheathment, in particular those associated with the removal of cytoplasm in areas where the glial sheaths undergo compaction. During compaction the two plasma membranes of the oligodendrocyte process come together while the cytoplasm between them is excluded. Presumably, the excluded cytoplasm flows to regions of uncompacted myelin, which in the early developing CNS alternate irregularly with compacted myelin sheaths along the individual axon (Remahl and Hildebrand, 1990; Storts and Koestner, 1969). Our results suggest that Cdc42 and Rac1 signaling are involved in the regulation of this process. Given the roles played by these molecules in the regulation of cytoskeleton dynamics in different cell types, we may speculate that cytoplasm exclusion is the result of an active process and that the forces necessary to drive it are likely to be generated by the cytoskeleton. In oligodendrocytes, the organization of the microfilament and microtubule array is likely to provide the driving forces for changes in plasma membrane conformation associated with process extension and branching (Richter-Landsberg, 2001; Simpson and Armstrong, 1999; Song et al., 2001). We found that Cdc42 is required for the activation of ERK, a protein thought to be important for oligodendrocyte differentiation (Stariha and Kim, 2001). Although our results show that Cdc42 is dispensable for OPC differentiation in culture, Cdc42 signaling appears to be limiting in more complex and stringent cellular events where extensive reorganization of the oligodendrocyte actin and microtubule cytoskeleton is required. This is the case during
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the transition from a completely unmyelinated to a fully myelinated status, a process thought to be gradual and non-uniform and most probably involving elongation and extensive remodelling of the uncompacted sheaths before compaction (Remahl and Hildebrand, 1990). During this process, the lack of Cdc42 may disturb normal cytoskeleton reorganization and eventually lead to the entrapment and consequent accumulation of cytoplasm in the inner tongue of the oligodendrocyte process (Figure 13B).

Noteworthy, perturbation of cytoskeletal reorganization and, eventually, of vesicle transport, also a putative function of Cdc42 (Larocca and Rodriguez-Gabin, 2002; Rodriguez-Boulan et al., 2005), may also explain the presence of large vesicles sometimes found in the cytoplasm of the mutant cells (Figures 12B and 13A).

5.5.3. Cytoplasm accumulation in the inner tongue does not halt myelination

Despite the extraordinary enlargement of the inner tongue in mutant Cdc42 and Rac1 oligodendrocytes, myelination was not halted. At P24, mutant spinal cord internodes were hypomyelinated, but they were still capable of producing sizeable compacted myelin sheaths whose relative protein composition and periodicity did not differ from that of controls. In addition, the myelin sheaths in P24 axon profiles with protruding inner tongues were thicker than those at P5. This indicates that the processes regulating the addition of further myelin wraps and their compaction were not arrested. These results challenge the commonly held view that the formation of the spiral multilamellar myelin sheath requires the continuous progression of the inner tongue over the axonal surface (Bunge et al., 1989; Speidel, 1964). Can enlarged inner tongues, sometimes more than several axon diameters in length, still be directed to spiral around their axons? Probably they cannot and thus our results raise the possibility that these two processes are uncoupled, i.e., that the formation of the multilamellar myelin sheath does not necessarily involve rotation of the inner tongue over the axonal surface.

5.5.4. Cdc42 and Rac1 synergize to regulate axon ensheathment

Cdc42 and Rac1 participate together in the regulation of important cellular functions in different cell types (Etienne-Manneville and Hall, 2002; Govek et al., 2005). Therefore, we analyzed the genetic interactions between Cdc42 and Rac1 in the regulation of CNS myelination. Combining conditional alleles for both genes revealed that the effects of the ablation of Cdc42 and Rac1 were cooperative in severity. Double heterozygous mutants
contained more outfoldings than wild type, single Cdc42 or Rac1 heterozygous mutants; Rac1 homozygous/Cdc42 heterozygous mutants contained more outfoldings than single Rac1 homozygous mutants and the double homozygous mutants had the highest number of myelin outfoldings of all genotypes analyzed. As the number of outfoldings in the single Cdc42 homozygous mutant were already much higher than in the single Rac1 homozygous mutant mice, only a modest and non-significant increase in the numbers of outfoldings was observed in the Cdc42 homozygous/Rac1 heterozygous mutants as compared to single homozygous Cdc42 mutant mice. Our results also suggest that Cdc42 does not directly regulate Rac1 activity. If Rac1 activity were regulated by Cdc42, no increase in the number of myelin outfoldings would have been observed in the double homozygote compared to the single Cdc42 homozygous mutant mice. Indeed, pulldown assays carried out in mutant Cdc42 OPCs confirmed that the levels of both Rac1 activity and expression were not significantly regulated (Figure 20).

Overall, our results show a stage-specific and critical requirement for Cdc42 and Rac1 in the regulation of myelination. The phenotype produced by their genetic ablation in oligodendrocytes is unique, and challenges the commonly held view that formation of the myelin sheath involves the continuous progression of the inner tongue of the oligodendrocyte process over the axon surface. An important task for the future will be to find and test alternative mechanistic models.

5.6. Acknowledgement

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5.7 Thesis author’s work

This study was performed in collaboration with Yves Benninger, who did the electron microscopic analysis of Cdc42/Rac1 mutant mice, including g-ratio measurements. I investigated the recombination of the conditional Cdc42 and Rac1 allele, the amount of myelin proteins, the distribution pattern of oligodendrocytes, regulation of downstream effectors and the crosstalk between different RhoGTPases. Using in vitro cultures, I analyzed oligodendrocyte differentiation, migration, proliferation and cytoskeletal organization.

Parts of the results shown in this section were submitted to the Journal of Neuroscience.

5.8 References


6. PART II

Different roles for Cdc42 and Rac1 in the regulation of Schwann cell biology and PNS myelination

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6.1. Abstract

Rho GTPases regulate a variety of important cellular processes including the assembly and organization of the actin and microtubule cytoskeleton, cell polarity, membrane trafficking and gene transcription. Therefore, they are good candidate molecules to regulate some of the cellular events associated with PNS myelination. Using a tissue-specific conditional targeting approach we show an essential role for the small Rho GTPases Cdc42 and Rac1 in PNS myelination. While Cdc42 is required for Schwann cell proliferation and probably, at a later developmental stage, for myelin sheath formation, Rac1 is activated by laminin/integrin interaction to mediate Schwann cell process formation during the radial sorting of embryonic axon bundles.

Key words: Cdc42, Rac1, integrin β1, Rho GTPases, myelin, PNS, Schwann cell
6.2. Introduction

Myelination allows the rapid propagation of action potentials along the axon and it is an important prerequisite for proper function of the nervous system. Schwann cells are the myelin-forming cells of the peripheral nervous system (PNS). During the development of the PNS neural crest cell-derived Schwann cell precursors populate outgrowing axon bundles, where they proliferate and differentiate into immature Schwann cells (reviewed by Jessen and Mirsky, 2005). These cells extend cytoplasmic processes into axon bundles to segregate and establish 1:1 relations with individual axons in a process called radial sorting (Webster, 1971). Myelination occurs only in Schwann cells that by chance contact large diameter axons. Schwann cells that contact small axons do not form myelin sheaths and become mature non-myelinating Schwann cells (Mirsky and Jessen, 1999).

To sense their environment and regulate their intrinsic developmental program accordingly, Schwann cells need to interpret instructive cues originating within the extracellular environment, among which growth factors and proteins of the extracellular matrix (ECM) are essential components. This process is likely to involve the GTPases of the Rho subfamily, of which Cdc42, Rac1, and RhoA are the most well studied members. These molecules, best known for their roles in regulating signaling pathways linking extracellular stimuli to the assembly and organization of the actin cytoskeleton (Hall, 1998), also control microtubule dynamics, cell polarity, membrane trafficking, and gene transcription (Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005).

Rho GTPases are expressed by Schwann cells (Terashima et al., 2001). In vitro experiments using dominant negative and constitutively active forms of Rac1 and Cdc42, suggested that these small GTPases together with focal adhesion kinase (FAK) may serve as linkers between growth factor activation (Insulin growth factor-I, IGF-I) and Schwann cell motility (Cheng et al., 2000). More recently, Yamauchi and colleagues, again using an in vitro approach, suggested that Cdc42 and Rac1 regulate the c-Jun N-terminal kinase (JNK) signaling cascade to enhance migration of Schwann cells in response to TrkC tyrosine kinase receptor activation by endogenous neurotrophin-3 (Yamauchi et al., 2003). Further work by the same authors proposed that NT3 activation by TrkC stimulates Schwann cell migration through two parallel
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signaling units. One involves the activation of Cdc42 by the guanine-nucleotide exchange factor (GEF), Dbl’s big sister (Dbs) (Yamauchi et al., 2005) while the other promotes the activation of Rac1 by the Rac1-specific GEF, T lymphoma invasion and metastasis (Tiam) 1 (Yamauchi et al., 2005). Precise regulation of Rac activity is also important to regulate Schwann cell morphology and to promote normal axonal interaction (Nakai et al., 2006). Cells derived from Schwann cell tumors (schwannomas) in which Rac activity is deregulated, showed a disorganized cytoskeleton structure (Pelton et al., 1998) and failed to interact with axons (Nakai et al., 2006). The re-establishment of normal Rac activity levels in these cells restored normal Schwann cell spindle morphology and their capacity to interact with axons (Nakai et al., 2006).

In this study, we examined the role of Cdc42 and Rac1 signaling in PNS myelination using tissue-specific conditional gene ablation in the Schwann cell lineage. Our data show critical but different roles for Cdc42 and Rac1 in Schwann cell biology and PNS myelination. While Cdc42 is required for Schwann cell proliferation and later in development for the acquisition of a fully mature myelinated status, Rac1 is required for radial sorting.

6.3. Material and methods

6.3.1. Generation of conditional knockout mice

The generation of conditional Cdc42 mutant mice has been described previously (Czuchra et al., 2005). Mice homozygous for the Cdc42 floxed allele (Cdc42^{lox/lox}), were crossed with mice heterozygous for the Cdc42 floxed allele, which expressed the Cre-recombinase under the control of the Dhh promoter (Dhh-Cre^{+} Cdc42^{lox/lox}) (Joseph et al., 2004), to obtain Dhh-Cre^{+} Cdc42^{lox/lox} mice (hereafter called mutant mice) and Dhh-Cre^{+} Cdc42^{lox/wt} (hereafter called control mice). To follow the fate of the recombined cells by detection of β-galactosidase expression, we also bred the conditional LacZ allele from the ROSA26 reporter mouse strain (Soriano, 1999) into control and mutant mice. Genotypes were determined by carrying out PCR on genomic DNA.

The generation of conditional Rac1 mice will be described elsewhere. The breeding strategy to produce mutant and control Rac1 mice was the same as for Cdc42 mice.
The generation of CNP-Cre Integrin β1 mutant and control mice has been described previously (Benninger et al., 2006).

For remyelination studies, the tamoxifen-inducible PLP-CreERT2 line was used (Leone et al., 2003). The breeding strategy to produce inducible Rac1 and Cdc42 mutant and control mice was the same as for Dhh-Cre Cdc42 mice.

6.3.2. Electron microscopy

Mice were deeply anesthetized and then perfused with 0.1M phosphate buffer (pH 7.4) followed by buffer containing 3% glutaraldehyde (GA) and 4% paraformaldehyde (PFA). Fixed tissues were post-fixed in 2% osmium tetroxide, dehydrated through a graded acetone series, and embedded in Spurr’s resin (Electron Microscopy Sciences). Semithin sections were stained with toluidine blue for analysis at the light microscope and ultrathin sections with 3% uranyl acetate and 1% lead citrate before observation in a Hitachi H-600 TEM at 75kV. For g-ratio calculations at least 150 myelinated axons were measured with MCID software (Imaging research Inc.).

6.3.3. Immunofluorescence, TUNEL staining, X-gal staining

Sciatic nerve sections were blocked for 1 hour with 10% goat serum, 0.1% Triton X-100 in PBS. Incubation with primary antibodies was carried out overnight at 4°C. Rat monoclonal antibodies against MBP (Serotec 1:50) and Ki67 (DAKO 1:50) and mouse monoclonal antibody against NF160 (Sigma 1:150) were used. On the following day, tissue sections were washed in PBS and incubated with the appropriate secondary antibodies for 1 hour at room temperature. Secondary antibodies conjugated to fluorescein or rhodamine were obtained from Jackson ImmunoResearch Laboratories. The sections were mounted in Citifluor (Citifluor Ltd) containing 4',6'-diamidino-2-phenylindole (DAPI) to stain the nuclei.

Mouse Schwann cell cultures were fixed in 4% PFA in MP buffer (65mM PIPES, 25mM HEPES, 10mM EGTA, 3mM MgCl2, pH 6.9) for 10 min at room temperature. The cells were permeabilized with 0.2% Triton X-100 in MP buffer for 5 min at room temperature. Primary monoclonal antibody against α-tubulin (Sigma 1:100) and Alexa Fluor 488 phalloidin (Molecular probes 1:100) were incubated in 1mg/ml BSA in PBS overnight at 4°C, followed by incubation with Cy3-conjugated anti-mouse (1:200, Jackson Immuno Research) at room temperature. Apoptotic cell death was analyzed by TUNEL staining using biotin-labeled UTP and
FITC-conjugated streptavidin complex according to the manufacturer’s instructions (Roche Diagnostics).

For X-gal staining, the sections were incubated overnight in PBS containing 5mM potassium ferrocyanide, 5mM potassium ferricyanide, 2mM magnesium chloride, 0.1% sodium deoxycholate, 0.02% NP40 and 2mM X-gal (AppliChem).

Images were acquired using a Zeiss fluorescence microscope equipped with a Zeiss Axiocam CCD camera. Images were processed using Photoshop 7.0 software (Adobe).

6.3.4. Primary cell culture

Primary mouse Schwann cell cultures were obtained from P0-P2 sciatic nerves. Briefly, nerves were digested in enzyme buffer (0.7mg/ml collagenase type I, 0.25% Trypsin (Invitrogen) in HBSS (Invitrogen)). After trituration, cells were grown on poly-D-lysine/laminin coated dishes in minimal medium plus 0.5% FCS. Minimal medium is DMEM/F12 (GIBCO), containing human apo-transferrin (100μg/ml), progesterone (60ng/ml), insulin (5μg/ml), putrescine (16μg/ml), L-thyroxin (400ng/ml), selenium (160ng/ml), triiodothyronine (10ng/ml) and BSA (300μg/ml (Fluka). Supplements were from Sigma unless stated otherwise.

6.3.5. Western blot

Sciatic nerve tissue was homogenized with a cilled mortar and pestle in lysis buffer (0.1% SDS, 10mM TrisHCl, 150mM NaCl, 50mM NaF, 1mM NaVO₄, 1mM EDTA, 0.5% sodium-deoxycholate, protease inhibitor cocktail (Sigma)). Extracts were processed using standard SDS-PAGE and western blotting procedures. The following antibodies were used: Krox20 (BabCO 1:100), Oct6 (Ghazvini et al 2002 1:100), β-actin (Sigma 1:1000), α-tubulin (Sigma 1:1000), GAPDH (HyTest 1:20'000), Rac1 (BD Biosciences 1:1000), Cdc42 (Santa Cruz 1:1000). Secondary antibodies were obtained from Pierce and Santa Cruz. Densitometry and quantification of the relative levels were carried out on scanned images of Western blots using Quantity one software (Biorad).

6.3.6. Rho GTPase activity assay

GST-PAK-CD construct was kindly provided by Dr. J. Collard. Rac1 or Cdc42 activity was measured similarly as described (Sander et al., 1998). Briefly, sciatic nerves from three P5 mutant mice or three P5 control mice were pooled, homogenized
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in FISH buffer (10% glycerol, 50mM Tris-HCl pH 7.4, 100mM NaCl, 1% NP-40, 2mM MgCl2, protease inhibitor cocktail (Sigma)), and centrifuged for 5 min at 21,000 g at 4°C. Aliquots were taken from the supernatant to determine the total protein amounts. The supernatant was incubated with bacterially produced GST-PAK-CD fusion protein, bound to glutathione-coupled Sepharose beads (Amersham Biosciences) at 4°C for 30 min. The beads and proteins bound to the fusion protein were washed three times in an excess of FISH buffer, eluted in Laemmli sample buffer (104mM Tris, pH 6.8, 5% sodium dodecylsulfate, 14.5% glycerin, 6mM bromphenol blue, 1.2M β-mercaptoethanol), and then analyzed for bound Cdc42 or Rac1 molecules by Western blotting.

6.3.7. Nerve crush/ Remyelination assay

Using the inducible PLP-CreERT2 transgene, Schwann cell specific recombination of Rac1 and Cdc42 was induced in three month-old mutant and littermate control mice by a daily intraperitoneal injection of 2mg Tamoxifen (Sigma) for five consecutive days. 4 weeks after the last Tamoxifen injection, mutant and control animals were subjected to the nerve crush induced remyelination assay. Mice were anesthetized by a single intraperitoneal injection of Ketamine (Ketaminol; Vetenaria; 65mg/kg bodyweight) and Xylazine (Narcoxyl; Vetenaria; 13mg/kg bodyweight). To prevent dehydration, a single intraperitoneal injection of 1ml of Ringer’s lactate solution (B. Braun Medical) was administered preoperatively. Sciatic nerves were crushed unilaterally at the sciatic notch using a forceps with a 0.3mm wide tip. The operations were performed under aseptic conditions. For the perioperative and postoperative analgesia, buprenorphinum (Temgesic; Essex Chemie; 0.1mg/kg bodyweight) was injected subcutaneously initially after the nerve crush before mice wake from narcosis and thereafter every 12 h for 2 days. 2 months after the nerve crush, sciatic nerves were analyzed 6 mm distally from the crush site.
6.4. Results

6.4.1. Inactivation of the Cdc42 or the Rac1 gene in Schwann cells

To study the role of Cdc42 and Rac1 signaling in Schwann cells, we conditionally ablated Cdc42 or Rac1 by expressing Cre-recombinase (Cre) under the control of the desert hedgehog (Dhh) gene regulatory sequences (Figure 22A). In this setting, Cre is active in Schwann cell precursors from embryonic day (E) 11 (Joseph et al., 2004). To identify the recombined cells, we bred the conditional LacZ allele from the ROSA26 reporter mouse (Soriano, 1999) into control, Cdc42 mutant, and Rac1 mutant mice. Recombination of the conditional Cdc42 or Rac1 alleles (Figure 22A) led to a strong reduction in Cdc42 and Rac1 proteins in lysates obtained from the sciatic nerves of postnatal (P) day 1 Cdc42 and Rac1 mutant mice, respectively (Figure 22B). The low Cdc42 and Rac1 level of expression detected in the respective mutant lysates was probably a result of the presence of unrecombined Schwann cells and of other Cdc42- and Rac1-positive cells present in mutant nerves. In acute Schwann cell cultures obtained from Cdc42 or Rac1 mutant nerves, over 90% of all Schwann cells were recombined as assessed by X-gal staining (data not shown).

At P14, the sciatic nerves of Cdc42 and Rac1 mutant mice were hypotrophic and hypomyelinated (Figure 22C). Cdc42 mutants displayed progressive muscular weakness that rapidly evolved in hind limb paresis by 4 weeks of age. Rac1 mutants were less affected.
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6.4 Results

Figure 22: Recombination of the conditional Cdc42 and Rac1 allele in the PNS of mutant mice

(A) Schematic representation of the conditional Cdc42 allele, the conditional Rac1 allele, and the reporter LacZ allele. Upon Dhh-Cre-mediated recombination the genomic region between the two LoxP sites is excised, inactivating the Cdc42 and Rac1 genes, and triggering the expression of the enzyme β-galactosidase in recombined Schwann cells. (B) Western blot analysis shows a marked decrease of Cdc42 and Rac1 protein expression in mutant sciatic nerve lysates. The remaining protein signal detected is mostly due to non-recombined endoneurial fibroblasts and axons. GAPDH levels confirm equal protein loading. Results are shown as the mean ± SD. Student's t-test was used to determine significance (n=5, *** p < 0.001). (C) Sciatic nerves from P14 control, Cdc42 mutant, and Rac1 mutant mice. Note the hypotrophy in both Cdc42 and Rac1 mutant sciatic nerves. For clarity, a needle is inserted behind the nerves. Scale bars represents 1mm.
6.4.2. Sorting and myelination of axons is impaired in \textit{Rac1} and \textit{Cdc42} mutant mice

During postnatal development Schwann cells segregate and myelinate individual axons from the axon bundles formed during embryogenesis (Figure 23g, j and m). We compared control and mutant sciatic nerves at different developmental stages. At E17.5, Schwann cells were present in similar numbers between bundles of tightly apposed axons in the sciatic nerves of control, \textit{Rac1} mutant, and \textit{Cdc42} mutant mice (Figure 23a, b and c). Although there were a few unsorted axonal bundles in control mice at P5 (Figure 23c), the majority of large caliber axons were already engaged in a 1:1 relation with Schwann cells and, at P14, very few unsorted bundles of axons remained (Figure 23j). By P24, myelination was virtually completed (Figure 23m).

In contrast, in the mutant nerves, axonal subdivision and myelination was impaired and unsorted axonal bundles with large caliber axons persisted throughout development. This impairment was much more dramatic in \textit{Cdc42} mutant nerves (Figure 23f, i, l and o) than in \textit{Rac1} mutant nerves, where a significant number of large caliber axons progressively became sorted and myelinated (Figure 23e, h, k and n). Occasionally, some axons were also myelinated in \textit{Cdc42} mutant nerves (Figure 23e and o). Bluogal EM (Figure 24) showed that myelinating Schwann cells were recombined suggesting that despite loosing Rac1 or Cdc42, these cells were able to establish 1:1 relations and myelinate axons.
Figure 23: Axonal sorting and myelination is impaired in *Rac1* and *Cdc42* mutant sciatic nerves

(a-o) Semithin cross sections of sciatic nerves from control (a, d, g, j, m), *Rac1* mutant (b, c, h, k, n), and *Cdc42* mutant (c, f, i, l, o) mice stained with toluidine-blue. In the controls, Schwann cells (arrowheads) gradually segregate and myelinate single axons from the axon bundles (arrows). In contrast, in *Rac1* and *Cdc42* mutant mice, axonal sorting and myelination is impaired and axonal bundles (arrows) persist throughout development. While axons get progressively sorted and myelinated in *Rac1* mutant nerves, only very few axons display a myelin sheath in P24 *Cdc42* mutant nerves (o). Scale bar represents 10 μm.
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6.4 Results

Figure 24: Myelinating Rac1 and Cdc42 mutant Schwann cells express β-galactosidase as a result of Cre-mediated recombination

Bluegal staining reports the Cre-mediated activation of the LacZ reporter gene in P24 Rac1 and Cdc42 mutant sciatic nerve cross sections. Bluegal precipitates (arrows) are present in myelinating Schwann cells (asterisks) showing that Cre-recombinase was expressed in these cells. Scale bar represents 5 μm.

6.4.3. Differentiation of Rac1 and Cdc42 mutant Schwann cells is delayed

The examination of Cdc42 and Rac1 mutant nerves suggested that Schwann cell differentiation was arrested at the promyelinating stage. To further investigate this, we examined the expression levels of the transcription factors Oct6 and Krox20 in Rac1 mutant, Cdc42 mutant, and control sciatic nerves at P2 and P14. Oct6 expression is required for the developmental switch from promyelinating to myelinating Schwann cells (Jaegle et al., 1996). The expression of Oct6 peaks at promyelinating and early myelinating stages and is downregulated at later stages of myelination (Jaegle et al., 1996).

At P2, control, Rac1 mutant, and Cdc42 mutant Schwann cells expressed Oct6, while at P14, Oct6 levels were downregulated in control Schwann cells but remained high in mutant Schwann cells (Figure 25A and B). Krox20 expression is required for Schwann cell differentiation, and it is thought that expression of Krox20 is needed for downregulation of Oct6 and for the activation of myelin genes such as PMP22, P0 and MBP (Topilko et al., 1994). At P2, Krox20 expression was detected in control but not in Rac1 and Cdc42 mutant nerves (Figure 25A and B). Later, at P14, Krox20 was present in control and Rac1 and Cdc42 mutant nerves (Figure 25A and B). The fact that Cdc42 and Rac1 mutant Schwann cells could upregulate Krox20 indicated that at least some mutant Schwann cells were able to differentiate and myelinate. This corroborated our morphological data, especially in the case of the Rac1 mutant.
nerves, where at P14 and P24, significant numbers of Schwann cells had established 1:1 relations with axons (Figure 23k and n).

Figure 25: Downregulation of Oct6 and upregulation of Krox20 is delayed in Rac1- and Cdc42-deficient Schwann cells
Western blot analysis of P2 and P14 Rac1 mutant (A), Cdc42 mutant (B), and control sciatic nerve lysates. At P14, Oct6 levels are higher in mutant nerves than in control nerves. Krox20 is expressed in P2 control nerves but not in P2 Rac1 and Cdc42 mutants. GAPDH and actin levels confirm equal protein loading. Western blots were performed at least twice.

6.4.4. Cdc42 is required for normal Schwann cell proliferation
Insufficient numbers of Schwann cells to ensheath axons, as a result of deficient proliferation or survival, could explain the persistence of aberrant bundles of unsorted mixed caliber axons observed in Cdc42 mutant and, to a lesser extent, in Rac1 mutant nerves. By gross observation, Cdc42 and Rac1 mutant nerves were thinner than control nerves, a potential sign of reduced Schwann cell numbers inside those nerves (Figure 22C). We compared the total numbers of DAPI-stained nuclei present within transverse sections of similar distal regions of P5 and P24 sciatic nerves of Rac1 mutant, Cdc42 mutant, and control mice (Figure 26A and B). While no difference was found between Rac1 mutant and control nerves (Figure 26A), the numbers of Schwann cells present in Cdc42 mutant nerves were significantly lower than those present in control nerves (Figure 26B).
Reduced Schwann cell proliferation could cause the decreased cell number. Therefore, we compared the percentage of proliferating, Ki67-positive cells in Rac1 mutant, Cdc42 mutant, and control nerves at E17.5, P0, P5 and P14. Our results show that Schwann cell proliferation was normal in the absence of Rac1 (Figure 26C), but was significantly lower in Cdc42 mutant nerves than in control nerves at E18 and P0. At P5, there was no difference between control and Cdc42 mutant Schwann cell proliferation and at P14, Schwann cell proliferation in Cdc42 mutant nerves was even higher than in control nerves (Figure 26D). Despite this continuing late proliferation, the total numbers of cells present in P24 Cdc42 mutant nerves were still less than half of those present in control nerves (Figure 26B).

To investigate if the reduction in cell numbers was influenced by increased cell death, we compared the percentage of TUNEL-positive cells in P0 and P5 Rac1 mutant, Cdc42 mutant, and control nerves. No significant differences were found in the numbers of dying cells in mutant or control mice (Figure 26E and F) at all developmental stages analyzed.
Figure 26: Total cell number, proliferation and apoptosis in Rac1 and Cdc42 mutant sciatic nerves

(A) DAPI-stained sciatic nerve cross-sections show similar numbers of cells in P5 and P24 control and Rac1 mutant mice. (B) The ablation of Cdc42 results in a significant decrease of the total cell number in mutant sciatic nerves at both P5 and P24 (n=3, *** p < 0.001). (C) At E18, P0, P5 and P14, the numbers of proliferating, Ki67-positive cells is not significantly different in sciatic nerves of control and Rac1 mutant mice. (D) In contrast, proliferation is significantly decreased in Cdc42 mutant nerves at E18 and P0 but at P14 it is significantly higher in Cdc42 mutant mice compared to control mice (n=3, ** p < 0.01). At P0 and P5, apoptotic, TUNEL-positive cell numbers are not significantly different in Rac1 mutant (E), Cdc42 mutant (F), and control nerves. Results are shown as the mean ± SD. Student’s t-test was used to determine significance. Scale bars represent 100μm.
6.4.5. Cdc42 but not Rac1 is required for the formation of myelin sheaths following sciatic nerve crush injury

We performed sciatic nerve crushes to examine the functional roles of Cdc42 and Rac1 during nerve regeneration and also to investigate if these proteins play a role in axonal myelination beyond the radial sorting stage.

Sciatic nerve crushes were performed in animals expressing CreERT2, a fusion protein containing the Cre recombinase and a tamoxifen-responsive mutant oestrogen receptor (Indra et al., 1999; Li et al., 2000), under the transcriptional control of the mouse proteolipid protein (PLP) gene regulatory elements (Leone et al., 2003). This PLP-CreERT2 transgene is transcriptionally active in Schwann cells and following injection of tamoxifen, the CreERT2 protein translocates to the nucleus, where it inactivates the floxed gene. This allowed us to adjust the timing of Cdc42 and Rac1 excision to our experimental requirements. We injected three month-old animals with tamoxifen over five consecutive days. One month later, sciatic nerves were crushed unilaterally. Western blot analysis of non-injured sciatic nerve lysates obtained from control and mutant mice one month after tamoxifen injection, confirmed that the expression levels of Cdc42 and Rac1 in mutant nerves were significantly reduced (data not shown). Two month after the crush, control and mutant nerves were dissected, embedded in resin blocks and analyzed in semithin cross sections 6mm distally from the crush site. Although a more thorough analysis at electron microscopic level and appropriate morphometric measurements are still missing (work in progress), the preliminary morphological examination of such nerves revealed that while in Rac1 mutant nerves remyelination appeared to be normal, it seamed profoundly impaired in Cdc42 mutant nerves (Figure 27). In contrast to control and Rac1 mutant nerves, Cdc42 mutant nerves contained a large proportion of Schwann cell-axon units that were still devoid of myelin sheaths. This suggests that, at least during remyelination, Cdc42 signaling is required for the production of myelin sheaths.
Figure 27: Remyelination is impaired in sciatic nerves of Cdc42 mutant but not Rac1 mutant mice

Toluidine-blue stained cross sections of PLP-CreERT2 Rac1 and PLP-CreERT2 Cdc42 mutant sciatic nerves. Nerves were collected 2 month after a nerve crush and sections were cut 6mm distal from the lesion. The majority of axons are remyelinated in control and PLP-CreERT2 Rac1 mutant nerves. Most axons in PLP-CreERT2 Cdc42 mutants are also engaged with a single Schwann cell but only few of them acquire a myelin sheath.

6.4.6. Integrin β1 regulates the activity of Rac1 but not of Cdc42 in Schwann cells of the developing PNS

Similar to Cdc42 and Rac1 mutant nerves, the conditional ablation of the Integrin β1 gene during Schwann cell development also results in the impairment of radial sorting (Feltri et al., 2002). This, and the fact that in a variety of different cell types, integrin signaling can regulate the activity of downstream Rho GTPases (Schwartz and Shattil, 2000), including Rac1 and Cdc42, prompted us to investigate if in Schwann cells, integrin β1 regulates the activity of these two proteins. Therefore, we performed pull down assays (see material and methods) in sciatic nerve lysates obtained from P5 CNP-Cre Integrin β1 mutant and control mice (Benninger et al., 2006). In these mice, the Cre recombinase is under the control of the 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) enzyme regulatory sequences and is expressed in Schwann cells from E12 on (Genoud et al., 2002, our own unpublished results, see section 7.5.2).
We show that integrin $\beta 1$ regulates the activity of Rac1 but not of Cdc42 (Figure 28). In Integrin $\beta 1$ mutant lysates the activity of Rac1 was significantly downregulated in comparison to control lysates. This result suggests that while Rac1 activity and function is directly under the regulation of ECM/integrin signaling, Cdc42 activity is likely to be regulated through other pathway(s). This hypothesis is supported by the fact that besides radial sorting, Cdc42 is also required for Schwann cell proliferation and remyelination following sciatic nerve crush, processes that are not dependent on integrin $\beta 1$ (Feltri et al., 2002) or Rac1 signaling (this work).

**Figure 28: The activity of Rac1 is significantly decreased in the absence of Integrin $\beta 1$**

Levels of active Rac1 (Rac1-GTP) and Cdc42 (Cdc42-GTP) were measured in a pulldown assay using GST-PAK-CK constructs. Sciatic nerve lysates from P5 CNP-Cre Integrin $\beta 1$ mutant mice show significantly decreased levels of active Rac1 compared to control lysates ($n=6$; ** $p < 0.01$). Total protein levels and activity of Cdc42 are not significantly different between control and mutant lysates ($n=4$). Values are shown as mean ± SD. Student’s t-test was used to determine significance.
6.4.7. Integrin β1 and Rac1 regulate Schwann cell process extension in culture

To initiate radial sorting and establish 1:1 interactions with axons, Schwann cells must send processes into axonal bundles. This process requires ECM/integrin signaling, and mutations in *Laminin* genes or the conditional ablation of the *Integrin β1* gene in Schwann cells are thought to affect the extension and targeting of Schwann cell process, causing radial sorting defects (Colognato et al., 2005; Feltri et al., 2002).

To investigate if Cdc42 and Rac1 are required to mediate Schwann cell process extension, we prepared primary Schwann cell cultures from sciatic nerves of PO-P2 control, Rac1 mutant, and Cdc42 mutant mice. In these experiments we also included Schwann cells prepared from sciatic nerves of CNP-Cre *Integrin β1* mutant mice. Cells were plated on PDL/laminin2 substrates and grown in defined media for 16 hours, a period of time sufficient for Schwann cells to extend several thin processes (Figure 29A). Labeling of microtubules by immunofluorescent staining and of the actin cytoskeleton by Alexa488-conjugated phalloidin enabled us to measure and compare the length of the Schwann cell processes formed. Our results show that *Integrin β1* and *Rac1* mutant Schwann cells produced significantly shorter processes than their control counterparts, whereas the length of the processes produced by *Cdc42* mutant Schwann cells was not significantly different from controls (Figure 29A). These results suggest that the radial sorting phenotype observed in the nerves of *Integrin β1* and *Rac1* mutant mice are likely to be a consequence of deficient Schwann cells process extension. They also imply that the radial sorting phenotype present in *Cdc42* mutant nerves is probably caused by insufficient Schwann cell proliferation rather than by an impairment of Schwann cell process extension. In *Rac1* mutant Schwann cells, smaller processes were accompanied by an almost complete absence of lamellipodia (Figure 29A and B; a’-d’). The formation of these actin-rich structures is Rac1-dependent (Nobes and Hall, 1995) and their inhibition in other cell types, such as neurons, has been linked with reduced process extension or reduced motility (Allen et al., 1998; Wheeler et al., 2006). However, in the case of the *Integrin β1* mutant Schwann cells, whose processes were also shorter than controls, lamellipodia were still formed (Figure 29B; a’-d’). One possible explanation is that in these cells, the level of activated Rac1 was sufficiently high to allow lamellipodia formation, but was still under the threshold needed for normal cytoskeletal dynamics. To be tested, this hypothesis requires the use of live time-lapse microscopy.
6. PART II, Rho GTPases in PNS myelination

6.4 Results

A

Integrin β1 mutant
Rac1 mutant
Cdc42 mutant

B

Merge
Tubulin
Actin

control

Integrin β1 mutant

Rac1 mutant

Cdc42 mutant
6.5. Discussion

Using tissue-specific conditional gene ablation strategies, we demonstrate an essential role for the small Rho GTPases Cdc42 and Rac1 during PNS myelination. We also show that these two proteins regulate different aspects of Schwann cell biology. Cdc42 is required for Schwann cell proliferation and probably, at a later stage, for myelin sheath formation, whereas Rac1 is important for Schwann cell process formation during the radial sorting of embryonic axon bundles. Our results also suggest that while Rac1 activity is regulated by ECM/integrin interactions, Cdc42 is not.

6.5.1. ECM/integrin interaction regulates radial sorting via a Rac1-dependent pathway

The loss of Cdc42 or Rac1 in Schwann cells led to the formation of a dysmyelinating phenotype in postnatal sciatic nerves of mutant mice characterized by the persistence of embryonic axon bundles throughout development. This defect in the radial sorting process was particularly pronounced in Cdc42 mutant mice. Although this process was also affected in the sciatic nerves of Rac1 mutant mice, many Schwann cells were able to establish 1:1 relations and form normal myelin sheaths, albeit with a delay, at later stages of development. These findings were also supported by analysis and comparison of the expression levels of the Oct6 and Krox20 transcription factors in mutant nerves at different stages of development.

Similar dysmyelinating phenotypes have been described in Laminin (Ln) mutant mice. Ln-2 is the major laminin of the PNS and Ln-2 deficient mice (dy, dy2J) show bundles of unsorted axons in spinal roots, cranial nerves and, to a lesser extent, in
Sciatic nerves (Bradley and Jenkison, 1975; Stirling, 1975; Weinberg et al., 1975). In these mice, however, brachial nerves myelinated normally what raises the possibility that other laminin isoforms, such as Ln-8 or Ln-10 can compensate Ln-2 function. Indeed, in Ln-2/Ln-8 null mice all nerves are almost completely dysmyelinated (Yang et al., 2005). The lack of the Ln\_1\gamma chain, present in all Ln isoforms expressed in the PNS, also led to the persistence of unsorted axon bundles throughout development (Chen and Strickland, 2003). Furthermore, the conditional ablation of the Integrin β1 subunit gene in Schwann cells also led to impaired radial sorting (Feltri et al., 2002). Integrins are the most important receptors for ECM proteins, including laminins. The fact that the loss of β1 integrin subunit, necessary for the formation of Schwann cell laminin receptors such as α1β1 and α6β1, resulted in radial sorting defects highlights the importance of the ECM/integrin signaling in this process. Several in vitro studies showed that ECM/integrin signaling, among other important cellular functions, control cytoskeleton dynamics via the regulation of Rho GTPase activity (Schwartz and Shattil, 2000). Using a biochemical assay, we demonstrated that in sciatic nerves, integrin β1 regulates the activity of Rac1 but not of Cdc42. To address the biological relevance of this regulation, we measured process extension in Integrin β1 and Rac1 mutant Schwann cells cultured on laminin2 substrates. Our results show that the ablation of these proteins resulted in a significant shortening of the Schwann cell processes, probably as a consequence of impaired cytoskeleton dynamics. Overall, and taking into account this data and the similarity of the phenotypes displayed by the laminin, integrin β1, and Rac1 mutants, we propose that the radial sorting of axons during PNS development is regulated in a Rac1 dependent way by a molecular pathway initiated by ECM/integrin interactions. Our results also indicate that Cdc42 is not directly involved in this pathway and its activation may be regulated by growth factor receptor signaling (work in progress).

Based on chemotactic Boyden chamber assays Yamauchi and colleagues postulated that Rac1 and Cdc42 activation regulates Schwann cell migration (Yamauchi et al., 2003; Yamauchi et al., 2005). Deficient migration, reduced proliferation or increased cell death are all factors that could reduce the number of Schwann cells present in the nerves and consequently impair radial sorting. Although we have not directly addressed Schwann cell migration in Cdc42 and Rac1 mutant nerves, our in vivo results suggest that migration does not play a major role in the phenotypes observed.
At E17.5, Schwann cells populate the nerves of control, Rac1 mutant, and Cdc42 mutant mice in similar numbers and, at least in the case of Rac1 mutant nerves, the numbers of cells in distal regions of P5 and P24 nerves is not significantly different from those in controls. In addition, it is not yet completely clear how Schwann cells migrate and to what extent their migration is driven by chemotactic cues. In contrast to oligodendrocyte progenitors that migrate autonomously in response to chemotactic cues, it is thought that during development, Schwann cell progenitors attach and migrate together with outgrowing nerves (Jessen and Mirsky, 2005). In this context, the various factors affecting Schwann cell migration are more likely to govern Schwann cell movements during radial sorting (Jessen and Mirsky, 2005) than during Schwann cell migration.

6.5.2. Cdc42 regulates Schwann cell proliferation

The fact that the loss of integrin β1 did not regulate the activity of Cdc42 and that process extension in Cdc42 mutant Schwann cells plated on laminin2 substrates was normal, supports the idea that Cdc42 is not required for radial sorting per se. Still, the dysmyelinating phenotype in these mutant mice was strikingly similar to the one observed in both Integrin β1 and Rac1 mutant mice. As the total number of Schwann cells present in Cdc42 mutant sciatic nerves was lower than in control nerves, we investigated Schwann cell proliferation and survival in Cdc42 mutant mice. We showed that Cdc42 is required for Schwann cell proliferation but not for their survival. We conclude that the lack of sufficient numbers of Schwann cells to ensheath axons is the likely cause for the radial sorting defects seen in the Cdc42 mutant nerves.

Proliferation has also been shown to be reduced in Schwann cells lacking Ln γ1 (Yu et al., 2005). In this mutant it was proposed that proliferation is reduced because Ln γ1-deficient Schwann cells fail to extend cytoplasmic processes and, as a consequence, to interact with axonal mitogens (Yu et al., 2005). Although we cannot exclude that proliferation in Cdc42 mutant nerves is affected by a similar mechanism, the loss of Cdc42 in Schwann cells did not affect process extension.

Growth factor signaling regulates the activation of Rho GTPases, including Cdc42, in several different cell types (Nobes et al., 1995; Ridley et al., 1992). In the PNS, growth factors such as neuregulin 1 (NRG1), are known to control multiple processes in Schwann cell development (Jessen and Mirsky, 2005), including proliferation and
the regulation of myelin thickness (Michailov et al., 2004). Some of these processes may be Cdc42-dependent. This hypothesis, however, has still to be supported by experimental evidence.

### 6.5.3. Cdc42 is required for myelin sheath formation during remyelination of adult sciatic nerves

As Rho GTPases are implicated in a multitude of cellular processes, it is possible that Cdc42 or Rac1 regulate additional aspects of Schwann cell myelination later in development and beyond the radial sorting stage. After a nerve crush, remyelination of axons in the adult sciatic nerve does not require radial sorting, migration or extensive proliferation of Schwann cells. Therefore, such studies, in combination with an inducible gene ablation system (Leone et al., 2003), provided us a valuable tool to address the role of Cdc42 and Rac1 in myelin sheath formation. In this context, we showed that while Rac1 is not required for the remyelination of the PNS, Cdc42-deficient Schwann cells established 1:1 relations with axons but were not able to form a myelin sheath. These findings indicate that in addition to the regulation of Schwann cell proliferation, Cdc42 may also be required for the transition of promyelinating Schwann cells into myelinating Schwann cells and for the formation of myelin sheaths. However, it must be stressed that the microenvironment of the remyelinating nerve is likely to be different from that of the developing nerve, and that the Cdc42 signaling requirements for nerve remyelination may differ from those needed during normal development. This issue warrants further investigation.

### 6.5.4. Lack of Rac1 inhibits lamellipodia formation of Schwann cells whereas Cdc42 deletion does not affect the formation of “filopodia-like” actin structures

Rho GTPases regulate the organization of the actin and microtubule cytoskeleton (Nobes and Hall, 1995; Rodriguez et al., 2003). In many cell types, activation of Cdc42 leads to the formation of actin-rich filopodia, whereas active Rac1 promotes actin polymerization at the cell periphery to form lamellipodial extensions. However, most of these studies were performed using dominant negative (DN) Rho GTPase mutants. If expressed at high levels such mutants may produce trans-dominant negative effects, (Braga et al., 2000) and inhibit the activation of other Rho GTPases, making it nearly impossible to discriminate between the specific contributions from different Rho GTPases. Recent work by some of us (Czuchra et al., 2005) shows that
the transfection of Cdc42-null fibroblastoid cells with a Cdc42 dominant-negative mutant (N17 Cdc42) elicits additional phenotypes that are not present in untransfected Cdc42-null fibroblasts. This observation raises important questions about the specificity of Rho GTPase dominant-negative approaches and is in line with previous reports where dominant-negative mutant expression induced more severe phenotypes than those produced by genetic loss-of-function approaches on identical cell types or organisms (Luo et al., 1994; Scott et al., 2003). Using a more specific conditional gene ablation approach, our results support a role for Rac1 in the formation of Schwann cell lamellipodia, but do not support a role for Cdc42 in the formation of filopodia. However, it is still possible that the “filopodial-like” protrusions present in Cdc42 mutant Schwann cells were only retraction fibres, which result from incomplete membrane retraction. To distinguish between these two structures, time-lapse microscopy would be needed. Alternatively, in the absence of Cdc42, other Rho GTPases such as Rif or TC10 (Neudauer et al., 1998, Vignal et al., 2000, Ellis et al., 2000) could induce formation of filopodia.

6.6. Thesis author’s work

This study was performed in collaboration with Yves Benninger, who did the electron microscopic analysis of Cdc42 and Rac1 mutant mice, including remyelination analysis. I demonstrated the loss of protein and investigated proliferation, total Schwann cell number, apoptosis and the expression of important transcription factors in sciatic nerves of these mutant mice. In addition I performed pulldown assays with Integrin β1 mutant mice and analyzed the cytoskeletal organization and process extension in Schwann cell cultures from the three different mutant mice. A manuscript including parts of these results is in preparation.
6.7. References


7. PART III

Profilin 1 is required for PNS myelination but dispensable for CNS myelination

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7.1. Abstract

During myelination of the nervous system glial cells proliferate and migrate extensively before undergoing the remarkable morphological changes associated with the ensheathment and myelination of axons. These processes are likely to require the dynamic reorganization of the actin and MT cytoskeleton. Profilin 1 is a G-actin binding molecule known to play an important role in actin dynamics. To study its role in myelination we conditionally ablate profilin 1 in oligodendrocytes and Schwann cells, the myelinating cells of the CNS and PNS, respectively. Our results show that profilin 1 is required for proper myelination of the PNS but is dispensable for CNS myelination. Schwann cells lacking profilin 1 migrate and proliferate normally, but differentiation into a myelinating phenotype is impaired.

Key words: profilin 1, myelin, CNS, PNS, oligodendrocyte, Schwann cell
7.2. Introduction

Myelination is carried out by oligodendrocytes in the CNS and by Schwann cells in the PNS. These cells proliferate and migrate extensively before extending processes that repeatedly wrap around the axon to produce myelin, a lipid-rich biological membrane. The resulting myelin sheaths act as insulators, enabling the rapid, saltatory conduction of electrical signals along the axons. The mechanisms controlling all these processes are poorly understood, but are likely to involve the dynamic reorganization of the actin cytoskeleton (Buttery and ffrench-Constant, 2001; Liu et al., 2003; Song et al., 2001).

Microfilaments are present in all regions of oligodendrocytes throughout their development (Song et al., 2001). They are enriched in the peripheral regions beneath the plasma membrane, particularly at the leading edges of processes and in areas where new branches are being formed. This cytoskeletal organization is reminiscent of that found during the growth of a neuron (Baas, 1999), and is consistent with the idea that microfilaments guide the local reorganization of microtubules for the elongation of oligodendrocyte processes and formation of new branches. Disruption of microfilaments by cytochalasin B treatment prevented the formation of processes in a majority of oligodendrocytes, and the small number of processes that did form showed no enrichment of microfilaments at their leading edge. Interfering with actin dynamics by the ablation of WAVE, a mediator of lamellipodia formation, impaired oligodendrocyte process outgrowth and myelination of the CNS (Kim et al., 2006).

Profilin (Pfn) is a small, ubiquitous actin-binding protein, thought to be a key regulator of actin dynamics in living cells. It has been found in all organisms examined to date, and was recently described to be expressed in oligodendrocytes (Liu et al., 2003). *In vitro* studies have shown that profilin can directly bind actin and inhibit actin polymerization by sequestering actin monomers in a 1:1 complex (Carlsson et al., 1977). However, when bound to G-actin, profilin functions as an ATP nucleotide exchange factor recharging ADP-actin with ATP, and can thus also enhance actin polymerization (Finkel et al., 1994; Sutherland and Witke, 1999). It has been demonstrated that profilin binds not only to actin, but also to phosphatidylinositol 4,5-bisphosphate (PIP2) (Lassing and Lindberg, 1985), poly-L-proline (Tanaka and Shibata, 1985), and to proteins with proline-rich sequences, which are thought to be important in regulating the actin cytoskeleton. Dissociation of
profilin from actin is stimulated by PIP2 and it is possible that profilin plays a role in transducing signals between the cell membrane and the cytoskeleton. Beside its well-studied role in the regulation of actin dynamics, profilin emerges as an important integrator of diverse signaling pathways (Witke, 2004). Through the poly-L-proline domain, profilin interacts with a variety of proteins including nuclear export receptors, putative transcription factors, and Rho GTPase effector molecules. However, the exact role of profilin in such signaling platforms has still to be defined.

In the mouse, four profilin isoforms have been described. They differ in their spatio-temporal expression pattern and in their isoform-specific interaction with certain ligands, suggesting unique functions of these isoforms in vivo. Profilin 1, the major profilin isoform, is expressed throughout development in all tissues (Witke et al., 1998). Profilin 2 is only expressed in neural cells (Lambrechts et al., 2000). Profilin 3 and profilin 4 are minor isoforms whose expression is restricted to the testis (Braun et al., 2002; Obermann et al., 2005).

In this study we show that profilin 1 is expressed by both oligodendrocytes and Schwann cells. To examine the role of profilin 1 signaling during myelination of the nervous system, we ablated the Pfn1 gene in oligodendrocytes and Schwann cells by tissue-specific conditional gene ablation. We found that profilin 1 is critical for proper PNS myelination but dispensible for myelination of the CNS.

7.3. Material and methods

7.3.1. Generation of conditional knockout mice

The generation of conditional Pfn1 mutant mice will be described elsewhere. Mice homozygous for the Pfn1 floxed allele (Pfn1\textsuperscript{lox/lox}), were crossed with mice that were heterozygous for the Pfn1 floxed allele and also expressed the Cre recombinase under the control of the either the 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) promoter ("knock-in") (CNP-Cre\textsuperscript{+} Pfn1\textsuperscript{lox/wt}) (Genoud et al., 2002; Lappe-Siefke et al., 2003; Saher et al., 2005) or the desert hedgehog (Dhh) promoter ("knock-in") (Dhh-Cre\textsuperscript{+} Pfn1\textsuperscript{lox/wt}) (Joseph et al., 2004). Progeny of interest were CNP-Cre\textsuperscript{+} Pfn1\textsuperscript{lox/lox} or Dhh-Cre\textsuperscript{+} Pfn1\textsuperscript{lox/lox} (hereafter called mutant mice), and CNP-Cre\textsuperscript{+} Pfn1\textsuperscript{lox/wt} or Dhh-Cre\textsuperscript{+} Pfn1\textsuperscript{lox/wt} mice (hereafter called control mice). To follow the fate of the recombinated cells by detection of \(\beta\)-galactosidase expression, we also bred the conditional LacZ allele from the ROSA26
reporter mouse strain (Soriano, 1999) into control and mutant mice. Genotypes were determined by PCR on genomic DNA.

7.3.2. Electron microscopy
Mice were deeply anesthetized and then perfused with 0.1M phosphate buffer (pH 7.4) followed by buffer containing 3% glutaraldehyde (GA) and 4% paraformaldehyde (PFA). Fixed tissues were post-fixed in 2% osmium tetroxide, dehydrated through a graded acetone series, and embedded in Spurrs resin (Electron Microscopy Sciences). Semithin sections were stained with toluidine blue for analysis at the light microscope, and ultrathin sections with 3% uranyl acetate and 1% lead citrate before observation in a Hitachi H-600 TEM at 75kV. For g-ratio calculations at least 150 myelinated axons were measured with MCID software (Imaging research Inc.).

7.3.3. Immunfluorescence, TUNEL staining, X-gal staining
Sciatic nerve sections were blocked for 1 hour with 10% goat serum, 0.1% Triton X-100 in PBS. Incubation with primary antibodies was carried out overnight at 4°C. Rat monoclonal antibody against Ki67 (DAKO 1:50) and mouse monoclonal antibody against NF160 (Sigma 1:150) were used. On the following day, tissue sections were washed in PBS and incubated with the appropriate secondary antibodies for 1 hour at room temperature. Secondary antibodies conjugated to fluorescein and rhodamine were obtained from Jackson Immunoresearch Laboratories. The sections were mounted in Citifluor (Citifluor Ltd) containing 4',6'-diamidino-2-phenylindole (DAPI) to stain the nuclei.
Mouse Schwann cell cultures were fixed in 4% PFA in MP buffer (65 mM PIPES, 25 mM HEPES, 10 mM EGTA, 3 mM MgCl₂, pH 6.9) for 10 min at room temperature. The cells were permeabilized with 0.2% Triton X-100 in MP buffer for 5 min at room temperature. Primary monoclonal antibody against α-tubulin (Sigma 1:100) and Alexa Fluor 488 phallloidin (Molecular probes 1:100) were incubated in 1mg/ml BSA in PBS overnight at 4°C, followed by incubation with Cy3-conjugated anti-mouse (1: 200, Jackson Immuno Research) at room temperature.
Apoptotic cell death was analyzed by TUNEL staining using biotin-labeled UTP and FITC-conjugated streptavidin complex according to the manufacturer's instructions (Roche Diagnostics).
For X-gal staining, the sections were incubated overnight in PBS containing 5 mM

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potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM magnesium chloride, 0.1 %
sodium deoxycholate, 0.02 % NP40 and 2 mM X-gal (AppliChem).
Images were acquired using a Zeiss fluorescence microscope equipped with a Zeiss
Axiocam CCD camera, and Axio VisionV4.5 (Zeiss) acquisition software. Images were
processed using Adobe Photoshop version 8.0.

7.3.4. In situ hybridisation
Twenty micrometer frozen tissue sections were collected and hybridized with digoxigenin-
labeled RNA probes overnight at 72°C in buffer containing 50% formamide. The bound
probe was detected using an anti-DIG-AP antibody (Roche Diagnostics).

7.3.5. Primary cell culture
Primary mouse Schwann cell cultures were obtained from P0-P2 sciatic nerves. Briefly,
nerves were digested in enzyme buffer (0.7 mg/ml collagenase type I, 0.25% Trypsin
(Invitrogen) in HBSS (Invitrogen)). After trituration, cells were grown on poly-D-
lysine/laminin-coated dishes in minimal medium plus 0.5% FCS. Minimal medium is
DMEM/F12 (GIBCO) containing human apo-transferrin (100µg/ml), progesterone
(60ng/ml), insulin (5µg/ml), putrescine (16µg/ml), L-thyroxin (400ng/ml), selenium
(160ng/ml), triiodothyronine (10ng/ml) and BSA (300µg/ml (Fluka). Supplements were
from Sigma unless stated otherwise.

7.3.6. Western blot
Sciatic nerve and spinal cord tissues were homogenized with a chilled mortar and pestle in
lysis buffer (0.1% SDS, 10 mM TrisHCl, 150 mM NaCl, 50 mM NaF, 1mM NaVO₄, 1
mM EDTA, 0.5% sodium-deoxycholate, protease inhibitor cocktail (Sigma)). Extracts
were processed using standard SDS-PAGE and western blotting procedures. The following
antibodies were used: Krox20 (BabCO 1:100), Oct6 (Ghazvini et al., 2002; 1:100), β-actin
(Sigma 1:1000), GAPDH (HyTest 1:20000), Pfn1 and Pfn2 (R. Fässler laboratory, 1:250
and 1:500). Secondary antibodies were obtained from Pierce and Santa Cruz. Densitometry
and quantification of the relative levels were carried out on scanned images of Western
blots using Quantity one software (Biorad).
7. PART III, Profilin function in myelination

7.4. Results

7.4.1. Profilin 1 expression is developmentally regulated in white matter areas of the CNS

Profilins are ubiquitous proteins that are found in almost all cell types and in all organisms examined to date. However, very little is known about the expression of profilin in oligodendrocytes or in white matter areas of the CNS. To characterize the spatio-temporal expression pattern of profilin 1 in the mouse, we performed in situ-hybridization on brain- and spinal cord sections at different developmental stages (Figure 30). Representative pictures were taken from different white matter areas including corpus callosum, cerebellum and spinal cord.

Profilin 1 was ubiquitously expressed in the spinal cord at embryonic day 14 (E14) and expression levels were highest in motoneurons. Abundant expression of profilin 1 was also observed in the spinal cord at postnatal day 5 (P5) and P21, whereas no profilin 1 mRNA-positive cells were found at P56. In the brain, we found a similar developmentally regulated expression of profilin 1. E14 brain sections showed a prominent staining in the subventricular zone of the lateral ventricles. Later, at P5 and P21, profilin was expressed in the whole brain, including cortex, hippocampus and the corpus callosum. At P56, profilin 1 mRNA was almost undetectable in the corpus callosum, but still significantly expressed in the hippocampus and caudate putamen. In the mouse spinal cord, myelination starts at birth. In the brain, it is completed in almost all regions around P45-60. Thus, the observed spatio-temporal expression pattern of Pfn1 is in line with our hypothesis that profilin 1 might be important for the myelination of the CNS.
Figure 30: *In vivo* expression of murine profilin 1

Transverse and sagittal sections of wild-type mice at different developmental stages show a spatio-temporally regulated expression of profilin 1. Scale bars represent 100μm. Abbreviations: cc, corpus callosum; cpu, caudate putamen; drg, dorsal root ganglion; he, hippocampus; lv, lateral ventricle; mn, motoneurons; svz, subventricular zone.
7.4.2. Recombination of the conditional \textit{Pfn1} allele in the CNS of mutant mice

Mouse embryos with a homozygously inactivated \textit{Pfn1} gene die before birth (Chen 2000). To study the role of profilin1 signaling in oligodendrocytes, we conditionally ablated \textit{Pfn1} by expressing Cre-recombinase (Cre) under the control of 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNP) gene regulatory sequences. CNP is expressed in the myelinating glia of the central and peripheral nervous system (Lappe-Siefke et al., 2003; Saher et al., 2005). To identify the recombined cells, we also bred the conditional \textit{LacZ} allele from the ROSA26 reporter mouse (Soriano, 1999) into control and mutant mice. Recombined cells (\textit{LacZ} expressing) were seen in pre-myelinating oligodendrocytes, Schwann cells and some spinal motoneurons from E12 on (Genoud et al., 2002) (Figure 31B). Recombination of the conditional \textit{Pfn1} allele was detected in the spinal cord, brain and sciatic nerve of mutant mice (Figure 31C). The concomitant down-regulation of profilin 1 protein was shown in mutant OPC cultures by western blotting (Figure 31D).
7. PART III, Profilin function in myelination

7.4 Results

**Figure 31: Recombination of the conditional Pfn1 allele in the CNS of mutant mice**
(A) Schematic representation of the CNP-Cre “knock-in” allele, the conditional Pfn1 allele, and the reporter LacZ allele. Upon Cre-mediated recombination the genomic region between the two LoxP sites is excised, inactivating the Pfn1 gene and triggering the expression of the enzyme β-galactosidase in recombined oligodendrocyte cells. (B) X-gal stained transverse sections of CNP-Cre+ LacZ reporter embryos at E14 (a) and E18 (b). Cre-recombinase is expressed in oligodendrocytes, Schwann cells and some spinal motoneurons. Abbreviations: drg, dorsal root ganglia.; mn, motoneurons; pn, peripheral nerves; sg, sympathetic ganglion. (C) PCR shows the recombination of the conditional Pfn1 allele in genomic DNA isolated from 1 day-old mutant (CNP-Cre+ Pfn1lox/lox) and control (CNP-Cre− Pfn1lox/wt) spinal cord (spc), brain and sciatic nerve (scn). Arrows indicate the fragments for wild-type (wt), Pfn1 floxed (flox), and Pfn1 deleted (KO) alleles. (D) Western blot analysis shows a marked decrease of Pfn1 protein expression in mutant OPC cultures. The remaining Pfn1 signal detected is likely due to some contaminating astrocytes and non-recombined OPCs in these cultures. Results are shown as the mean ± SD. Student’s t-test was used to determine significance (n=3, *** p < 0.001).

Pfn1 mutant mice develop a slight paresis of the hind limbs and are generally smaller compared to control littermates. The severity of the different symptoms varies considerably between different mutant mice. Mutant mice are born at a much lower frequency than expected, and, if born, most animals die within the first four weeks.

7.4.3. Profilin 1 is not required for proper myelination and ensheathing of CNS axons

Spinal cord, corpus callosum and optic nerve of P25 control and mutant littermates were analyzed at electron microscopic level to assess myelination of the CNS. No malformations of the myelin sheaths were observed in any of these fibre tracts (Figure 32A, data not shown). The thickness of myelin was also not different in mutant and control spinal cords (Figure 32B). Although our results cannot exclude a developmental delay in CNS myelination, they show that profilin 1 is not an absolute requirement for CNS myelination.
7. PART III, Profilin function in myelination

7.4 Results

Figure 32: Myelin sheath formation and thickness are not altered in pfn1-deficient mice
(A) Electron micrograph of white matter regions of control and mutant spinal cord at P25 show no major differences in myelination. (B) The graph shows the linear regressions of fiber measurements carried out in the spinal cord of one control (blue) and one mutant (red) mouse at P25. The linear regression is similar in control and mutant mice.

It is possible that profilin 1 function is partially compensated by other profilin isoforms. Profilin 2 is a likely candidate, as it is also expressed in neural tissues. In contrast to the steady expression levels of profilin 1, profilin 2 expression is upregulated from E14 and increases until birth (Lambrechts et al., 2000). However, profilin 2 was not significantly up-regulated in the absence of profilin 1 (Figure 33).

Figure 33: Levels of Pfn2 are not increased in the absence of Pfn1
Western blot analysis of P24 mutant and control spinal cord lysates show no differences in the relative amounts of Pfn2. Actin levels confirm equal protein loading. Western blots were performed at least twice.
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7.4.4. Recombination of the conditional Pfn1 allele in the PNS of mutant mice

To study the role of profilin 1 in Schwann cells, we conditionally ablated the Pfn1 gene by expressing Cre-recombinase (Cre) either under the control of the CNP gene regulatory sequences (see Figure 31A) or the desert hedgehog (Dhh) gene regulatory sequences (Figure 34A). Dhh-Cre is active in Schwann cells from embryonic day E11 on (Joseph et al., 2004). In addition, we bred the conditional LacZ allele from the ROSA26 reporter mouse (Soriano, 1999), into control and mutant mice to identify the recombined cells. Recombination of the conditional Pfn1 allele was detected in the sciatic nerve and dorsal root ganglia of mutant mice (Figure 34B). The concomitant downregulation of profilin 1 protein was shown in mutant sciatic nerve lysates (Figure 34C).

Figure 34: Recombination of the conditional Pfn1 allele in the PNS of mutant mice

(A) Schematic representation of the Dhh-Cre allele, the conditional Pfn1 allele, and the reporter LacZ allele. Upon Dhh-Cre-mediated recombination the genomic region between the two LoxP sites is excised, inactivating the Pfn1 gene and triggering the expression of the enzyme β-galactosidase in recombined Schwann cells. (B) PCR shows the recombination of the conditional Pfn1 allele in genomic DNA isolated from 1 day-old mutant (Dhh-Cre° Pfn1lox/lox) and control (Dhh-Cre° Pfn1lox/lox) sciatic nerve (scn), lung, dorsal root ganglia (drg) and brain. Arrows indicate the fragments for wild-type (wt), Pfn1 floxed (flox) and Pfn1 deleted (KO) alleles. (C) Western blot analysis shows a marked decrease of Pfn1 protein expression in mutant sciatic nerve lysates. The remaining Pfn1 signal detected is mostly due to non-recombined endoneurial fibroblasts and axons. Actin levels confirm equal protein loading. Results are shown as the mean ± SD. Student’s t-test was used to determine significance (n=5, *** p < 0.001). (D) Sciatic nerves from P14 control and Pfn1 mutant mice. Note the hypotrophy of the Pfn1 mutant sciatic nerve. For clarity, a needle is inserted behind the nerves. Scale bar represents 1mm.
7.4.5. Profilin 1 deficiency in peripheral nerves leads to motor dysfunction and paralysis

The phenotype of \textit{Dhh-Cre Pfnl} mutant mice resembled that of \textit{CNP-Cre Pfnl} mice. These mice developed a slight paralysis of the hind limbs, and were smaller and weaker than their control littermates. This phenotype was variable in severity, even among littermates. During development, peripheral nerves of control mice became successively myelinated, resulting in a typical, whitish appearance of the nerves. However, sciatic nerves of P14 mutant mice remained translucent, a sign of impaired myelination (Figure 34D).

![Figure 35: Dhh drives Cre expression outside the PNS](image)

X-gal stained transversal sections of \textit{Dhh-Cre+ LacZ} reporter embryos at E18 (A; assembled from multiple photomicrographs) and longitudinal section of a P1 sciatic nerve (B). Expression of Cre-recombinase was found in peripheral nerves as expected but also in some cells of the heart, liver, kidney etc. Abbreviations: drg, dorsal root ganlion; pn, peripheral nerve; spc, spinal cord.

A high percentage of \textit{Pfnl} mutant mice died during early postnatal stages. A closer analysis of the recombination patter in perinatal \textit{Dhh-Cre LacZ} mice revealed additional recombination in cells of the heart, liver, kidney etc. (Figure 35). It is possible that Dhh-Cre is already expressed by migrating neural crest cells. Crest cells in the trunk give rise to
melanocytes, sensory and autonomic neurons and glia. The cardiac crest in more anterior parts of the trunk generate fibroblasts and smooth muscle cells, and the cephalic crest in the head region forms cells of the cartilage and bone. Targeting of crest cells would therefore explain, at least partially, the widespread recombination pattern of Dhh-Cre lacZ mice. Earlier studies showed that Dhh+ cells derived from embryonic mouse nerves gave rise not only to Schwann cells but also to endoneurial fibroblasts (Joseph et al., 2004).

7.4.6. Profilin 1 mutant mice develop a dysmyelinating phenotype

During postnatal development, premyelinating Schwann cells extend processes into bundles of axons. Myelinating Schwann cells then establish a 1:1 ratio with axons and form a myelin sheath around them, while small caliber axons remain engaged with a nonmyelinating Schwann cell. To assess the role of profilin 1 in Schwann cell myelination, we examined cross sections of CNP-Cre Pfn1 control and mutant sciatic nerves at different developmental stages. In P5 control nerves, most axons had already formed a 1:1 relationship with Schwann cells and acquired a thin myelin sheath (Figure 36B). In contrast, axonal sorting was impaired in mutant nerves where large bundles of mixed caliber axons were present throughout postnatal development (Figure 36B, D;a). Myelination of the sorted axons was also affected as not all of them obtained a myelin sheath (Figure 36B, D;b). In addition, myelin sheaths of similar sized axons were thinner in mutant nerves than in control nerves. However, this observation has to be confirmed by appropriate g-ratio measurements.

Although the absence of profilin 1 affected axonal sorting, a significant number of axons acquired a myelin sheath. To prove that these Schwann cells were recombined, we performed Bluogal staining on sciatic nerves of P30 control and mutant mice. Bluogal precipitates were present in myelinating Schwann cells of mutant nerves (Figure 36C), indicating that profilin 1 is not required for the production of myelin per se.
7.4 Results

Figure 36: Cross sections of sciatic nerves of *CNP-Cre Pfn1* control and mutant mice

(A) Toluidine Blue-stained semithin cross sections of sciatic nerves of P30 control and *Pfn1* mutant mice. Myelination is completed in control nerves, whereas mutant sciatic nerves still contain large bundles of unsorted axons (arrowheads). (B) Electron micrographs of *CNP-Cre Pfn1* control and mutant sciatic nerves at different developmental stages. In the controls, at P5 most Schwann cells already established a 1:1 ratio with the axons and gradually myelinate them until P30, when myelination is completed. In contrast, in the mutant mice the axonal sorting and myelination was impaired, and there were unsorted axon bundles present in all stages (arrowheads). In addition, many Schwann cells engaged with a single axon did not start to myelinate it (asterisks) or formed only very thin myelin sheaths compared to the sheaths of similar caliber axons of control mice (white box). (C) Bluogal staining of reporter *LacZ*-positive P30 control and mutant sciatic nerve sections show that precipitates are present also in myelinating Schwann cells (arrows) showing that Cre recombinase was expressed in these cells. (D) Enlargements of single Schwann cells in P30 mutant sciatic nerves. a) This Schwann cell engages a bundle of axons wherein at least 3 large diameter axons that should have been already sorted out (asterisks). b) A Schwann cell, forming a 1:1 relationship with a large caliber axon but unable to myelinate it.
7.4.7. Mutant Schwann cells proliferate normally but show increased apoptosis

To investigate if the number of Schwann cells was altered, we performed DAPI-staining on cross sections of distal regions of sciatic nerves of mutant and control mice. The total number of cells was not significantly different in control and mutant nerves (Figure 37A). This also indicates that the absence of profilin 1 did not affect Schwann cell precursor migration.

To determine Schwann cell proliferation, the percentage of Ki67+ cells was measured at different developmental stages. We found no significant difference in the number of Ki67-positive cells in control and mutant nerves, including at E18, the peak of Schwann cell proliferation (Stewart et al., 1997) (Figure 37B). Apoptosis of Schwann cells was analysed by measuring the percentage of nuclei stained by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling (TUNEL). Mutant nerves showed a slight but significant increase in the number of apoptotic cells at P0 and P5 (Figure 37C). However, this increase did not significantly reduce the total number of cells present in P5 sciatic nerves (Figure 37A). Although apoptosis was slightly increased in mutant Schwann cells, it is not likely to be the main cause for the axonal sorting and myelination phenotype we observed in P5 mutant nerves.
7.4 Results

Figure 37: Proliferation and total cell number in Dhh-Cre Pfn1 mutant sciatic nerves is not altered although apoptosis is slightly increased
(A) DAPI-stained sciatic nerve cross-sections show similar numbers of cells in P5 control and mutant mice. (B) At E18, P0 or P5, the numbers of proliferating, Ki67-positive cells is not significantly different in sciatic nerves of Pfn1 control and mutant mice. (C) At P0 and P5, apoptotic, TUNEL-positive cell numbers are significantly increased in mutant sciatic nerves compared to control nerves (*p<0.05, ***p<0.001). Results are shown as the mean ± SD of three independent experiments. Student’s t-test was used to determine significance. Scale bar represents 100µm.

7.4.8. Impaired differentiation of profilin 1-deficient Schwann cells
If mutant Schwann cells populate the nerve and their number is not significantly altered, why is the sorting and myelination of the axons impaired? One possibility was that Schwann cells that lack profilin 1 were not able to differentiate properly. To address this question, we investigated the expression of transcription factors known to be important for the onset of myelination. Myelination in Schwann cells is governed by several transcription factors, including the POU proteins Oct6 and Brn2 and the zinc-finger protein Krox20. Oct6 is transiently expressed in Schwann cells, peaking at the promyelinating stage (Jaegle et al., 2003). The analysis of Oct6 loss-of-function alleles indicated that mutant Schwann cells show a transient delay in myelination (Jaegle et al., 1996). Another pivotal factor for the onset of myelination is Krox20, which is expressed continuously and specifically in
myelinating Schwann cells (Topilko et al., 1994). Mutation of Krox20 blocks Schwann cells at the promyelinating stage (Topilko et al., 1994). During the promyelin-myelin transition, Oct6 and Brn2 cooperate with Sox10 in driving Krox20 expression, and thereby controlling myelination in Schwann cells (Ghislain and Charnay, 2006).

To detect whether protein levels of Oct6 and Krox20 are altered by profilin 1 deficiency, we performed western blot analysis in protein lysates obtained from P2 Dhh-Cre Pfn1 control and mutant sciatic nerves. The expression of Oct6 was slightly higher in mutant lysates (Figure 38A). In contrast, the expression of Krox20 was significantly lower in mutant lysates (Figure 38A).

Although mutant mice show a strong phenotype, it is possible that profilin 2 partially compensates for the lack of profilin 1. However, western blot analysis of P2 sciatic nerve lysates did not show a difference in Pfn2 expression levels in control and mutant mice (Figure 38B).
7.4.9. Process extension and cytoskeletal organization is not affected in mutant Schwann cells

Schwann cells are highly motile cells that undergo dramatic morphological changes when forming the myelin sheaths around the axons. These processes require extensive rearrangements of the cytoskeleton and profilins are key regulators of the actin cytoskeleton. Therefore, the radial sorting deficiency observed in Pfn1 mutant mice may be caused by a deficient reorganization of the actin cytoskeleton.

To test if the cytoskeleton is affected by the lack of profilin 1, we prepared primary Schwann cell cultures from neonatal mutant and control sciatic nerves. The cells were cultured on PDL/laminin-2 coated dishes for 16 hours, a period of time sufficient for the extension of several Schwann cells processes. The presence of such processes clearly distinguished them from the more spread fibroblasts also present in these cultures. Immunostaining of these cultures showed that all cells with thin processes were positive for the Schwann cell marker S100, whereas fibroblasts were not labelled (data not shown). Recombination efficiency in these cultures was close to 100% and no X-gal positive fibroblasts were found (data not shown). This confirms that Dhh targets mainly Schwann cells. We did not observe any obvious alterations in the organization of either the actin cytoskeleton or microtubules in control and mutant Schwann cells (Figure 39A). They all show the presence of microfilaments beneath the plasma membrane and an array of actin filaments at the tip of the process followed by loose microtubules that compact in the process. In addition, we did not observe a significant difference in the length of the processes formed by mutant Schwann cells (Figure 39B). These results suggest that at least in vitro, profilin is not required for the reorganization of the actin cytoskeleton during Schwann cell process formation.
7. PART III, Profilin function in myelination

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Figure 39: Loss of Pfn1 does not affect the organization of the Schwann cell cytoskeleton or the length of their processes

Mixed primary Schwann cell cultures were obtained from sciatic nerves of P1 Dhh-Cre Pfn1 control and mutant mice and cultured for 16 hours. (A) The actin cytoskeleton (green), MT (red) and nuclei (blue) were visualized by phalloidin, tubulin antibodies and DAPI, respectively. The tip of a Schwann cell process is enlarged (b-d; b'-d'; white box in a, a'). Actin filaments are enriched in the peripheral regions beneath the plasma membrane, particularly at the leading edges of processes. Microtubules form tight bundles in Schwann cell processes and splay as they enter the process tips. (B) The mean length of Schwann cell processes is not significantly different (n=3, p=0.52) in control and mutant Schwann cell cultures. Results are shown as the mean ± SD. Student's t-test was used to determine significance. Scale bars represent 50μm.
7.5. Discussion

During development of the vertebrate nervous system, glial cells proliferate and migrate extensively before undergoing the remarkable morphological changes associated with ensheathment and myelination of the axons. As the cytoskeleton is central for key cellular processes such as migration, cytokinesis, and shape changes, it is very likely that signaling pathways regulating the dynamic reorganization of the cytoskeleton are crucially involved in myelination. In an effort to find out more about the molecular mechanisms underlying myelination of the nervous system, we specifically ablated the key regulator of actin dynamics, profilin1, in glial cells using the Cre/loxP system. We found that profilin 1 is essential for proper myelination of the PNS but it is not required for CNS myelination.

7.5.1. Requirements for Pfn1 in myelination of the CNS and PNS are different

The ablation of Pfn1 in oligodendrocytes did not affect myelination of the CNS. In the spinal cord, all large caliber axons were myelinated properly and the thickness of myelin sheaths was normal. In contrast, myelination of the PNS was severely delayed in Pfn1 mutant mice. Sciatic nerves of these mice were much more transparent than control nerves, as they contained many dysmyelinated axons. Electron microscopic analysis revealed the presence of large bundles of unsorted mixed caliber axons even during late development. In addition, many axons were captured in a 1:1 relationship with a Schwann cell and stayed unmyelinated, although their diameter had already significantly increased. With time, many axons were myelinated. However, their myelin sheaths were much thinner than those observed in control nerves. We showed that myelinating Schwann cells did recombine, and that the absence of profilin 1 was not compensated by an upregulation of profilin 2 either in the CNS or in the PNS. We cannot exclude that profilin 2 is taking over some functions of profilin 1 without increasing its expression levels, but the impairments in myelination clearly indicate that at least some functions must be unique to profilin1. Different roles for profilin isoforms have been proposed already in earlier studies, and this hypothesis is supported by the fact that, in mouse brain, profilin 1 and profilin 2 bind different sets of ligands (Witke et al., 1998). Analysis of myelination in Pfn1/Pfn2 double mutant mice could provide more informations about the redundancy of these isoforms. Our finding that profilin 1 is important for PNS myelination but dispensable for myelination of the CNS suggests different requirements for profilin signaling in oligodendrocytes and Schwann cell.
myelination. This is in line with previous studies that have shown that the function of profilin is dependent on the cell type or organism studied. Deletion of profilin in *Saccharomyces cerevisiae* results in severe growth reduction (Haarer et al., 1990), whereas ablation in the amoebae *Dictyostelium discoideum* leads to defects in cytokinesis (Haugwitz et al., 1994). Pronounced differences between Schwann cells and oligodendrocytes have also been seen for the ECM receptor β1 integrin: In the CNS, β1-integrin signaling mediates survival of premyelinating oligodendrocyte but is not required for myelination (own unpublished results), whereas the conditional disruption of β1 integrin in Schwann cells impairs PNS myelination (Felti et al., 2002).

### 7.5.2. Increased apoptosis in sciatic nerves of *Pfn1* mutant mice

Survival of Schwann cell precursors depends on axon-derived β-neuregulin 1, which binds to ErbB3 receptors on the precursor cells (Dong et al., 1995; Riethmacher et al., 1997). After this stage, Schwann cells establish an autocrine survival loop involving neurotrophin-3, insulin-like growth factor, and platelet-derived growth factor BB (Meier et al., 1999). In addition, the PI3-kinase/Akt pathway is important for Schwann cell viability (Cheng et al., 2000; Maurel and Salzer, 2000).

We investigated apoptosis in *Pfn1* mutant sciatic nerves using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). The number of TUNEL-positive cells in mutant nerves was significantly higher than in control nerves at P0 (1.7% vs. 0.3%) and P5 (2.4% vs. 0.5%). The slight increase in cell death was not compensated by proliferation, which was similar in control and mutant nerves at E18, P0 and P5. In addition, total cell numbers were not significantly different in P5 control and mutant nerves, suggesting that the increase in apoptosis is physiologically negligible. Currently, there exists no evidence for a link between profilin signaling and apoptosis/cell survival pathways, and further analysis is required at embryonic stages to determine whether β-neuregulin 1/ErbB signaling is affected, and at later postnatal stages to investigate PI3kinase/Akt signaling and whether the phenotype persists or even worsens.

### 7.5.3. Profilin 1 deletion does not affect the global organization of the glial cytoskeleton

Extensive biochemical analysis has established profilin as a key regulator of the actin
cytoskeleton. Profilin impacts actin dynamics by (i) sequestering actin monomers (Carlsson et al., 1977), (ii) enhancing nucleotide exchange on actin (Goldschmidt-Clermont et al., 1992), (iii) promoting barbed-end assembly of actin filaments (Kang et al., 1999; Pantaloni and Carlier, 1993), and (iv) by increasing the rate of actin filament turnover in a synergistic manner with cofillin (Didry et al., 1998). However, these findings resulted exclusively from in vitro studies and the significance of each of these activities needed to be tested in vivo. A single study in Saccharomyces cerevisiae confirmed the in vivo importance of actin nucleotide exchange catalysed by profilin (Wolven et al., 2000).

In our study, we did not observe any obvious changes in the organization of the cytoskeleton in Schwann cells lacking profilin 1. Actin filaments were enriched beneath the plasma membrane and particularly at the leading edge of the processes. Microtubules were present in the cell body and splayed before extending into the leading edge and branches, following the tracks led by microfilaments. This organization pattern was consistent with earlier descriptions of the cytoskeletal architecture of glial cells (Song et al., 2001) and was reminiscent of that found during neuronal growth (Baas, 1999). In addition, we did not find a significant difference in the length of Schwann cell process in control and mutant cultures. In the CNS, oligodendrocytes formed proper myelin sheaths around the axons, and therefore defects in the reorganization of the cytoskeleton are unlikely. Still, due to the neurological phenotype we observed in the PNS of mutant mice, we do not exclude that the lack of profilin 1 affects the dynamics of cytoskeletal changes. Further studies using time lapse microscopy of mutant Schwann cells are needed to investigate the dynamic behaviour of the cytoskeleton in these cells. Microfilaments are regulated by a variety of actin binding proteins, raising the possibility that some of these molecules can partially take over the function of profilin. For example, thymosin-β also sequesters G-actin monomers and is often much more abundant than profilin (Safer et al., 1991).

Although the most well defined function of profilin is its role in regulating actin dynamics, the discovery of more than 50 ligands suggest that it acts as an important integrator of diverse signaling pathways (Witke, 2004). Except for actin, the structural basis and functional relevance for these profilin-ligand interactions are not well understood (Witke, 2004; Zorick et al., 1999). Pfn1 mutant mice might therefore provide a good model system to test which profilin-ligand interactions are important in vivo.
7.5.4. Differentiation of *Pfn1*-deficient Schwann cells is impaired

Ablation of *Pfn1* did not prevent the formation of myelin sheaths *per se*, but impaired the differentiation of Schwann cells, as many axonal bundles were not sorted. The observed delay in myelination is likely a result of the impaired radial sorting. Schwann cells express several transcription factors that are pivotal for initiating myelination. These include the zinc finger protein Krox20/Egr-2 and the POU protein Oct6 (Zorick et al., 1999). We found that profilin 1-deficient Schwann cells have increased Oct6 protein levels and fail to up-regulate Krox20, indicating that the initiation of myelination was affected. Krox20 is also known to suppress c-Jun-mediated TGFB-induced Schwann cell apoptosis (Parkinson et al., 2004). Thus, increased Schwann cell apoptosis could result from the failure of Krox20 to inhibit c-Jun activation.

Overall, this work reveals a novel role for profilin 1 in Schwann cell myelination. Interestingly, lack of profilin 1 in oligodendrocytes did not affect CNS myelination, suggesting that the requirements for profilin are different for oligodendrocyte and Schwann cell myelination. An important task for the future will be to elucidate the nature of the differences between CNS and PNS myelination.

7.6. Thesis author’s work

In this study, I investigated the developmental expression pattern of *Pfn1* in white matter areas of the CNS. Using the conditional *Pfn1* mutant mouse, I analyzed recombination of the conditional allele in the CNS and PNS, the expression of profilin isoforms, proliferation, apoptosis, total cell number and expression of important transcription factors in sciatic nerves. I also established primary Schwann cell cultures to assess the organization of the cytoskeleton and process extension.

A manuscript including parts of these results is in preparation.
7.7. References


8. FINAL DISCUSSION AND OUTLOOK

8.1. Novel function for Cdc42 and Rac1 in CNS and PNS myelination

8.1.1. Rho GTPases and myelin sheath formation

Rho GTPases are likely candidates to regulate CNS and PNS myelination. We chose a genetic route to study the role of these molecules during myelination. Using Cre/loxP technologies, we demonstrated a role for the small Rho GTPases Cdc42 and Rac1 in myelination of the CNS and PNS.

Ablation of Cdc42 and Rac1 in oligodendrocytes produced an extraordinary enlargement of the inner tongue of the oligodendrocyte process, probably as a result of abnormal accumulation of large amounts of cytoplasm in this region. This phenotype challenges the commonly held view that the formation of the spiral multilamellar myelin sheath involves the continuous progression of the inner tongue over the axon surface. In addition, we showed that Cdc42 and Rac1 synergize to regulate myelination in a "gene-dosage" dependent way. The relatively late and specific myelin phenotype in the CNS of Cdc42/Rac1 mutant mice was somewhat unexpected, as earlier in vitro studies using dominant negative (DN) or constitutively active (CA) mutants pointed to an essential role of these molecules in cell proliferation, migration, differentiation and cytoskeletal reorganization (Etienne-Manneville and Hall, 2002; Liang et al., 2004; Nobes and Hall, 1999). The reason for such discrepancies may lie in the mutants used to alter Cdc42 and Rac1 activity. While CA mutants remain GTP-bound because the GTPase activity is inhibited, DN mutants act by binding the corresponding activating GEFs with higher affinity than endogenous Rho proteins. As many GEFs are not specific for a single Rho GTPase, DN molecules may also inhibit the activation of other Rho GTPases (Schmidt and Hall, 2002). Moreover, the degree of inhibition is dependent on the expression level of the DN mutant (Braga et al., 2000). These observations suggest that DN mutants of Rho GTPases are likely to inhibit whole subfamilies rather than single Rho proteins. In addition, the expression of DN constructs in Cdc42 null cells elicited additional phenotypes.
that were not present in untransfected cells (Czuchra et al., 2005). Therefore, the use of conditional gene ablation techniques provides a more stringent system to study the roles of Rho GTPases in the nervous system, circumventing the potential drawbacks of a dominant negative mutant approach.

In sciatic nerves of Cdc42 and Rac1 mutant mice, axons were hypomyelinated and large bundles of unsorted axons were present throughout development. In contrast to the outfolding defects in CNS myelin in Cdc42/Rac1 mutant mice, morphologically normal myelin sheaths were formed in the PNS once myelination started. Our findings indicate that there are different requirements for Cdc42/Rac1 signaling myelination by oligodendrocytes vs. Schwann cells. Different requirements for CNS and PNS myelination have been observed before in integrin β1 and dy/dy mutant mice (Bradley and Jenkison, 1975; Chun et al., 2003; Feltri et al., 2002). An important task for the future will be to elucidate the nature of the differences between CNS and PNS myelination.

Over the past few years, it has become evident that in addition to the obvious differences in oligodendrocyte and Schwann cell myelination, also regional differences in myelination occur within the CNS and PNS. Myelin deficits, including dysmyelination of axons and reduced myelin thickness, have been observed in the forebrain (corpus callosum and optic nerve) but not in the spinal cord of several mutant mice. These include mice deficient in the ECM component laminin-2 (dy/dy) (Chun et al., 2003), the src family kinase fyn (fyn−/−) (Sperber et al., 2001) and the Rac1 target WAVE1 (WAVE1−/−) (Kim et al., 2006). In the PNS, regional differences in myelination are observed in dy/dy mutant mice, which display severe dysmyelination in spinal roots and cranial nerves, whereas sciatic nerves are only partially affected and brachial nerves are nearly normal (Bradley and Jenkison, 1975; Stirling, 1975; Weinberg et al., 1975). In Laminin-8-deficient mice, only distal nerves showed myelin deficits whereas spinal roots were unaffected (Lu et al., 2002; Richardson et al., 2000; Yang et al., 2005; Zhou and Anderson, 2002). These regional differences in myelination may be explained by differences in the local extracellular environment and/or by intrinsic differences in myelinating cells. In the CNS, there is evidence for the presence of different lineages of oligodendrocytes (Cai et al., 2005; Richardson et al., 2000; Spassky et al., 2000; Vallstedt et al., 2005), which differentially respond to developmental cues and also might exhibit different behaviours.
8.1.2. Rho GTPases and in vitro myelination studies

The relative inaccessibility of the vertebrate nervous system makes it challenging to extensively examine and manipulate glial development in situ. Although providing a somewhat artificial environment, the use of cell cultures may help to circumvent this problem. Such experiments can support the in vivo data and aid in identifying the different molecular components involved in myelination.

OPCs can be grown as purified cultures, and their timing of proliferation, migration, differentiation, and apoptosis appears to correspond well to that seen in vivo (Raff, 1989). OPC differentiation in culture involves process extension, the production of specific myelin proteins, and the formation of myelin membranes. Ablation of Cdc42 in cells of the oligodendrocyte lineage did not affect OPC proliferation, survival, directed migration and morphological differentiation. However, it is still possible that Cdc42/Rac1 signaling is only limiting in more complex cellular events when the oligodendrocyte process wraps around the axon.

We also took advantage of the oligodendrocyte culture system to study the cross-activation between different Rho GTPases. Several modes of crosstalk have been described for Rho family proteins. Cdc42 can activate Rac, which in turn stimulates RhoA (Nobes and Hall, 1995). Rac can also antagonize RhoA (Nimnual et al., 2003), and RhoA has been shown to activate Rac under some conditions (Tsuji et al., 2002). In contrast to other cell types where Cdc42 regulates Rac1 activity, we did not observe any difference in the expression levels or the activity of Rac1 in Cdc42 mutant OPCs. However, pulldown assays provide no spatial information and give only limited temporal resolution. The ablation of a particular Rho GTPase might not only affect the overall activity of other family members, but might also influence the site at which they are activated. It is still possible that the ablation of Cdc42 can influence the localization and local activation pattern of other Rho GTPases within a cell. To test this hypothesis I would perform FRET studies in mutant and control OPC cultures (see Chapter 8.3).

In comparison to oligodendrocytes, Schwann cells are less suited for in vitro analysis, as they do not express myelin proteins or form myelin membranes under most conditions, and because purification and expansion of these cells in culture is very difficult. Still, cultured Schwann cells extend several processes, which allowed us to study the role of Rho GTPases in cytoskeletal reorganization during early morphological differentiation. We
showed that Schwann cells lacking Rac1 had significantly shorter processes and did not form lamellipodia. In contrast, Cdc42-deficient Schwann cells did not show any obvious alterations in the organization of either, MFs and MTs. Although dominant negative Cdc42 inhibits the formation of filopodia in primary fibroblasts and Swiss 3T3 cells (Kozma et al., 1995; Nobes and Hall, 1995; Nobes and Hall, 1999), filopodia-like extensions were present in Cdc42 mutant Schwann cells. However, to distinguish these spike-like protrusions from retraction fibres, which result from incomplete membrane retraction, time-lapse microscopy would be needed. If the protrusions in Cdc42-deficient cells could unambiguously be identified as filopodia, our data would prove that Cdc42 is not required for filopodia formation in Schwann cells. Instead, their production could be mediated by other Rho GTPases such as Rif (Ellis and Mellor, 2000), TC10 (Neudauer et al., 1998; Vignal et al., 2000), TCL (Vignal et al., 2000), or Wrch-1 (Tao et al., 2001).

8.1.3. Rho GTPase signaling pathways in myelination

In an attempt to identify the intracellular signaling pathways involved in the formation of the different phenotypes in our transgenic mice, I performed western blotting analysis for candidate effector proteins of Cdc42 and Rac1, using lysates from primary oligodendrocytes (CNS) and sciatic nerves (PNS) of control and mutant mice. Cdc42-deficient oligodendrocytes showed a significant decrease in the activity of ERK, while the activities of p38 and JNK were normal. However, for most of the analyzed downstream proteins, either the expression levels and activities were not altered or the measurements were highly variable and did not allow any conclusions. Analysis of proteins by western blotting has several disadvantages: 1) it requires high amounts of proteins, especially for the detection of phosphoproteins (only 1-2% of total proteins are phosphorylated); 2) it is time consuming as only single proteins are analyzed; 3) it needs prior knowledge of potential targets; 4) highly specific antibodies for these proteins have to be available (also for their phosphorylated forms) and 5) it fails to detect changes in the localization of effector proteins. This last point should not be underestimated, as changes in the localization of effector proteins can be an important element in their activation. To circumvent some of these pitfalls I would perform differential display proteomics, using techniques such as difference gel electrophoresis (DIGE) or isotope-coded affinity tagging (ICAT), to compare protein profiles of control and mutant samples (Figure 13 and 14).
These approaches allow the simultaneous comparison of thousands of different proteins involved in the multiple pathways and networks that regulate biological processes such as myelination. Differentially regulated proteins could then be tested for a myelination phenotype in co-culture systems using iRNA technology. If their ablation results in myelin abnormalities, these candidate proteins could be considered for the generation of conditional knockout mice to verify their role in myelination.

Several knockout mice with various myelination phenotypes have been described, and the list of molecules implicated in oligodendrocyte and Schwann cell myelination in vitro is rapidly growing. By comparing these studies with our observed phenotypes, we may be able to make "educated guesses" in relation to potential Cdc42 and Rac1 effector proteins involved in myelination.

For example, potentially similar myelin sheath outfoldings have been observed specifically in the CNS of MAG and galactosphingolipid-deficient mice (Dupree et al., 1998; Li et al., 1994; Montag et al., 1994). However, these outfoldings occurred with a much lower frequency and were accompanied by various other myelin abnormalities.

Rho GTPases are key regulators of the actin cytoskeleton. They regulate WASP and WAVE, which activate Arp2/3 and therefore lead to the nucleation of new MFs. WAVE1-/- mice displayed regional dysmyelination in the corpus callosum and to a lesser extent in the optic nerve (Kim et al., 2006). As these alterations are completely dissimilar to the myelin defects we observed in Cdc42/Rac1 mutant mice, WASP/WAVE proteins are unlikely to be directly involved in the pathways regulated by Cdc42 and Rac1.

The lack of Cdc42 and Rac1 might also affect the dynamic reorganization of the MT network. It is generally believed that vesicles move along MT and MF using different motor proteins (DePina and Langford, 1999; Nelson, 1991). The presence of aberrant vesicles in the inner tongue cytoplasm of Cdc42/Rac1 mutant mice might indicate a potential role for the MT network in myelination. It was shown that active Cdc42 and Rac1 capture microtubules through IQGAP1 and CLIP-170 (Fukata et al., 2002). However, CLIP-115/CLIP-170 mutant mice did not display any myelination deficits (our own unpublished results). Another link between Cdc42, Rac, and the microtubule network is provided by stathmin, which can be phosphorylated by the Cdc42 and Rac1 downstream target PAK (Daub et al., 2001), or by CIP4, which interacts with both WASP and MT (Tian et al., 2000). Therefore it might be interesting to analyze the distribution and protein
level of stathmin in *Cdc42* and *Rac1* mutant mice.

In the PNS, *Cdc42* and *Rac1* mutant mice displayed a dysmyelinating phenotype that closely resembles the one observed in several laminin mutant mice. Laminins (Ln) are heterotrimeric extracellular matrix proteins and main structural components of the basal lamina that surrounds Schwann cells (Yurchenco et al., 2004). Ln-2 (α2, β1, γ1) is the major laminin in the PNS and mutant mice lacking this laminin (dy, dy2J) show dysmyelination in spinal roots, cranial nerves, and in the spinal cord (Bradley and Jenkison, 1975; Stirling, 1975; Weinberg et al., 1975). Brachial nerves were not affected and it was hypothesized that other laminin isoforms such as Ln-8 and Ln-10 might compensate. Indeed, Ln-2/Ln-8 mutant mice displayed a nearly complete dysmyelination of all nerves and reduced Schwann cell proliferation (Yang et al., 2005). Ablation of Ln γ1, which is present in all Ln isoforms of the PNS, also led to the complete inhibition of Schwann cell differentiation and to the presence of unsorted axon bundles throughout development (Chen and Strickland, 2003). In addition, the observed deficiency in Schwann cell-axon interaction prevented proliferation and increased apoptosis of Schwann cells (Yu et al., 2005).

Schwann cells express several laminin receptors including dystroglycan and the heterodimeric integrins α1β1, α6β1 and α6β4 (Colognato et al., 1997; Einheber et al., 1993; Previtali et al., 2001). Whereas *Integrin β4* mutant mice showed normal myelination (Frei et al., 1999), ablation of *Integrin β1* in Schwann cells led to impaired radial sorting (Feltri et al., 2002), indicating that this integrin is playing a central role in laminin signaling. Proliferation and apoptosis were not altered in *Integrin β1* mutant mice between E15.5 and P5 (Feltri et al., 2002). Schwann cells lacking dystroglycan receptors passed the pro-myelinating stage but their myelin sheaths were abnormally folded (Saito et al., 2003). It was concluded that dystroglycan is more likely to regulate organized myelin formation and myelin maintenance rather than radial sorting. Several *in vitro* studies showed that integrins regulate Rho GTPases and vice versa (Schwartz and Shattil, 2000), and we proved that integrins regulate the activity of Rac1 in Schwann cells. I therefore propose that a signaling pathway from laminin to integrins to Rac1 is important for PNS myelination. Possible links between integrins and GTPases are described in epithelial cells (proposed pathway: integrin → ILK → β-parvin → αPIX → Cdc42/Rac1) and endothelial...
cells (proposed pathway: integrin → ILK → βPIX → Rac1) (Boulter et al., 2006; Mishima et al., 2004). Interestingly, ILK (integrin linked kinase) mutant mice display a very similar dysmyelinating phenotype and appear to regulate the activities of both Cdc42 and Rac1 (personal communication, J. Pereira). However, β-parvin and αPIX mutant mice show no apparent neurological phenotypes (personal communication, J. Pereira), indicating that the pathway previously mentioned for epithelial cells is probably not essential for Schwann cell myelination. Other mechanisms linking integrin engagement with activation of Rho GTPases in various cell types include signaling by focal adhesion kinase (FAK) (Schaller et al., 1999; Schlaepfer et al., 1997), p130Cas (Cheresh et al., 1999; Klemke et al., 1998), Vav family GEFs (Schwartz and Shattil, 2000), and the Src family kinase Fyn (Liang et al., 2004).

In addition to ECM engagement, growth factor receptor signaling is known to activate Rho GTPases (Nobes et al., 1995; Ridley et al., 1992) and to regulate multiple processes in Schwann cell development (Jessen and Mirsky, 2005). In fibroblasts and epithelial cells, the Rho GEF Vav2 activates Rac1, Cdc42 and RhoA in response to growth factor receptor activation (Liu and Burridge, 2000). This pathway is independent of EMC/integrin signaling. To dissect if a deficient response to certain growth factors could account for the phenotypes observed in the Cdc42 and Rac1 mutants, we could treat Schwann cell cultures with different growth factors and assess the activation of Rho proteins.

The present knowledge of downstream signaling pathways of Rho GTPases in Schwann cells is very limited. *In vitro* studies of myelination showed that activation of p38 by signaling pathway(s) involving laminin and appropriate integrin receptor(s) is required for the alignment of Schwann cells with axons (Fragoso et al., 2003). Therefore, it would be interesting to investigate if MAP kinase signaling is altered in our mutant mice. Another candidate protein is the transcription factor NF-κB (nuclear factor κB), which is essential for the upregulation of Oct6 and the progression of axon-associated Schwann cells into a myelinating phenotype (Nickols et al., 2003). Interestingly, Rho family GTPases activate NF-κB in response to multiple signaling pathways (Montaner et al., 1998; Perona et al., 1997).
8.2. Novel function for profilin 1 in CNS and PNS myelination

8.2.1. Profilin 1 and myelin sheath formation
Glial cells proliferate and migrate extensively before undergoing dramatic morphological changes and sending out processes to wrap around the axons. The mechanisms controlling all these processes are poorly understood but are likely to involve the dynamic reorganization of the actin cytoskeleton (Buttery and ffrench-Constant, 2001; Liu et al., 2003; Song et al., 2001). We ablated profilin 1, a key regulator of actin dynamics, using tissue-specific conditional gene ablation. Profilin 1 was required for proper myelination of the PNS but was dispensable for CNS myelination. Schwann cells lacking profilin 1 did migrate along the axons and proliferate normally, but differentiation into a myelinating phenotype was impaired. Our results propose different requirements for profilin signaling in oligodendrocyte and Schwann cell myelination.

8.2.2. Profilin 1 and in vitro myelination studies
To test if the organization of the cytoskeleton is affected by the lack of profilin 1, we used primary Schwann cell cultures from neonatal mutant and control sciatic nerves. We did not observe any obvious alterations in the organization of either the actin cytoskeleton or microtubules in control vs. mutant Schwann cells. In addition, we did not find a significant difference in the length of the processes formed by mutant Schwann cells. Still, due to the neurological phenotype we observed in the PNS of mutant mice, we do not exclude that the lack of profilin 1 affects the dynamics of cytoskeletal changes. Further studies using time lapse microscopy of mutant Schwann cells are needed to explore the possibility that profilin 1 affects the dynamic behaviour of the cytoskeleton.

8.2.3. Profilin 1 signaling pathways in myelination
Rho GTPases are key regulators of the actin cytoskeleton, and considering the diverse functions of profilin in actin dynamics, it is likely that profilin plays a role in small Rho GTPase-mediated actin filament reorganization. This hypothesis was supported by in vitro studies showing that profilin is required for the formation of Cdc42-induced actin microspikes and Rac-induced membrane ruffles (Suetsugu et al., 1998; Suetsugu et al., 1999). Although a direct interaction between profilin and small Rho GTPases has not yet
been reported, several profilin ligands are well-known effector molecules for Cdc42, Rac, and Rho. In the mouse brain, active RhoA recruits the Rho-dependent coiled-coil kinase (ROCK), which, in turn, binds and phosphorylates profilin 2, thereby hindering neuritogenesis due to hyperstabilization of the actin cytoskeleton (Da Silva et al., 2003). The Cdc42 ligand WASP is usually in a closed inactivated conformation and, upon binding of Cdc42, the buried actin-related protein (Arp)2/3-binding site is exposed and actin nucleation is induced by the WASP-Arp2/3 complex (Kim et al., 2000). Interestingly, profilin seems to synergize with Cdc42 in activating WASP, probably by inducing the open conformation (Yang et al., 2000).

In contrast to the severe myelination phenotype observed in Cdc42/Rac1-deficient mice, animals lacking profilin 1 displayed completely normal myelination of the CNS. It is possible that profilin 1 is compensated by other actin binding proteins, thereby still allowing a major role for actin dynamics during myelination. To address this issue, I would analyze the expression level and activity of actin binding proteins such as thymosin-β or cofilin in Pfn1 mutant oligodendrocytes.

We hypothesized that actin dynamics are central to many biological events occurring during Schwann cell myelination. Consistent with this view, disruption of MF by low concentrations of cytochalasin D prevented spiralling of the myelin process around the axon and the expression of genes encoding myelin proteins (Fernandez-Valle et al., 1997; Fragoso et al., 2003). To investigate the role of actin dynamics in PNS myelination, we conditionally ablated profilin 1 in the Schwann cell lineage. The highly similar myelination phenotype in the PNS of Cdc42, Rac1 and profilin 1 mutant mice suggest a signal transduction pathway from Rho GTPase to profilin 1 to the actin cytoskeleton, thereby controlling the differentiation of Schwann cells. Therefore, it would be interesting to test whether protein levels, activity, or localization of profilin 1 is altered in Cdc42/Rac-deficient Schwann cells. Although we did not observe major defects in the organization of the MF network in mutant Schwann cells in vitro, it is possible that actin dynamics are slightly altered or that a defect only manifests itself in the challenging situation of in vivo myelination.
8. Final discussion and outlook

8.3. Future perspectives

In line with the previous discussion, I propose several experiments to further clarify the role of Rho GTPases and profilin in myelination of the nervous system:

- To obtain more insights into the formation, the abundance, and the localization of the observed myelin figures in the CNS of Cdc42 and Rac1 mutant mice, we aim to achieve 3D profiles of "native" myelin figures by injecting single oligodendrocytes in slice cultures with a fluorescent dye and image the cells using 2-photon confocal microscopy (collaborative work with F. Kirchhoff, MPI).

- To pursue the issue of regional differences in myelination, I propose a detailed analysis of the optic nerve at early developmental stages. The optic nerve provides a well-defined and challenging example for directional oligodendrocyte migration. In the mouse, PLP+ OPCs enter the chiasm from E14.5 onward and migrate in response to the chemokines netrin-1 and semaphorin 3 toward the retina, which they reach at E17.5 (Spassky et al., 2002). A second wave of PDGFRα+ OPCs enters the nerve at birth (Wallace and Raff, 1999). Both profilin and Rho GTPases are candidate molecules for the regulation of cell migration. Although we did not observe a migration deficit either in Boyden chamber assays or in the spinal cord of mutant mice, it might be that such a phenotype manifests itself in the optic nerve. To analyze migration capacity I would cut cross sections of optic nerves from E17 mutant and control mice at several levels and compare total numbers of PLP+ oligodendrocytes.

- As discussed before, I would perform differential display proteomics, using techniques such as difference gel electrophoresis (DIGE) (Figure 40) or isotope-coded affinity tagging (ICAT) (Figure 41), to compare protein profiles of control and mutant samples. In contrast to western blotting, where only single proteins can be analyzed, these approaches allow the simultaneous comparison of thousands of different proteins. ICAT technology has the potential to detect proteins expressed at lower levels, and is more likely to detect hydrophobic and large proteins than is DIGE. However, the two methods complement each other, as DIGE preferentially detects low molecular weight proteins and those proteins with few or no cystein residues (which ICAT requires for labelling). As an alternative to these methods, protein chips/arrays could be used for parallel detection and quantitation of specific proteins in complex solutions (Haab, 2001).
Many important cellular processes in the nervous system do not rely on changes in mRNA or protein abundance, but rather on modifying proteins in a cell. Presently, phosphorylation of proteins is the most studied post-translational modification. However, the analysis of phosphoproteins is still on its infancy. In the last few years, isotopic labelling procedures have been developed for differential quantitative analysis of phosphoproteins (Oda et al., 2001; Zhou et al., 2001). Alternatively, two-dimensional electrophoresis and phosphotyrosine immunodetection by western blotting could be used to investigate signaling pathways activated in transgenic mice (Semenov et al., 2006).

Figure 40: Schematic illustration of difference gel electrophoresis (DIGE) technology
Two different protein samples are derivatized with two different fluorophores, combined, and then run on a single 2D gel. Different excitation and emission wavelengths are used for successive scanning of each particular sample. Differentially expressed proteins can be excised from the gel and, after proteolytic digestion can be identified by mass spectrometry. Figure adapted from Patton, 2002.
8. Final discussion and outlook

Figure 41: Schematic illustration of isotope-coded affinity tagging (ICAT) technology
(A) The tag has three functional elements: 1) a biotin tag, used during affinity capture; 2) the isotopically labelled linker chain; and 3) the reactive group, which binds to and modifies cystein residues of the protein. (B) For differential analysis, two different samples are derivatized with two different ICAT reagents, heavy (deuterated) and light (normal). The samples are combined, enzymatically digested, and the labelled cystein-containing peptides are captured using avidin affinity chromatography. The captured peptides are then analyzed by LC-MS, which can determine the relative abundance for each peptide pair. Any peptide-pairs that are significantly different can be further sequenced using MS/MS. Figure adapted from Gygi et al., 1999.
8. Final discussion and outlook

- So far, all the in vitro analysis has been done in primary oligodendrocyte and Schwann cell cultures. To further study myelination, which is depending on a complex interplay between axons, glial cells and the extracellular environment, a reliable coculture system is needed. Therefore I would aim to establish dissociated DRG cultures from E13 mice and dissociated brain cultures from E15 mouse embryos to study Schwann cell and oligodendrocyte myelination, respectively (Bermingham et al., 2006; Cosgaya et al., 2002) L.Dimou, personal communication). These cultures would also provide a fast screening system for myelination phenotypes using iRNA technology. Such functional assays are essential, as high throughput proteomic approaches are likely to generate enormous quantities of data from which it will not always be easy to extract relevant information.

- To investigate the dynamic behaviour of the cytoskeleton I would perform live imaging studies on primary oligodendrocyte and Schwann cells. Subtle differences in the dynamic behaviour of MT and MF might contribute to the anomalous myelination observed in our mutant mice. Several MT binding proteins, including CLIP-170 (cytoplasmic linker protein-170), CLIP-115, EB1 (end binding protein 1), and EB3, have been shown to associate specifically with the plus ends of growing MT. When fused to GFP (green fluorescent protein), these proteins are powerful tools to visualize and quantify cytoskeletal dynamics in different cell types (Stepanova et al., 2003). To explore the dynamics of the actin cytoskeleton, mutant oligodendrocytes and Schwann cells could be transfected with a plasmid containing an EGFP β-actin fusion construct (Ballestrem et al., 1998). The cytoskeletal organization of cells that are grown in cultures is often very different from that of cells in a living organism (Walpita and Hay, 2002). Therefore, it would also be interesting to transfec cells in a coculture system to monitor cytoskeletal dynamics during the early phases of axon engagement.

- The ablation of a particular Rho GTPase might not only affect the overall activity of other family members, but also the site where they are activated. To decipher the dynamics of Rho family GTPase activation in living cells, multiple biosensors have been designed (Pertz and Hahn, 2004). Most of them rely on fluorescence resonance energy transfer (FRET) technology, where energy is transferred non-radioactively from an excited donor to an acceptor fluorophore, which then emits at its characteristic wavelength. However, these FRET biosensors often require overexpression of
RhoGTPases or modification of their C-terminus, which might interfere with their normal function and regulation. Recently, novel biosensors using solvatochromic dyes whose fluorescent intensity or wavelength is strongly dependent on the surrounding environment have been developed (Nalbant et al., 2004). This approach is advantageous in that it monitors the activation of endogenous, untagged Rho proteins and due to the high sensitivity of such dyes, reduces the amount of biosensor required to detect low levels of endogenous activity.

FRET was successfully used to follow Rac1 activity in human Schwann cells and schwannoma cells during interaction with neurons (Nakai et al., 2006). It has been shown that normal Schwann cells elongated processes along neurites under low Rac activity whereas schwannoma cells displayed high Rac activity at distal regions of the cells and failed to align processes with neurites. These results indicate that regulation of Rac activity is required for successful Schwann cell-neuron interaction.

8.4. References


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**Publications**


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Manuscript in preparation

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